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Bacteriophages: A Revisited Strategy for the Treatment of Severe Bacterial Infections

Roberto Badaro
Deputy Editor; Director Health Institute of Technologies SENAI CIMATEC; Salvador, Bahia, Brazil

Bacteriophages are viruses that infect and parasitize bacteria [1]. Phages were discovered by Frederick Willian Twort in 1915, an England microbiologist, based at the St. Thomas Hospital in London, during his tentative to grow a virus in culture media. He found water looking areas in a bacteria culture tubes that later he correlated with some virus that made impossible colonies of bacteria grow on any medium. He had inoculated an agar medium with some fluid (unfiltered), commonly used for smallpox vaccinations [2]. He noticed that although the vaccinia virus did not grow, a bacteria (micrococcus) did grow. The bacteria, however, appeared to be affected with some disease - inoculated agar tubes often showed watery-looking areas, and in cultures that grew micrococci, it was found that some of these colonies could not be subcultured, but if kept, they became glassy and transparent.

In retrospect, this phenomenon, which came to be known as the glassy transformation of Twort, seems exceedingly strange, but indeed was the bacteriophage. An observed agent with Brownian motion present in unfilterable, heat-labile culture media activity against *Vibrio cholerae*, was independently investigated by a French microbiologist, Felix d'Herelle, in 1917. D'Herelle noticed an anomaly shown by some cultures of the coccobacillus that consisted of clear spots, quite circular, two or three millimeters in diameter, speckling the cultures grown on agar of patients with isolates from the bloody stools dysentery bacillus. Still, the spreading on agar of a broth culture, to which had been added a filtrate from the feces spread, was devoid of all growth and lead him to conclude that what caused that clear spot was, in fact, an invisible microbe, a filterable virus, but a virus parasitic on bacteria. Later, called bacteria eaters, the bacteriophages [1].

From 1920 to 1940, prior penicillin discovery, Phage therapy was the only one antimicrobial available treatment in western Europe [3].

Bacteriophage multiplies using two different mechanisms: a lytic cycle that determines the lysis of the infected bacteria and the lysogenic cycle that depending on whether they contain integrase and repressor genes. Those that contain these genes can become integrated into the bacterial chromosome and replicate with the bacterium without killing it. They are termed "temperate" or "lysogenic" phages (as opposed to "lytic" phages that lack this characteristic) [4]. Phages replication have and important implications for their therapeutic application. Virulent or obligate lytic phages infect and quickly kill their bacterial host cell, whereas temperate or lysogenic phages may either stably integrate into their host's genome or enter into the lytic life cycle [3]. Each phage recognizes a specific molecular motif on the surface of its bacterial target. Once bound to their receptors, they pin themselves to the membrane and inject phage DNA into the interior of the bacterium. Bacterial metabolism is taken over by the phage, and the bacteria make 100 – 1,000 phage copies as well as phage-specified lysin proteins that blow up the bacterial target. Progeny phage then attacks additional susceptible bacteria in the population [5]. These types of phages can pick up bacterial genes when they are reactivated and move bacterial resistance genes, pathogenicity genes to other bacteria. Besides, they create defensive "repressor" molecules that inhibit the ability of new phages to attack the bacterium into which they

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are integrated. Natural phages for certain types of bacteria are almost exclusively lysogenic (e.g., mycobacterial phages). Each phage is specific to a particular bacterial genus or species [6].

The current increase in the incidence of antibiotic resistance in human bacteria has favored the study of phages as a therapeutic alternative (phage therapy) [4]. Phage therapy is defined as the administration of virulent phages directly to a patient to lyse the bacterial pathogen that is causing a clinically severe infection [7]. Previous studies have shown the efficacy that revisiting phages therapy patients with severe antibiotic resistance infections were successfully treated [8]. Today it is definitively an alternative treatment for multidrug resistance microorganisms (MDR) that antibiotic failure [9]. Bacteriophage therapies re-enter clinical trials [10]. Different clinical trials are underway to establish the safety, reactogenicity, and therapeutic efficacy of multiple phages. As active elements, phages must undergo rigorous quality controls to ensure the absence of undesirable effects. The bacterial lysis that they cause is of magnitude inferior to what antibiotics do. The future problem must be solved as the possibility of using mixtures of several phages, establish the ideal route of administration and modify them genetically to deactivate bacterial resistance genes, thus antibiotic recovery sensitivity of MDR organisms.

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Reliability Analysis for the Food Manufacturing Industry

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The present study aims to analyze the main reliability measures for packing equipment used in the production process of corn chips. The Anderson-Darling test was used to evaluate the best distribution of the data collected, showing the lognormal distribution as the best model for the data. We used the Minitab v.17 software for the probability functions of some statistical models. The results showed an MTTF indicator at 2.5 days and R(t)=0.47. Furthermore, the packaging equipment depends on maintenance actions having only a 47% chance of not failing when in operation.
Keywords: Reliability. Lognormal. MTTF. Hypothesis Test.

The globalized economy and the relentless chase of recovery from the economic crisis have demanded companies to have a higher degree of control. So, the organizations have sought new management tools that point to greater competitiveness through the quality and productivity of products, processes, and services [1].

The goal of better management is always to seek higher profitability, cost-reduction, increase productivity, promoting the company's growth and competitiveness in a short-time. So, growing productivity implies a better use of employees, machines, energy, and fuel consumed raw materials, and other issues [2].

The data presented implies the vulnerability of the productivity indicator. The impact caused by equipment failures was discussed in this paper, evaluating the Reliability Centered Maintenance (RCM) management scenario. The data was organized in Pareto graphs to determine the most significant impact per machine and higher call opening demand; then, the data was applied to the probability of failure functions to know which model has the best data behavior and, hence, apply the data to the reliability function R(t) and MTTF.

This article aims to analyze the reliability and the meantime of the machine's flaw that most imply the failures of the production line. So, the maintenance department will be able to direct the efforts and actions to correct the points in order to re-measure the indicators and confirm their evolution or not.

Reliability-Centered Maintenance

Reliability-centric maintenance refers to a maintenance program designed to return the equipment's inherent production capacity [3]. The primary purpose of maintenance is to maintain and improve the reliability and regularity of the production system's operation [4].

Siqueira [5] reports that the RCM incorporates new maintenance and monitoring techniques, as well as absorbs modern statistical optimization methods developed by production engineering. One of the advantage of this system is the establishment of a structured way to select maintenance activities.

The production system has a few failures. So, the reliability and availability of machines and equipment increase the point to be solved. For Xie and colleagues [6], the failures in a regular operation system are random events caused by a sudden increase in stress or human error.

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Key Reliability Indicators and Functions

The time to fail, measured from the time the unit is commissioned to fail, is defined by the equation:

$$F(t) = P(T \leq t) = \int_0^t f(u)\,du\,, t > 0 \qquad (1)$$

The equation that defines the reliability is:

$$R(t) = \frac{n_s(t)}{n_s(t) + n_f(t)} = \frac{n_s(t)}{n_0} \qquad (2)$$

The equation for risk is following (the risk is also known as failure rate or risk-rate, and it is associated with the conditions under which the unit is subjected):

$$h(t) = \lim_{\Delta t \to \infty} \frac{R(t) - R(t + \Delta t)}{R(t)\Delta t} = \frac{-R(t)}{R(t)} \qquad (3)$$

$$= \frac{f(t)}{R(t)}\,, t \geq 0$$

The meantime to fail:

$$MTTF = E(t) = \int_0^{+\infty} tf(t)dt \qquad (4)$$

Methods

According to Fogliatto and Ribeiro [4], the main models used to describe reliability functions are exponential probability distributions, Weibull, gamma, lognormal, and reasonable. In the definitions of reliability analysis, it is necessary to determine which probability distribution best fits the data.

We presented a case study in which the data was collected from a company, and the reliability-centered maintenance technique was applied. We used Minitab v. 17 software for the graphs of the probability functions of some statistical models.

Case Study

The company studies is established in the State of Bahia, Brazil, in an industrial headquarters in Salvador city. This organization is a leader in the northeast region in the production of corn products, snacks, popularly known as “salty snacks”. The factory is comprised of production lines and has approximately 200 employees working directly in production. The production line studied was “salty,” following some definitions that characterize the process for the operation of the line.

Workday: Monday to Saturday from 10 PM to 6 AM; starting on Sunday at 10 PM and stopping on Saturday at 10 PM;

Three work shifts: Night, morning and afternoon;

The line consists of 4 machines;

Work directly in line production: 60 employees and 20 per shift;

The average day of 25 production days per month;

The start of the line production: Sundays in the night shift that starts at 10 PM, and the time is scheduled for 1h to prepare the machines;

Every Tuesday: Service is held at 11AM for the staff, and all employees of the morning shift are released 1h earlier so that they can attend. On Saturdays, the morning shift ends the operation of the line at 1 PM;

On Saturdays: The line is cleaned, so there is no production operation in the afternoon shift.

Table 1 shows the hours in daily hours of each work shift and defines the production operating hours of the line. It is possible to observe that the snack line operates at 19.33h/day.

Table 1. The shifts' workday.

Shift	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Average hours (round/day)
Night	6	7	7	7	7	7	7	6.83
Morning	7	6	7	7	7	7	6	6.67
Evening	7	7	7	7	7	0	0	5.83
Factory	20	20	21	21	21	13	0	19.33

Table 2 presents the data on the meantime between failures of the machine that compromises the snack food production line (hours per month).

Table 2. MTTF (h) of the snack line machine.

	Jan	Feb	Mar	Apr	May	Jun	Jul
Supply Mat	136	11	82	9	11	6	9
Extruder	367	217	22	29	32	35	96
Packager	211	269	420	101	158	124	180
Oven	80	50	30	20	15	14	10

Table 3 provides information on cumulative data on maintenance service order quantities opened each month. Table 4 provides crucial information for directing the actions of the maintenance department, thinking of planning the workload for each available staff-hour.

Table 3. Number of occurrences.

	Jan	Feb	Mar	Apr	May	Jun	Jul
Supply Mat	31	46	28	19	13	15	18
Extruder	39	24	10	25	20	9	20
Packager	115	125	80	93	87	25	30

Table 4 and Figure 2 guide the prioritization of maintenance department actions for the equipment that most affects failures and to plan their workforce, staff-hours regarding the type of maintenance.

