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Development of a physicochemical method to quantify the extracellular liquid volume: Application to the transformation of cassava into gari

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Liquid volume localisation, Extracellular liquid, Cassava processing, poly(4-styrenesulfonate) sodium salt

Abstract

In fresh vegetal materials, liquid is mostly located in cells. During processing, this liquid is distributed in the entire vegetal matrix volume including exctracellular volume. According to its location, it will be more or less available for physico-chemical mechanisms that takes place during processing of vegetal matrices. This availability greatly influences the quality of the final products. This work consisted in developing a method for determining the quantity of extracellular liquid in a cell-component food matrix. The method developed is spectrophotometric-based and consists in adding a known mass of

polymer (poly(4-styrenesulfonate) sodium salt, PSS) to a rasped vegetal material and defining its concentration in the supernatant separated from the solid phase. The PSS molar mass prevents it from penetrating into plant cells. The PSS concentration measured makes it possible to calculate the extracellular liquid volume. The method validation and its application to rasped and processed (fermentation, pressing) cassava makes it possible to obtain results in agreement with those obtained with other methods.

1. Introduction

During processing of plant or animal raw materials into food, cell-component food matrices can be subjected to liquid flows at cell element level. The understanding and the quantification of this phenomenon represents a major challenge in understanding mechanisms and controlling the products elaboration (Sehy, Banks, Ackerman & Neil, 2002). With regard to meat matrices, much work has been devoted to the post-mortem evolution of intra- and extracellular spaces in relation to liquid movements in the muscle and with water holding capacity (Offer & Knight, 1988). Meat juiciness is assumed to be directly related to the progressive intracellular water efflux occurring during the acidification of postmortem muscle, a change in good agreement with the parallel increase in the extracellular space. These movements of intracellular liquid towards the extracellular space are assumed to be due to pH decrease close to the pHi of myofibrillar proteins that induces a release in bound water (Guignot, Vignon & Monin, 1993; Ouali *et al.*, 2006).

During apoptosis, cells swell up with liquid to the point where lysis of their plasmic membrane occurs. It is a genuine cellular explosion which leads to the release of the cytoplasmic content in the surrounding medium. Liquid availability also plays a fundamental role in manufacturing or value-adding processes (such as agglomeration, cooking, drying) of food and non-food plant matrices. This availability is closely linked to the histological structure of the plant walls (Lima *et al.*, 2013). Plant cell walls are complex structures with central roles in plant form, growth, development and responses to environmental stresses (Sørensen, Domozych, & Willats, 2010). Composed of polysaccharides, proteins and phenolic compounds, cell walls generally belong to either primary or secondary cell wall categories. Primary cell walls are composed of cellulose, pectins, hemicelluloses, and protein or phenolic compounds. They generate turgor pressure (resisting to tensile forces), accommodate cell expansion, mediate cell adhesion, and occur at the surface of most plant cells. Secondary cell walls are

restricted to specific types of differentiated cells. They are produced after the cessation of cell expansion in certain specialized cells. They tend to be thicker than primary walls, and resist to compressive forces. These two wall types are composed of cellulose and hemicelluloses and are often impregnated with lignin (Cosgrove, 1993). Pectins are involved in the control of cell wall porosity and are the major adhesive material between cells (Willats, McCartney, Mackie & Knox, 2001). Hemicelluloses form strong hydrogen-bonded complexes with cellulose fibers. The most important biological role of hemicelluloses is to strengthen the cell wall.

A recent study conducted on the processing of gari, a powdered food generated from cassava and very widely consumed in west Africa, highlighted the major role played by intracellular liquid mobilized during the manufacturing process on the final product quality (Escobar *et al.*, 2018). Some of the final quality descriptors of gari (compactness, diameter distribution, or gelatinization rate) are highly dependent on liquid availability during processing. Liquid distribution in the total available volume can be described in two compartments: (i) the liquid volume present between the tissue fragments generated after the preliminary step of rasping of the cassava root, (ii) the liquid volume inside the tissue fragments (in tight intercellular junctions and inside the cells *i.e.* in the cytoplasm or vacuole). Depending on this distribution, liquid is more or less available to establish capillary bridges between the tissue fragments during the subsequent steps of the manufacturing process (such as pressing or sieving) thereby modulating agglomeration phenomenon. Similarly, liquid availability might greatly affect the gelatinization process occurring during gari roasting insofar as it is highly dependent on the hydrothermal conditions. The hydrotextural approach developed to track the processing pathway from raw material to end product describes the liquid and air proportions within the solid matrices (Ruiz, Delalonde, Bataille, Baylac, Dupuy de Crescenzo, 2005; Escobar *et al.*, 2018). However, the liquid contents considered are only overall values, which do not take into consideration the different locations (outside the tissue fragments or inside the cell) of the liquid.

In the field of human or animal physiology, measurement of compartment volumes is of major importance. Quantification methods of intracellular or extracellular liquid volumes by using radioactive tracers (tritiated water, deuterium, ^{18}O) or ionic elements (bromide) are often employed (Fielding *et al.*, 2003; Simpson *et al.*, 2001). In the field of health, dual-frequency impedance measurement methods have also been used to determine the amount of intracellular or extracellular liquids (Ellis & Wong, 1998; Moreno, 2007). At low frequencies (1 - 5 kHz) the capacitive nature of the cell membranes does not allow the current to penetrate the cell, so impedance is only related to the

extracellular compartment. At high frequencies (200 - 500 kHz) the current penetrates the cell membranes and the recorded impedance is a measure of the combined intracellular and extracellular spaces. Recently, these impedance measuring methods were used in the food sector to detect the lesions sustained by membrane cells of potatoes (Ando, Mizutani & Wakatsuki, 2014) and carrot (Ando *et al.*, 2016) during drying and freezing processes. While these two studies were able to identify the presence of liquid flows *via* variation in impedance value, to our knowledge no works in the literature have quantified extracellular and intracellular liquid volumes.

The objective of this work was to develop an easily-deployable physico-chemical method (low-tech, smart tech), able to quantify the evolution in the proportion of extracellular liquid during processing of cell-component food matrices. This method is developed in the specific framework of cassava processing into gari and is based on the use of a soluble polymer whose molar mass prevents it from diffusing in the cell through the membrane. The method is first validated and used for the measurement of the extracellular liquid in different cassava pulps (fermented or non-fermented, before and after pressing). The new method for determining liquid location can be used to describe the impact of unit operation on the spacial distribution of liquid in the vegetal tissues. This will result in a better understanding of phenomenom occuring during processing as, for example, agglomeration which has been observed during cassava pulp pressing-sieving (Escobar *et al.*, 2018).

2. Materials and methods

2.1 Raw Material

Cassava roots from Costa-Rica were purchased in a French supermarket for this study and analyzed/processed within the following 24 h. They were first manually peeled and then rasped in a kitchen robot (Thermomix®, Vorwerk, Germany) for 1 min at speed 6 so as to generate a pulp. After rasping, the water content of the cassava pulps (1.88 ± 0.04 g water / g dry basis) was measured after drying a 5 g sample in an oven at 105 °C for 24 h.

The poly(4-styrenesulfonate) sodium salt (PSS) is a water soluble polymer with an average molecular weight of 70 kDa. It is a strong polyelectrolyte completely dissociated from pH 3 (Balastre, Persello, Foissy & Argillier, 1999). It was purchased from Alfa Aesar (USA). A PSS solution of 70 g/L was generated and maintained under stirring until complete dissolution (approx. 24 h at 25 °C). Successive

dilutions were used to generate solutions