Table 4. The calls by maintenance type.

	Jan	Feb	Mar	Apr	May	Jun	Jul
Electrical	15	22	15	19	21	3	2
Mechamical	90	88	60	66	58	20	35
Pneumatic	10	15	5	8	8	2	3
Total	115	125	80	93	87	25	3

Figure 1. Pareto of MTTF of the machine of the snack line.

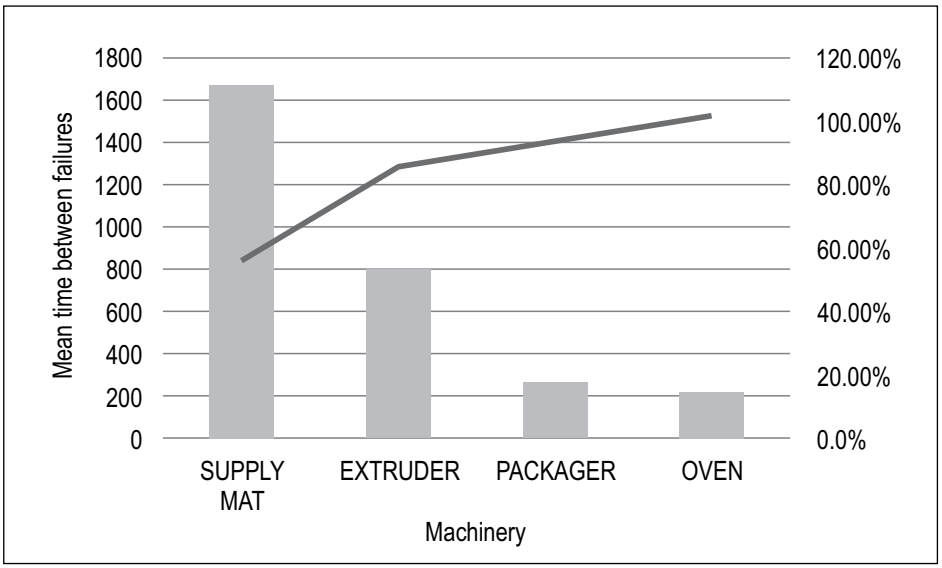
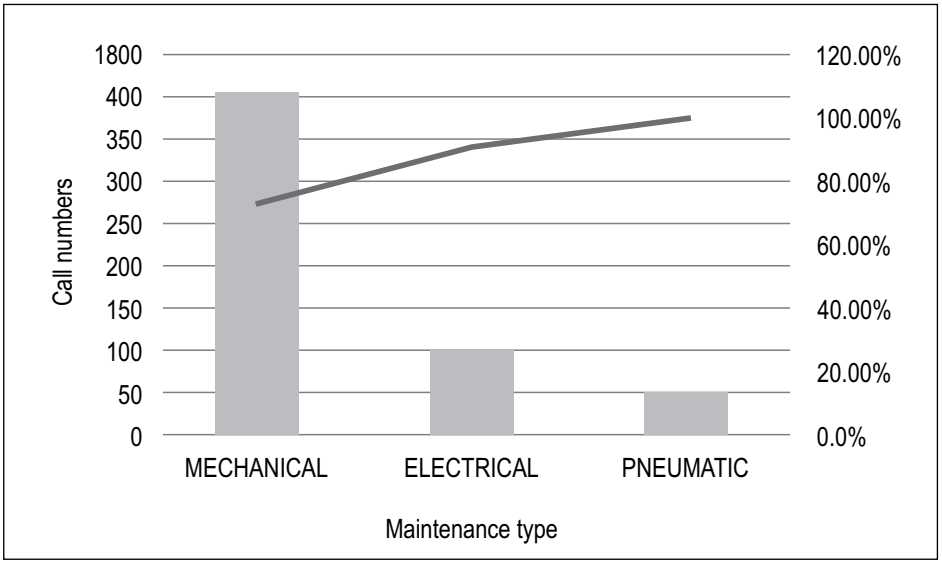


Figure 2. Priority Pareto by maintenance type.



Based on Table 3 and Figure 1, the efforts should be addressed to the packing equipment with the robust performance of the mechanical maintenance team.

Table 5 presents the weekly MTTF (h) of the snack line wrapper due to the analysis of the graphical representation of Figure 1, demonstrating the importance of prioritizing the actions on the wrapper.

By applying the data from Table 5, we get to know the probabilistic model, which presents the best behavior.

For analysis and definition of the model, we used the Anderson-Darling test that serves to verify how well the data follows a distribution. By analyzing the values expressed in Table 6 for the Anderson-Darling test, we found that Lognormal is the probability model that best presents the distribution of data.

Table 5. Weekly MTTF of the wrapper.

Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
40	35	45	51	40
Week 6	Week 7	Week 8	Week 9	Week 10
68	67	67	68	70
Week 11	Week 12	Week 13	Week 14	Week 15
130	100	120	50	20
Week 16	Week 17	Week 18	Week 19	Week 20
20	11	35	46	48
Week 21	Week 22	Week 23	Week 24	Week 25
30	35	37	40	13
Week 26	Week 27	Week 28	Week 29	Week 30
39	25	28	31	30
Week 31				
27				

The probability presented in the graphs (Figures 1 and 2) allowed the hypothesis test for suitability to a given distribution and made the analysis of the corresponding ρ value necessary. If the value of ρ is less than or equal to α, which is the significance level (α = 0.05), then the null hypothesis that the data followed the distribution is rejected. Minitab software for some cases does not always converge mathematically, so the Anderson-Darling test is used to calculate the p-value.

Based on the Anderson-Darling statistic values expressed in Table 6 and the analysis of the Figure 3, we verified that the lognormal distribution presents the best approximation for the data. So lognormal is the distribution that represented the best model for the data.

Defining the probability distribution as lognormal, we apply the values to the reliability model using Eq. (5) to μ=3.70168 and σ=0.56858.

$$R(t) = 1 - \sigma\left(\frac{\ln(t) - \mu}{\sigma}\right) \quad (5)$$

Therefore, considering t = 100 hours, we have R(t) = 0.47, i.e., for every 100 hours, so we have a probability of 47 hours of the wrapper not breaking.

We calculated the meantime to fail, as Eq. (6) to μ = 3.70168 and σ = 0.56858, resulting MTTF = 47.62 hours.

$$MTTF = e^{\mu+0,5.\ \sigma^2} \quad (6)$$

As the working day of the snack line is 19.33 hours, we have an MTTF corresponding to 2.5 days.

Conclusions

The present study sought through the technical application of the concepts of reliability and the use of real data, to support the national industry and demonstrate the importance of integration between companies and universities in order to develop technology and give durability and capability to industrial processes.

The machine analyzed in the packaging process (wrapper) had a reliability of 47%, which is very low, as well as an MTTF indicator of 2.5 days. The machine is operated for three shifts for six days,

Figure 3. Probability chart for failure.

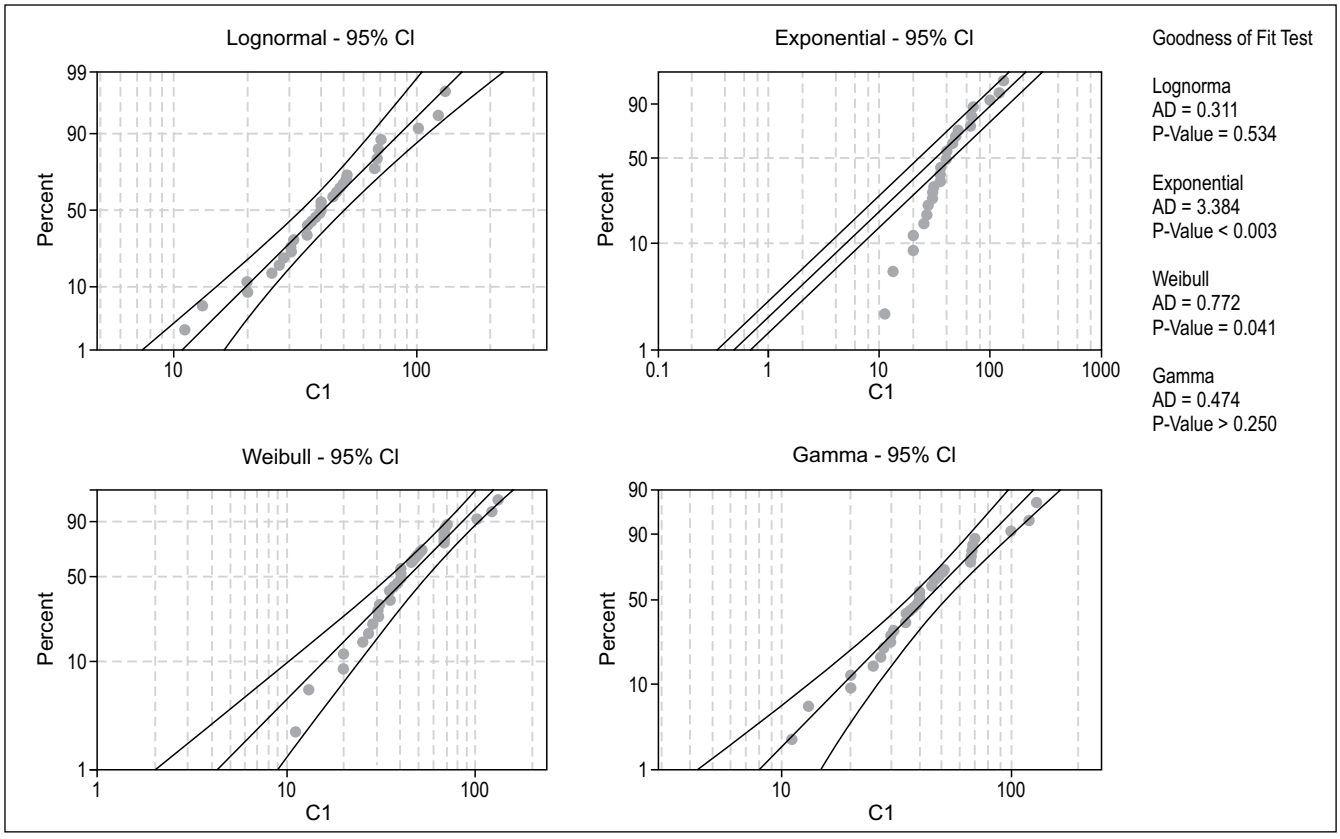


Table 6. Anderson-Darling statistic values for various distributions.

Distribution	AD
Normal	1.512
Box-Cox Transformation	0.311
Lognormal	0.311
3-Parameter Lognormal	0.305
Exponential	3.389
2-Parameter Exponential	1.502
Weibull	0.772
3-Parameter Weibull	0.497
Smallest Extreme Value	2.851
Largest Extreme Value	0.448
Gamma	0.474
3-Parameter Gamma	0.540
Logistic	0.976
Log-logistic	0.235
3-Parameter Log-logistic	0.233
Johnson Transformation	0.183

meaning that there will be probably two possible occurrences of mechanical failure in a working week.

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Manufacturing 4.0: Discussion on Application in the Extractive Industry of Essential Oils

Carlos Alberto Tosta Machado[1]*; Matheus Antonio Nogueira de Andrade[2]; Herman Augusto Lepikson[3]
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Yield uncertainties characterize the industrial extraction of essential oils. The variable plant contents, undetected and uncorrected operational issues such as production in small batches, with a significant variation in the process cycles, productivity, and quality, compromise process performance, and the value of the finished product. The technological advances of industry 4.0 provide an opportunity to achieve economic, environmental, and productivity gains, even with the wide variety of products manufactured. This study aimed at improving the performance of such processes using the pillars of industry 4.0 as a method, highlighting the techniques of data acquisition and processing for intelligent analysis, based on automated and systemic learning, generating improvements for the overall performance of the operation as well as enhancing good manufacturing practices (process safety, quality, repeatability and traceability) and supply chain management. Our findings showed the adherence of 4.0 vision to this industry.
Keywords: Manufacturing 4.0. Essential Oils. Supply Management.

Essential oils are natural products extracted from plant parts (flowers, bark, stem, leaves, roots, fruits, and seeds) with essential applications in the food, beverage, cosmetics and pharmaceutical industries, and as alternative treatments for aromatherapy (baths, inhalation, massage and topical applications), as well as biological fungicides, pesticides and antioxidants [1]. An essential oil consists of a range of components, from the most volatile to the heaviest.

According to Research and Markets, March 2019, the global market for essential oils is expected to reach $ 16.9 billion by 2026, growing at an average rate of 10.8% during this period [2].

Besides the diversity of species from which essential oils are extracted, seasonal variations also impact the products obtained, which may vary significantly in composition and quantities, with evident impacts on agronomic and economic aspects [3]. Therefore, the extractive technology appears like an essential asset since not only the methods and equipment used have importance over the obtained products, but also the embedded control technology and the self-configuration of the production facilities, adapting the process to the specific content in the binomial plant and period of its harvest.

The industry segment of essential oils presents new opportunities for technological upgrades [4]. The introduction of manufacturing 4.0 pillars aimed in this study the improvement of the overall performance of the essential oil extraction industry.

The multidisciplinary aspect of this industrial sector leads to the need to integrate the knowledge of Chemistry, Biology, and Technology (Figure 1) [5].

The chemical aspects are related to the integrity of the volatile products obtained; the biological aspects refer to the behavior of the plant in the extractive process, which enable understanding where the essential oils were deposited in the cellular structure; and the technology ensures the previous aspects as well as productivity and process safety.

Among the essential extractive methods, steam distillation, subcritical carbon dioxide application, cold pressing, and solvent application, concentrate the majority of worldwide production. This manuscript focused on steam distillation, providing insight into the technological possibilities in

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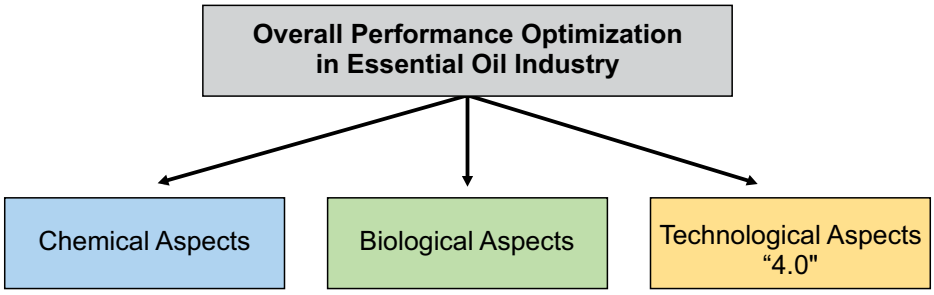
Figure 1. Multidisciplinary aspect of performance in the essential oil industry.



quality, productivity, and sustainability, that providing opportunities for improvements in the business operating margins.

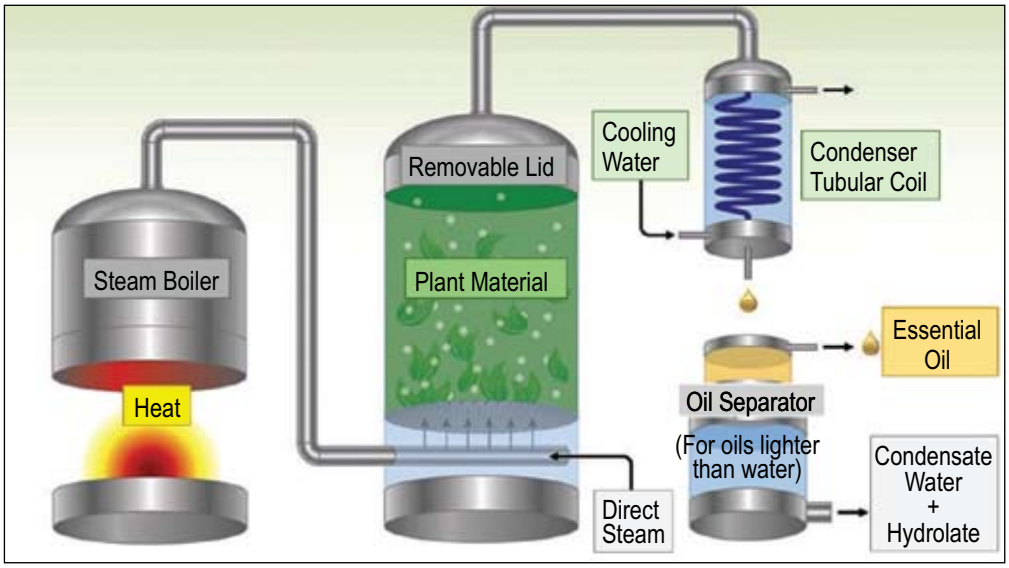
The steam crosses the plant material inside the processing vessel and the product is, then, condensed. The density difference separates this water-oil mix. The supernatant is the essential oil, and the rest of the condensate is called hydrolate, which also has commercial value (Figure 2).

The application of water/steam as a solvent brings environmental benefits in the extraction and purification processes. Nonpolar solvents (hydrocarbons) have significant negative environmental impact and require careful handling during production and subsequent separation [5].

This manuscript creates a 4.0 perspective such as connectivity and real-time process' visibility, which allows identification and correction of deviations and failures, where the control system acquires parameters and optimizes procedures, incorporating them into standard operations [6], autonomously and, in an adaptive way, generating a database for traceability and verification of process repeatability.

The backbones of industry 4.0 comprise a set of technological possibilities that aim to integrate the operational areas of companies. Such composition creates a Cyber-Physical System (CPS), in which the physical and computational components will be deeply braided [10].

Figure 2. Schematic representation of water steam distillation.



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This higher technological level has been demonstrated in this article, resulting in additional enhancements for several operational and business areas. It is a consistent competitive opportunity in a growing and increasingly demanding market [7]. The opportunities' areas brought by the authors address the 4.0 mainstays considered appropriate, as a technological situation in the reading of this business segment.

Methods

The method tried to understand the product (essential oils) and the current state of the industry in this area through visits, and conference calls with national and international researchers - Prof. Ph.D. Farid Chemat - Avignon University, France; Prof. Dr. Maria Angela Meireles - UNICAMP and Prof. Dr. Martins Dias de Cerqueira - UFBA) and visiting companies in this field (LINAX, DIERBERGER) - from February / 2019 to July / 2019.

Systematic bibliographic research completed the formation of a proposal for 4.0 industry's improvement.

Also, good manufacturing practices (process safety, quality, repeatability, and traceability) and supply chain management were discussed in the supplier's and customer's interfaces, as the industrial process is not dissociated from these significant links.

This paper focused on industrial processes of extracting essential oils by hydrodistillation with a proposed operational and control model, applying the concepts of manufacturing 4.0: data acquisition systems through the intensive intelligent application of sensors - analytical processes that generate learning automatic and systemic with advantages for the branch concerned.

Results and Discussion

Describing the Opportunities

One point has become unanimous among all the researchers and companies: empiric approaches base the essential oil production process, using facilities still not modernized, applying the same set of operating practices to all types of products, without the necessary differentiation [4,5]. Such procedures bring yield uncertainties and unwanted variations in quality, and others process.

So, it is necessary to improve:

- Material Yield Improvement: Plants from the same crop (raw material) generate different yields of essential oil.
- Process Time: Lack of repeatability even with similar materials. Dependence on empiricism-based interventions.
- Variable Purity and Quality: Turbidity, coloration, and - especially - quantification of all essential oil components.
- Variable energy consumption from batch to batch.
- Variation in solvent consumption (water steam).
- Non-automatic cleaning procedures bringing uncertainties to the next batch, facility readiness and contamination risks.
- Degradation of essential oil components by overexposure to overheated points, reflecting lack of control.
- Effluent generation beyond the minimum possible.
- Impacts on product operating margins as a consequence of all previous items.

Good Manufacturing Practice Aspects

The production of essential oils will be subject to legal requirements based on good manufacturing practices, according to ANVISA RDC 17. (Collegiate Board Resolution of October 16, 2010), as they are used as raw materials for cosmetics and pharmaceuticals. Physical facilities should be following this regulatory guideline, also meeting repeatability and traceability requirements.

Supply Chain Aspects

The reliability of supply – timeliness, quality, and product integrity (meeting physicochemical specifications) – clearly brings recognition from

customers, which goes far beyond the negotiated price: the reliability in service (safety, quality, and timeliness) of companies in the cosmetics and pharmaceutical industries. Hence the importance of robust industrial processes that achieve their global operational objectives for the first time. An eventual correction, in view 4.0, will be assimilated by the process (machine learning), incorporating the improvements for future batches.

Discussion of a Method for Overall Performance Improvement

We proposed a set of 4.0 recommendations in this manuscript, whose applicability is appropriate in the items: Describing the Opportunities; Good Manufacturing Practice Aspects; and Supply Chain Aspects.

Yield Variations

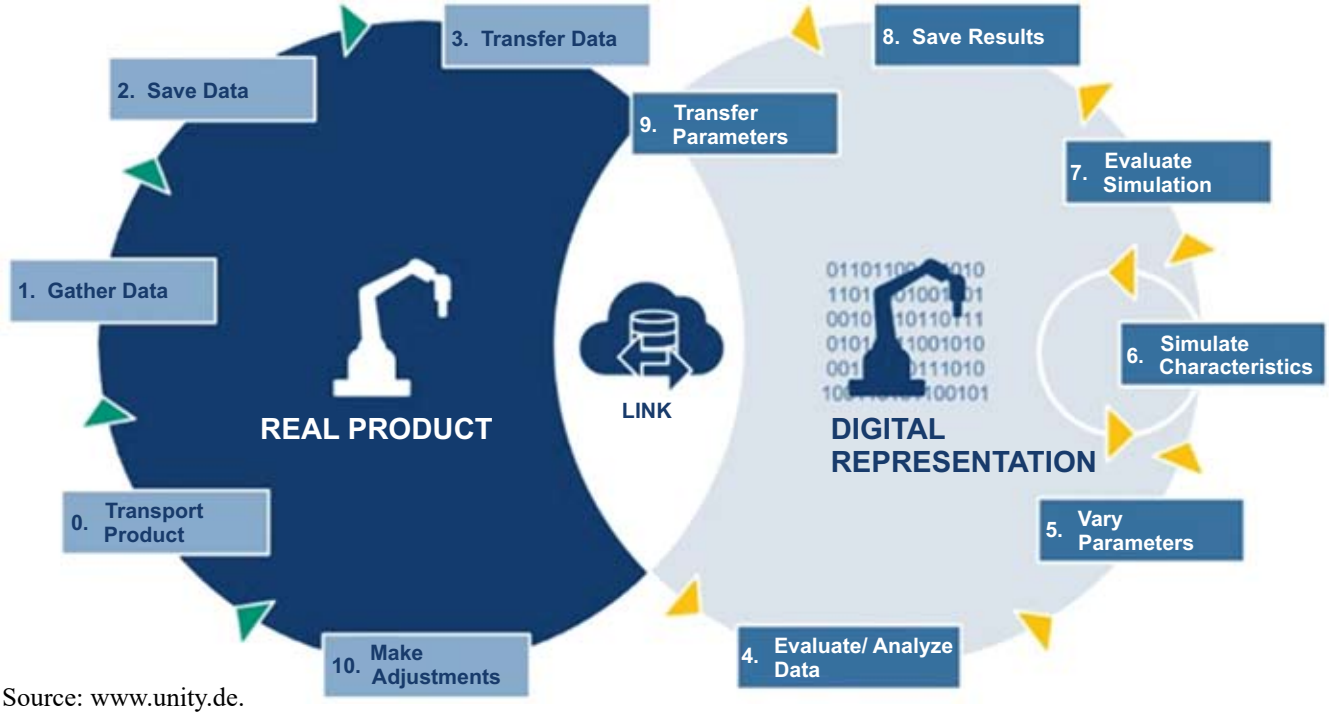
Variations in material balance, from batch to batch (material yield or yield), occur primarily due to lack of effective control of vapor flow through the plant bed. Except for differences in particle size of the ground plants, variations in the amount of product obtained come from either the quality of the plant material or the production process. If we assumed that the materials are from the same harvest, then the process would be the source of variation considered.

Numerous articles show mathematical models (References). However, the simplifications lack representativeness, based on the reality of the manufacture (for instance, minimal, non-representative quantities), such as the repeated simplifying hypothesis of an isotropic plant distribution (with homogeneous and compacted porosity without any variations), the hypothesis of smooth and perfectly radial flow of steam (regardless of preferred paths, called channeling) [3,8,9], and other issues.

The proposed action is a digital twin (Figure 3), which receives signals from field sensors, in parallel with the actual process, and adapts itself to repeat process variables reliably: yield, time, and energy consumption.

The question on how to detect channeling is crucial, i.e., the shortcut of the steam stream through the raw material. This phenomenon has

Figure 3. Functions of a digital twin.



Source: www.unity.de.

two main negative impacts. First of all, some of the raw material is unreached by the steam-generating yield losses; and the overexposure of some spots partially degrade the product. The best approach to detect this uncertainty is by applying external temperature detection (infrared detection or those called "skin point" sensors) in the extractor vessel. Several temperature transmitters distributed within the diameter and depth would be an expensive alternative. The suitable instrumentation and a reliable digital twin lead to the autonomous learning process (machine learning) and early detection of any deviations.

Process Time

It is not acceptable that plants from the same crop generate different process times, nor that different plants have the same treatment indiscriminately. These facts reflect a lack of monitoring and control of the process variables: steam distribution, flow, and pressure, raw material degree of compaction within the vessel. Thus, the process should be repetitive, without requiring intermediate inspections, with process interruptions by the operator.

Real-time data (measurements of condensed quantities, for instance) should follow a pattern (set in previous standard batches) to the digital twin. This database, physical or in the cloud, is subject to analysis and applied to dynamically and continuously improve the overall performance.

Purity and Quality Variations

Density, viscosity, and turbidity should be monitored in real-time. The trend of these indicators creates databank for the digital twin, providing automatic learning and consistency.

Variations in Energy and Solvent Consumption (Water)

The variations in energy and solvent consumption (water) directly impact product cost and yield, depending on how much they go up or down. The steam consumption curve must be monitored to ensure yield and quality. Energy losses, in addition to the environmental impact, reduce the operating profit margin, decreasing the company's competitiveness.

Cleaning Processes

Cleanliness becomes a significant risk-factor when the critical process is applied. Cross-contamination both from previous batches and due to unwashed cleaning products may lead to unwanted losses, failing the customer service agreement (delay) and financially. The manual cleaning process, widespread in this industry, indeed introduces such risks. The introduction of an automatic cleaning system using spray balls, with pressure, temperature and flow controls as well as the additive dosing, washing, rinsing and drying with proper monitoring, not only by the time factor but through indicators of the output fluids (pH, conductivity, among other possible examples). CIP (cleaning in place) systems are widely used in the pharmaceutical and cosmetics industry. The database allows analysis and optimization when virtually controlled, in real-time, by the digital twin. For instance, if the final rinse water parameters are related to the wash temperature, the control system may suggest feasibility tests for shortening times and using smaller amounts of water, cleaning products, and energy.

Degradation of Essential Oils

Product degradation or denaturation occurs due to overexposure to high temperature, oxidation, light exposure, contact with products left from previous batches, or improper cleaning process. The use of an inert atmosphere and reduction of light exposure (closed processes) can solve the mentioned factors.

Effluent Generation

The environmental consequence of uncontrolled processes is direct: solvent and energy usage beyond the optimal. All devices and methods aforementioned will have a positive effect. The optimization of material usage (raw material consumption) will generate less processed material (called bagasse) and less planted areas.

Operating Margins Impact

Higher material yields overwhelming boost business financial results in an industry characterized by insufficient material yields (for instance, 100 mL of product from 3 tons of plants). These products are refined, but increments of a few percentage points are welcome. Process time gain directly increases production capacity, eliminating downtime, waiting, and other issues, improving market response speed, and somehow delaying capacity investments.

Conclusion

The extraction process of essential oils, when improved by the application of industry 4.0 concepts, can deliver significant advantages from material and energy yields to positive impacts on environmental and financial results. It is not a case of "one size fits all"; therefore, each company will plan investments in the specific 4.0 areas, depending on the technological, economic-financial, compliance and expected benefits returns. It is, therefore, a matter of an oriented choice, developing the maturity to select projects to comprise the technological future of the business. Enhanced operational and financial results will provide new days to the organization, allowing the creation of a relentless behavior of pursuing new technical and operational horizons of excellence. Nevertheless, this is a subject for further studies.

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Development of Active Biodegradable Films: Starch Films Incorporated with Starch Nanoparticles and Oregano Essential Oil

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The present study aimed to develop biodegradable starch films with starch nanoparticles incorporated with oregano essential oil – OEO (*Origanum vulgare*), for application as active packaging in food preservation. Film production was performed using the casting method. Three concentrations of oregano essential oil (0.0%, 5.0% and 10%) were studied. The physical and mechanical properties of the films produced were also evaluated. For analysis of antimicrobial efficiency, the methodologies of CLSI and APHA were used. The incorporation of essential oregano oil influenced the performance of the films. The films with 5.0% and 10% OEO showed antimicrobial efficiency when compared to the control.
Keywords: Packaging. Nanoparticles. Antimicrobial. Essential Oil.

In recent years, the use of biodegradable packaging has increased 5% each year [1], with starch, alginate, cellulose ethers, chitosan, carrageenan, or pectins being some of the most commonly used biopolymers in packaging production [2]. Starch has been highlighted for being abundant and having the lowest cost, besides presenting possibilities of chemical, physical, or genetic modification, resulting in resistant and biodegradable films [3].

However, many of these biodegradable polymers have performance-related problems, such as low mechanical and thermal resistance, brittleness and low deformation temperature, and low moisture barrier, limiting the use of biodegradable packaging by industries [4]. Nanotechnology comes through the use of nanoparticles to improve the mechanical and thermal characteristics of these films as an alternative in solving the performance problems of starch films. Due to their nano size, they may have new and improved physical, chemical, and biological properties [5], and better functionality when compared to more extensive material [6].

In addition to improving the properties mentioned above, we also seek to enhance the safety, quality, and shelf life of ready-to-eat foods. According to the Ministry of Health [7], the bacteria *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* are the principal cause of the foodborne disease (FBD). With this, built-in packaging with antimicrobials aims to have higher protection and increase the shelf life of foods. Plant essential oils are an example of natural antimicrobial substances widely studied for food applications [8]. Foods commonly used in the diet of the population, like cheese, are generally present in all social classes [9]. It is characterized as a highly perishable product, contains high levels of protein and lipids, undergoes many deterioration reactions, some of them with high humidity, and has essential nutrients for microbial development [10]. Proper packaging for this type of food is of great importance to maintain the quality during storage. The objective of the present study was to develop biodegradable starch films with starch nanoparticles and oregano essential oil (*Origanum vulgare*), for application as an active packaging for preservation, aiming at controlling the microbial population in mozzarella cheese.

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Methods

The analyses were performed in the Fish and Applied Chromatography Laboratory (LAPESCA). Corn starch (DURYEA®), starch nanoparticles, glycerol (SYNTH®) were used as a plasticizer, and oregano essential oil was used as an antimicrobial agent (FERQUIMA®). Corn starch nanoparticles were prepared by the ultrasound method in a Q55 Sonicator (United States), according to the methodology of Pereira and colleagues [11]. The colloidal suspension was frozen and freeze-dried to obtain nanoparticles with diameters of approximately 400 nm.

Preparation of the Films

The films were prepared according to the casting technique, which consists of the preparation of a filmogenic solution by dissolving the cornstarch in distilled water, starch nanoparticles, and the glycerol plasticizer and oregano essential oil as antimicrobial agent. The films were developed with a concentration of 4.0g starch, 0.25g starch nanoparticles and 2.1 g glycerol/100 g filmogenic solution. The films were prepared with three different concentrations of oregano essential oil (0.0, 5.0, and 10.0%), respectively, concerning the total filmogenic solution.

Mechanical Properties

The films were characterized for thickness upon reading with a manual micrometer (Mitutoyo Corp.) and subjected to maximum tensile strength, rupture deformation, and modulus of elasticity load according to ASTM D882-09 [12] using the Universal Testing Machine. (INSTRON Corporation, Norwood, MA, USA). Ten specimens of each formulation with dimensions of 10 cm long and 2.5 cm wide were tested. The machine was operated at 1kN load at a pulling speed of 5 mm /min.

In vitro Antimicrobial Analysis

The antimicrobial activity of the essential oil films was evaluated using the agar diffusion test of the prepared films. We used *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp' cultures. According to the methodology of the Clinical and Laboratory Standards Institute (CLSI) Manual [13], inocula of each microorganism were prepared by direct suspension, in sterile saline, of isolated colonies selected from Trypticase Soy Agar (TSA) plates, and incubated at 35°C for 18-24h.

In vivo Antimicrobial Analysis

The films were cut to a sufficient size to cover the surfaces of the mozzarella cheese slices. The food covered in the antimicrobial films was stored at 8±2ºC. Microbiological analyses of cheese were performed according to the methodology described by the American Public Health Association (APHA) [14] at 0, 7, 14, 21 days.

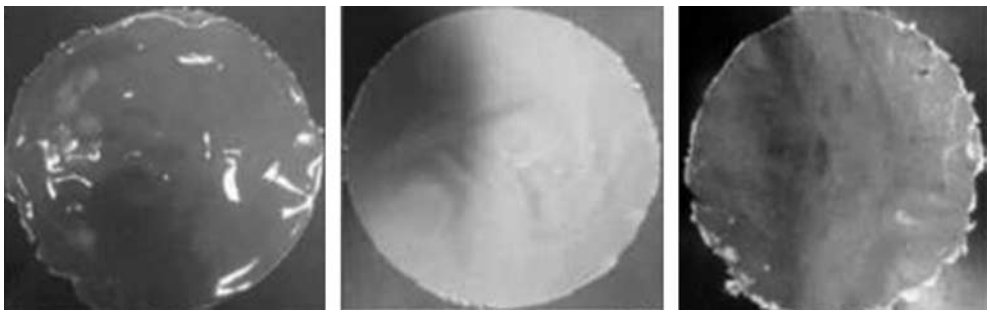
Results and Discussion

The visual aspect of the 0% film (Figure 1A) composed of starch, 0.25 g of starch nanoparticles, and 0 g of OEO (control film) showed the best homogeneity, transparency, brightness, flexibility, and no difficulty in removing the drying plate. The 5% film (Figure 1B) had slightly lower homogeneity when compared to the control film. Besides, it presented great flexibility, transparency, brightness, and little difficulty to remove in the drying plate. The 10% film (Figure 1C) showed less homogeneity when compared to the other films, showed the change in color due to a higher concentration of OEO, and with greater difficulty to remove the drying plates.

Mechanical Properties

The results of the mechanical tests show that the increase of OEO concentration decreased the maximum tensile strength, rupture deformation, and modulus of elasticity of the films (Figure 2). The formulations 5% and 10% presented inferior results when compared to the control (F0). Starch nanoparticle studies by Li and colleagues [15] showed that films with 5.0% nanoparticles had

Figure 1. Embedded starch films of starch nanoparticles, glycerol, and 0% (A), 5% (B), and 10% (C) oregano essential oil as antimicrobial agent.



(A) (B) (C)

a maximum tensile strength of 9.96 MPa, an increase of 72.9% compared to the control film without nanoparticles, and the rupture deformation decreased 12.58%, showing that the addition of OEO contributed to the reduction of these parameters.

Dai and colleagues[16] found that the addition of 10% starch nanoparticles increased the maximum tensile strength to 2.87 MPa, the justification for the improvement of the mechanical properties of the film was due to the high specific surface area provided by the nanoparticles, causing higher resistance. The concentration of nanoparticles used has a strong influence on the properties of the films [17].

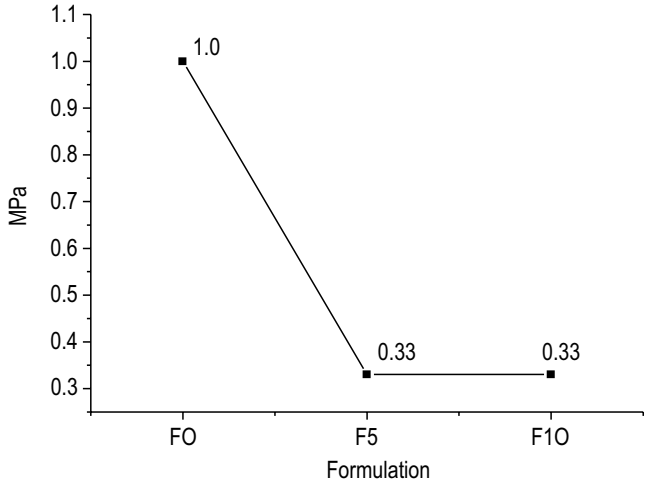
Results similar to the present study were presented by Martucci and colleagues [18], in which the mechanical properties of biogenic gelatin films with essential oregano oil as an antimicrobial agent were evaluated. According to the authors, the addition of oil significantly affected the tensile strength and the stretching ability of the films obtained. The incorporation of the oil possibly resulted in decreased interaction between gelatin monomers, preventing polymer chain-chain interactions, and consequently, compromised mechanical properties. Similarly, in the starch films produced, the incorporation of OEO possibly resulted in decreased interaction between the starch glucose monomers, resulting in decreased strength.

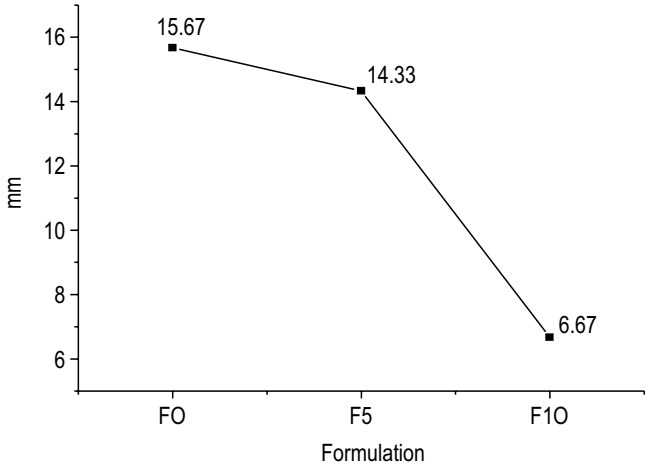
In vitro Antimicrobial Activity

We observed that the higher concentration of essential oregano oil was the sensitivity of *in vitro* antimicrobial activity (Table 1). *S. aureus* was the microorganism that presented the highest sensitivity to the film, with halos of 26 mm and 36 mm in concentrations of 5% and 10% of oregano essential oil, respectively (Table 1). In a study by Botre and colleagues [19], antimicrobial activity against *S. aureus* was also found in films with 50% oregano essential oil. The authors found that the film incorporated with 25% oregano essential oil formed a larger inhibition halo, demonstrating that the higher the essential oil concentration, the greater the antimicrobial activity.

According to Medeiros [20], the cellulose acetate film incorporated with 50% oregano essential oil showed antimicrobial activity on *S. aureus*, obtaining an average inhibition halo of 20 mm. Also, showing antimicrobial activity against *Staphylococcus aureus*. Javidi and colleagues [21] analyzed the microbiological effectiveness of PLA (polylactic acid) films obtained by the casting method, incorporated with OEO, against *S. aureus*, *Listeria monocytogenes*, *E. coli* and *S. enteritidis*. They obtained a better antimicrobial action against Gram-negative bacteria. Different from the present study, where antimicrobial films showed better effectiveness against Gram-positive bacteria (*S. aureus*), and it may be related to the

Figure 2. Mechanical Properties - Maximum tensile strength (A), Rupture deformation (C). Modulus of elasticity of starch films incorporated with starch nanoparticles and with 0% (F0), 5% (F5), and 10% (F10) of oregano essential oil.





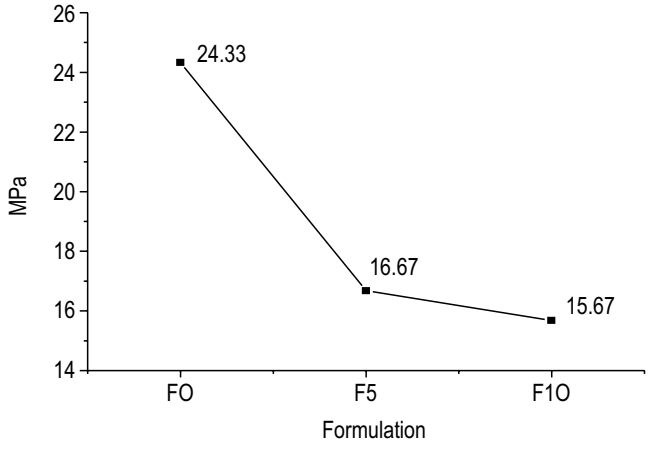


Table 1. Measurements of microbial growth inhibition halos of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp, under different percentages of oregano essential oil in starch films, starch nanopaticles.

	***Escherichia coli* (mm)**	***Staphylococcus aureus* (mm)**	***Salmonellas* pp (mm)**
Film 0%	0	0	0
Film 5%	21	26	21
Film 10%	28	36	24

Means from duplicates.

form of obtaining the films and the interaction between the polymer and the essential oil.

According to Silva [22], the antibacterial action of the essential oils varies significantly based on the bacteria, being the sensitivity higher for Gram-positive than Gram-negative bacteria. One possible explanation for the observed differences in bacterial sensitivity may be differences in the bacterial wall structure, such as the presence of lipopolysaccharide in Gram-negative bacteria and the absence of Gram-positive bacteria, which may allow substances to enter or not in the bacterial cell, thus interfering with the action on the microorganism.

Antimicrobial Activity in the Food Matrix

Microbiological analysis of the sliced mozzarella cheese was performed at time 0 (Table 2), which followed the microbiological limits established by RDC-12, which establishes *S. aureus* limits of 10^3 CFU/g, Thermotolerant Coliforms of 5 $X10^2$ CFU/g and no *Salmonella* spp. 25 g in mozzarella cheese [24].

Films containing 10 g OEO had different variations in their antimicrobial effectiveness when compared to films containing 5 g OEO *in vivo* and *in vitro* tests. However, films containing 5g and 10g of OEO were active until the 14th day. It extended the shelf life of the cheese by 12 days, following current RDC legislation No. 12. Melo [25] failed to extend the shelf life of chilled chicken meat by storing added cellulosic base film. 20% rosemary essential oil within nine days of storage, although inhibition has been proven *in vitro* tests. Thus, we believed that the type of food matrix is an important factor that can interfere in this process, as interactions between antimicrobial compounds and food components, such as proteins and fats, may influence the effectiveness of these compounds.

Table 2. Microbiological analysis of cheese within 0 days.

Thermotolerant Coliforms	**Absent**
Total Coliforms	$2.0x10^{-1}$
S.aureus	$1.7x10^{-2}$
Salmonella spp	Absent
Mesophiles	$2.0x\ 10^{-2}$

According to Cardoso an colleagues [26], PBAT films incorporated with different OEO concentrations, in which fish fillets were packed, were also effective in decreasing total coliform counts, *Staphylococcus aureus* and psychrotrophic microorganisms. It led to an increase in fillet life for up to 10 days, which shows us that OEO has a useful *in vivo* antimicrobial action. Botre and colleagues [19], who analyzed 25 and 50% OEO-added cellulosic resin films packaged in ready-made pizzas, did not obtain inhibition of psychrotrophic growth. However, the growth of filamentous fungi and yeast showed little inhibition after 15 days of storage at 7ºC when submitted to 50% oregano treatment. In contrast, a study by Soares and colleagues [27], revealed a reduction of 0.5 and 1.0 log cycle in *Listeria innocua* growth in fresh cheese packaged in the presence of OEO-

containing films, in relation to control, at the end of the storage period (12th day), confirming the potential use of OEO as an antimicrobial agent in active packaging.

Conclusion

The incorporation of essential oregano oil interferes with the performance of starch films and starch nanoparticles, reducing the mechanical resistance. The 5% OEO and 10% OEO films showed antimicrobial efficiency *in vitro* and *in vivo* analyses, thus demonstrating their potential as a tool for maintaining food quality. The films represent an alternative for reducing the use of conventional plastics in the packaging of some foods. However, there is a need for further studies to evaluate the action of these films in other food matrices.

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Preparation of Panification Products Using Passion Fruit in Replacement of Wheat Flour

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Passion fruit (fruit of significant domestic production) has 52% of its weight comprised of peel (albedo), is rich in pectin, and has essential technological properties for bakery products. In the present coursework, the production of passion fruit albedo flour (FAM) was carried out, later used in different concentrations to produce pasta (4%, 8%, 12%, and 16%) and cookies (3%, 6%, 9%, and 12%). Physical and technological analyses were performed to characterize the products. The data treatment showed that the ideal concentration of FAM for pasta is 15%, and for the cookie, the maximum incorporation did not present a significant difference. We concluded that FAM has excellent potential for use in baking, being necessary further adjustments.

Keywords: Pasta. Cookie. Passion Fruit Albedo Flour.

Passion fruit (*Passiflora edulis*) is a fruit that has been gaining headway in the Brazilian agricultural market in the last four decades. The fruit cultivation has been explored and is already seen as an alternative for coffee producers because the faster financial return [1]. Today, Brazil is the largest producer of passion fruit in the world, yielding one million tons/year [2].

The genus Passiflora is represented by 530 species, being 140 natives of Brazil. Of these, only one is used for commercial purposes in the food production of nectars, juices, pulps, edible ice cream, jellies, and candies [3].

The productive chain uses the fruit pulp to manufacture the products, generating as residue: the bark, which represents ~52% of the passion fruit weight; and the seeds, which represent 6%-12% [4,5]. The residue created from the passion fruit processing is vast and presents a high potential uses for enrichment other food products because it is rich in pectin (a soluble fiber that aids in lowering blood glucose levels; besides presenting gelling properties of great interest to the industrys' foods), protein, B3 vitamin, and minerals [6].

Another use for the passion fruit shell is in the form of flour, which can be used in the bakery industry as an alternative to substitute the wheat flour [3]. According to ABIT (Associação Brasileira da Indústria de Panificação e Confeitaria) [7], the bakery segment creates around 800 thousand direct jobs and 1,8 million indirect in the approximately 70 thousand bakeries and patisseries in Brazil. Emphasizing the use of solid waste from agribusiness contributes to environmental conservation. Besides, it increases the nutritional value of food products [3], minimizing the occurrence of malnutrition due to lack of food, which is one of the aims of the program ONU 2030 - No Hunger and Sustainable Agriculture in Brazil.

The present study aimed to evaluate the technological and physical characteristics of passion fruit albedo flour, species genetically improved by Embrapa, as well as the biscuit and pasta produced from the increment of flour produced.

Methods

Flour Preparation

Passion fruit albedo (purple and yellow) was

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cut into slices and intended for drying in a tray dryer with forced air circulation at a temperature of 80ºC for 8.5 h. In order to determine the drying curves of the flour, moisture measurements were taken every 2 hours, until the albedo humidity was between 10% and 13%, below the maximum limit of 15% [8]. They were then ground in a knife mill (TECNAL, TE-651) and sieved with a 30 mesh opening vibrating sieve, packed in 50µm thick of high-density polyethylene plastic containers, and stored at room temperature, 25 ± 2ºC for storage, following analyzes. Flours were characterized by grain size [9] humidity, by the use of infrared scales and water absorption (ABS), with results expressed as a percentage (%) of absorption.

Product Development

Pasta

Indian extruder model Pasta Mini 5" (Água Rasa – São Paulo) processed the fresh pasta, containing 1: 1 compression ratio thread and noodles with 23 rectangular 1 mm x 5 mm holes, internally coated with Teflon. The dough was kneaded, mixed and extruded, and then dried in a forced-air dryer for further analysis. The parameters evaluated were: cooking time method 66-50 [10], weight gain, volume gain, and loss of soluble solids. The texture of the cooked pasta was determined using the TA-XT plus Texturometer equipment with A / LKB-F, HDP / PFS and A / SPR accessories, to evaluate the firmness and shear force parameters [10].

Four noodle formulations were incorporated with passion fruit albedo flour (FAM) of the purple variety, with concentrations of 4; 8; 12 and 16%, in addition to the standard formulation (without additional passion fruit albedo flour) for comparison. The formulation consists of 3% oil, 1.5% salt, and water.

Cookie

The cookies were made with the incorporation of the yellow variety FAM in the proportions of F1 3%, F2 6%, F3 9%, and F4 12%, in addition to the standard formulation (F0), in which FAM is exempt for comparison purposes. All the ingredients, i.e., wheat flour, passion fruit albedo flour, sugar, butter, salt, and eggs, were mixed in a planetary mixer (Arno), its dough rolled and cut into 2cm wide, 0.8cm thick rectangles and 9cm long, for baking and other analyzes. The analyzes were made of texture profile of the raw mass (hardness, adhesiveness, flexibility, and cohesiveness) and the texture of the cookie (hardness and fracturability) in texturometerTA.XT.plus. Also, weight, diameter, thickness, expansion factor, and specific volume were evaluated. AACC, Method 10-50 D [10].

Statistical Analysis

The experiments were conducted in a completely randomized method, designed with three replications and evaluated by Tukey's range test, with a 5% probability, using SAS software, version 9.0.

Results and Discussion

Particle Size of Passion Fruit Flour of the Purple and Yellow Varieties

The results obtained in the particle size characterization of passion fruit albedo flours of purple and yellow varieties were respectively 44.40% in 42 mesh (0.355 mm) and 47.05% in the same aperture, indicating that both Flours, despite the different varieties, have similar grain size.

The particle size of the food after grinding is an essential aspect in the preparation of flour derivative, since a more excellent uniformity of the granulometry allows the preparation of a final product with better sensorial quality, mainly texture, taste and visual aspect. The food absorbs water evenly, resulting in uniform cooking of the product. Therefore, the grain size of the FAM of the two varieties does not present satisfactory uniformity for the preparation of bakery products [11].

Moisture of Passion Fruit Albedo Flour (Purple and Yellow) Varieties

Passion fruit albedo flour had a moisture content of 10.78 ± 0.3% for the purple variety and 11.56 ±

0.3% for the yellow variety. Moisture is a crucial aspect for the preservation of food quality, as it can directly influence the chemical composition, microorganism development, chemical, and enzymatic reactions, causing, among other damages, the decrease in the shelf life of the product [12].

Flour Water Absorption Capacity

Absorption analysis was performed on the two varieties of passion fruit albedo flour present in triplicates. It was also made with wheat flour incorporated with passion fruit albedo in the proportions 5%, 10%, 15%, 20%, and 25%. The results obtained for yellow, purple, and wheat flour AMF were respectively 559.83 ± 1.36, 549.84 ± 0.17, and 58.39 ± 0.20.

Both varieties are similar when we compared the results between the absorption values found in passion fruit albedo flour. Also, passion fruit flour has a much higher absorption compared to wheat flour. This superior FAM absorption value can be explained by the high fiber content present in passion fruit peel, especially pectin, when compared to the fiber content present in cereal bran and pulses. Furthermore, this high content of soluble fiber increases the nutritional value of foods, providing a reduction in glycemic response, helping in problems such as diabetes, cardiovascular problems, and obesity [13].

Optimum Cooking Time

Increasing FAM incorporation in the dough formulation led to a decrease in cooking time with a mean of 7.00 ± 0.01 min. Cooking time depends on dough cohesion through the interaction of gliadin and glutenin proteins and starch present [14].

Absorption of the Water

Absorption occurs in a significant model described as 0.011 [FAM] + 2.03 with $R^2 = 0.83$, and its increase was already an expected factor due to the high fiber content found in FAM. Flours with a higher fiber and protein content retain a higher absorption [15]. Optimum cooking time was reduced, and absorption increased, leading to faster moistening and swelling of the starch, increasing gelatinization in a shorter time.

Loss of Solids

The loss of solids occurs by a significant model with -0.03 [FAM] ² +, 72 [FAM] + 6.24 with $R^2 = 0.99$, the results show that from the incorporation of 5% of FAM, poor quality flour is already obtained, and the addition only increases the loss of solids and the maximum loss with the incorporation of 11.91% of FAM. Flours with greater grain size and fibrous parts, increase the loss of solids [11].

Increase of the Volume

For this parameter, we obtained a significant model described as 0.01 [FAM] +2.42 with $R^2 = 0.86$, an increase in the incorporation of FAM in the mass formulation generated a similar volume increase. The parameters weight gain and volume increase are related to the water absorption capacity of the pasta during cooking and depend on the shape of the pasta. The volume increase is classified as good with values in the range of 200 to 300% [16], which did not occur, the values below were found, and only the incorporation of 12% was satisfactory.

Texture Analysis of Pasta Samples

Firmness

The results show a significant model described as 0.401 [FAM] ²-12.36 [FAM] + 332.6 with $R^2 = 0.85$ (Figura 1).

The incorporation of the FAM generated a decrease in the firmness parameter, with its minimum point of 15.41% of the addition of passion fruit albedo flour. The use of nontraditional products interferes with the formation of the gluten matrix, weakening its bonds, making the system more fragile, and facilitating water penetration [17].

Shear Strength

The shear strength parameter has a significant

mathematical model, described as 0.041 [FAM] ²-1.161 [FAM] + 23.587 with $R^2 = 0.94$; as well as firmness, this parameter has its value reduced with the increase of the addition of passion fruit flour to the pasta, being its minimum value with the incorporation of 14.23% of FAM in its formulation (Figura 2).

Texture Analysis of Cookie Samples

Expansion and Thickness Index, Weight Loss, and Specific Mass

The results obtained from the expansion and thickness indices, weight loss and specific mass did not present a significant model, having as mean and standard deviation results 0.96 ± 0.01; 0.94 ± 0.38; 0.18 ± 0.01 and 0.97 ± 0.15 respectively.

Cookie Texture: Hardness and Fracturability

The hardness test results do not show a significant model, with average and deviation of 5791.26 ± 692.48. We observed the increase in fiber content in the product with the increase in the value of parameters related to food texture, such as hardness [18].

The fracturability, which forces the cookie to fracture, has a significant model with Fra = 0.0042 [FAM] ² + 0.072 [FAM] + 0.69 with R^2 = 0.92. As hardness, it grows with the increased incorporation of passion fruit flour into the cookie dough formulation. It was expected, as a harder cookie is more difficult to break, the tensions of the forces required for the fracture become larger, having its peak with the addition of 8.57% of FAM (Figure 3).

Conclusion

We observed that the yellow and purple FAM did not present significant differences in the analyzed parameters. For the noodles, the best results were in the addition of 15% of FAM, where the shear strength and hardness were minimal, and the water absorption was close to the expected. Regarding the cookie, the addition of FAM did not present significant results. FAM presented technological problems, but improvements such as the uniformity of granules would be beneficial, significantly improving the quality of baked goods.

Acknowledgment

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Figure 1. Firmness parameter in pasta, in different percentages of passion fruit flour.

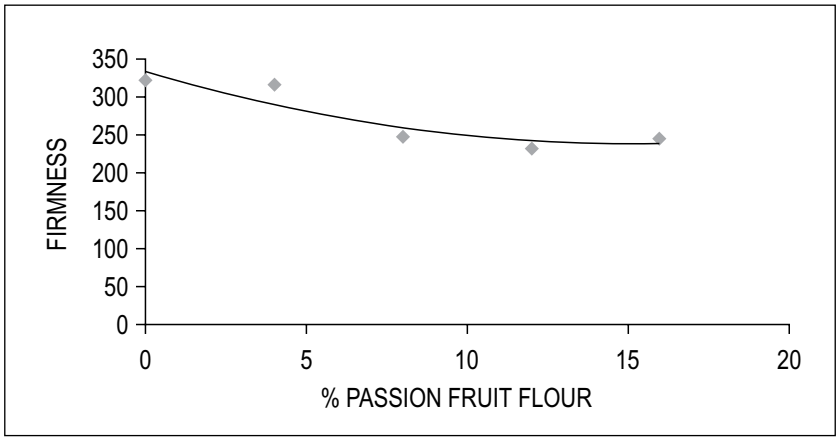
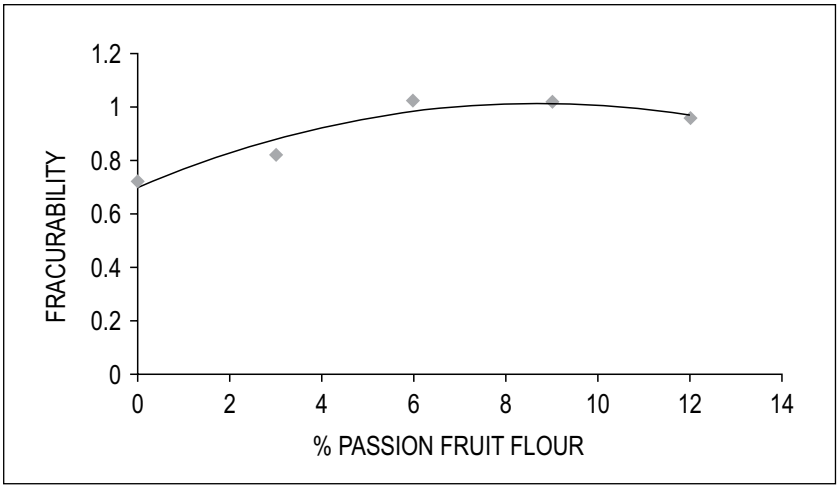


Figure 2. Shear strength parameter in pasta, with different percentages of passion fruit flour.

Figure 3. Parameter Fracturability in the cookie, in different percentages of passion fruit flour.



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Influence of Different Solvents on the Phenolic Compounds Content Grape Bark Extracts (*Vitis vinifera*) from Syrah Variety

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Grape bark from the wine industry still contains significant amounts of phenolic compounds. Specific characteristics of the applied solvents influence the extraction of phenolic compounds directly. This study aimed to evaluate the influence of different solvents obtaining phenolic compounds in Syrah grape bark extracts by High-Performance Liquid Chromatography (HPLC). Grape peel extracts were prepared with three different solvents (water, 50%, and 80% grain alcohol), and evaluated using standard curve with 15 phenolic compounds. It was not possible to quantify the phenolic compounds, but the chromatograms suggest the presence of some of them. The solvent that provided the best interaction with grape peel was 50% grain alcohol.

Keywords: Antioxidants. Waste. Grape Peel. Chromatography.

Numerous researches over time have shown interest in studying the beneficial health effects attributed to phenolic compounds present in most foods. Studies highlight the multiple biological and clinical effects related to these diet compounds such as antioxidant, cardioprotective, anticancer, antibacterial, antiviral, vasodilatory, antidiabetic, and anti-inflammatory actions [1].

Phenolic compounds constitute one of the largest groups of secondary metabolites in plants with over 8000 already known. They are important compounds of foods. Grapes contain significant amounts of these substances compared to other fruits and vegetables [2]. The total phenolic content of grape skins changes through cultivar, such as topography, geology, drainage, climate, microclimate, grape variety, human intervention [3].

Researches show outstanding effectiveness in the reuse of grape marc because it contains significant amounts of phenols and antioxidants. The bark has components such as flavonols (kaempferol, quercetin and myricetin) [4], anthocyanins (cyanidin, delphinidin, peonidin, petunidin, malvidin), stilbene (resveratrol), phenolic acids (caftaric acid and p-coumaroyl tartaric acid), as well as a wide variety of tannins [5-7].

We used different solvent systems for the extraction of phenolic compounds from different matrices [8]. The solvent extraction is the most common method for separating natural antioxidants. These, when serially in foods with diversified solvents, may be more efficient in extracting bioactive compounds, since it takes into account the diversity of each sample [9]. The solvent system used in the extraction of grape marc directly influences the contents of total phenolic, anthocyanin, and antioxidant activity of the extracts [6].

The type and polarity of the solvent are characteristics that affect the transfer of electrons and hydrogen atoms, a vital aspect in the extraction of polyphenols, and, consequently, in the antioxidant capacity [10]. Beyond the yield, the choice of solvent system to be used also influences the composition of the extract [11].

The present study aimed to evaluate the influence of different solvents on the phenolic compounds obtained in Syrah grape bark extracts by High-

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Performance Liquid Chromatography, considering the importance of phenolic compounds for human health.

Methods

We used methanol, DMSO (dimethyl sulfoxide) (Sigma-Aldrich Chemical Co.- St. Louis, MO, USA) and acetic acid for HPLC grade, and grain alcohol (92.8% - Anidrol); a 0.20 μm regenerated cellulose membrane filter (Agilent Captiva, California, USA); caffeic acid (CAS number 331-39-5), gallic acid (CAS number 149-91-7), trans-cinnamic acid (CAS number 205-398-1), crystalline p-coumaric acid (CAS number 501-98-4), biochanin A (CAS number 91-80-5), catechin (CAS number 7295-85-4), epicatechin (CAS number 490-46-0), formononetin (CAS number 485-72-3), isoliquiritigenin (CAS number 961-29-5), myricetin (CAS number 529-44-2), narigenin (CAS number 67604-48- 2), quercetin (CAS number 117-39-5), resveratrol (CAS number 501-36-0), and hydrated rutin (CAS number 207671-50-9) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and trans-ferulic acid (CAS number 537-98-4) from Fluka.

Obtaining Extracts

The samples of Syrah grape marc (*Vitis vinifera L.*) were obtained from wine industries in the San Francisco Valley region in August/2018. The barks were separated from seed and stalk and stored in an ultra-freezer (-20 °C) until the time of use. Three Syrah grape peel extracts with solvent variation were obtained: aqueous extract (EA), 50% grain alcohol extract (EAC50), and 80% cereal alcohol extract (EAC80). The peel was crushed with the solvent in proportion 1:5 m/v. Then, the mixture was placed in an ultrasonic bath (Elma Sonic, S40H, Germany) at 30 min/60°C. Subsequently, it was homogenized in a shaker incubator for 120 min/180 rpm, filtered and concentrated in a sample concentrator (Genevac, MiVac Concentrator, United Kingdom) at 50°C. The extracts were stored at freezing temperatures (-27 °C).

Chromatographic Analysis of Extracts

High-Performance Liquid Chromatography (HPLC) performed the identification of the phenolic compounds (caffeic acid, gallic acid, p-coumaric acid, trans-cinnamic acid, trans-ferulic acid, biochanin a, catechin, epicatechin, formononetin, isoliquiritigenin, myricetin, naringenin, quercetin, resveratrol and rutin hydrate) in the Syrah grape bark extracts. Initially, solutions of 10 $mg.mL^{-1}$ were prepared and dissolved in methanol. Methanolic solutions of grape extracts were prepared (5500 ppm). The samples were filtered with a 0.20 μm nylon filter (Agilent Captiva) for subsequent injection into an HPLC system (Shimadzu, LC-20AT, Japan) equipped with an automatic injector and diode array detector (DAD) (Shimadzu, SPD-M20, Japan). The chromatographic separation was performed according to the methodology proposed by Salgueiro and Castro [12] and Cabral and colleagues [13]. A NUCLEODUR 100–5 C18 EC column (150 x 4 mm ID, 5-μm particle size) was used in conjunction with a ZORBAX Eclipse Plus C18 4.6 x 12.5 mm precolumn (Agilent, USA).

Analysis conditions were performed with an elution gradient with a mobile phase of 5% acetic acid and methanol in different proportions, and with a total analysis time of 42 minutes (from 0 to 35 min (0-92% B); 35 to 40 min (92-0% B); 40 to 42 min (0% B)). The injection volume was 20 μL, and the flow 1 $mL.min^{-1}$. The machine was operated at a temperature of 40±2°C. The DAD reading was adjusted in the range of 190 to 800 nm, and the chromatographic acquisition was defined between 260 and 370 nm. The compound identification was made by comparing the retention time, and the ultraviolet spectrum between samples and standards (Table 1), the working range of the compound was 0.5 to 30 $mg.L^{-1}$.

In order to ensure the reliability of the results obtained, the validation was performed according to the methodologies of the National Health Surveillance Agency (ANVISA) [14] and the National Institute of Metrology, Quality and Technology (INMETRO) [15]. This analysis was performed according to the parameters of

selectivity, linearity, precision, detection limits, and quantification limits.

Results and Discussion

The phenolic compound investigated in different grapes bark extracts are shown in Table 2.

It is noteworthy that, although no compounds are quantified, the extracts are shown in their chromatograms peaks that suggest the presence of some compounds in their phytochemical composition, which are flagged with <LD (below detection limit) in Table 2.

According to the results presented, it is suggested that the solvent that provided the best interaction with the grape peel was the 50% grain alcohol. Below, is the chromatogram (Figure 1) for rutin at a wavelength of 260 nm.

The myricetin is an isoflavonoid with antioxidant activity, and it is commonly distributed in fruits, vegetables, nuts, berries, tea, and red wine [16]. It is believed that mirecetine has multiple therapeutic effects, the potential for health as antioxidant properties [17,18], anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, anti-thrombotic, anti-diabetic and antiviral [19], these have anti and pro-oxidants effects, as well as exhibiting mutagenic effects and antimutagenic potential [18,20-22].

The rutin, a natural flavone, has several properties, including antiallergic, anti-inflammatory, antitumor, antioxidant, antidiarrheal, antihypertensive, antimutagenic and protection against nitrosative stress and hepatocellular injury [18,23,24].

Wang and colleagues [25] analyzed grape extract obtained from solutions of HCl and methanol (1:1) and acetone (70%), and analyzed by the same chromatographic method, and found values of 34.8±3.3 μg.mg and 9.0±0.8 μg.mg to myricetin and rutin.

Table 1. Limit of detection and quantification of active compounds by HPLC DAD.

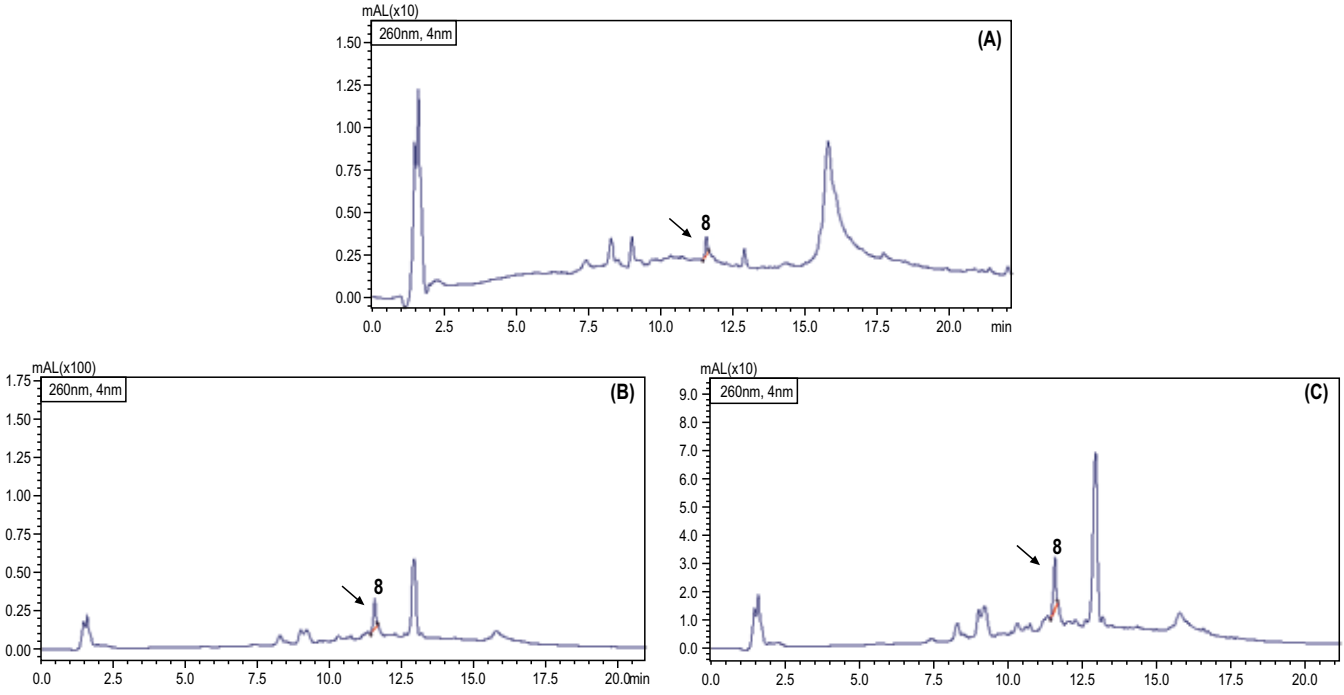
Phenolic Compound	tR (min)	ʎ (nm)	Detection Limit ($mg.g^{-1}$)	Limit of Quantification ($mg.g^{-1}$)
Coffee acid	8.20	320	0.29	0.96
Gallic acid	2.32	280	0.96	3.21
p-coumaric acid	10.35	300	1.04	3.48
Trans cinnamic acid	16.02	280	0.28	0.93
Trans-Ferulic Acid	11.33	320	1.99	6.64
Biochanin A	22.00	280	0.41	1.38
Catechin	6.59	280	1.07	3.58
Epicatechin	8.45	280	0.81	2.69
Formononetin	19.28	300	0.28	0.94
Isoliquiritigenin	18.70	370	0.34	1.14
Myricetin	13.06	370	0.72	2.39
Naringenin	16.54	280	0.24	0.79
Quercetin	15.30	370	0.77	2.57
Resveratrol	13.79	300	0.28	0.93
Rutin Hydrate	11.23	260	0.29	0.97

Table 2. Qualitative evaluation of the active compounds present in Shiraz grape extracts obtained with different solvents.

Phenolic Compound	EA	EAC50	EAC80
Coffee acid	<LD	<LQ	<LQ
Gallic acid	ND	<LD	<LD
p-coumaric acid	<LD	<LD	<LD
Trans cinnamic acid	<LD	<LQ	<LD
Trans-Ferulic Acid	ND	<LD	<LD
Biochanin A	ND	ND	<LD
Catechin	<LD	ND	<LD
Epicatechin	<LD	<LD	<LD
Formononetin	ND	ND	ND
Isoliquiritigenin	ND	ND	ND
Myricetin	<LD	<LQ	<LQ
Naringenin	<LD	<LD	<LD
Quercetin	ND	ND	ND
Resveratrol	ND	<LD	<LD
Rutin Hydrate	<LD	<LQ	<LQ

ND = Not detected; < LD = below the limit of detection; <LQ = below the limit of quantification.

Figure 1. (Chromatogram of grape extracts obtained with different solvents. EA (A), EAC50 (B) and EAC80 (C).



Conclusion

We observed that the extracts have phenolic compounds in their composition. However, the concentration analyzed of the samples was relatively low compared to the analytical curve used for the analysis method. Thus, it is inferred that the matrix since this study (grape peel) has phytochemical compounds, but a new analysis with a higher concentration is necessary to quantify these substances effectively.

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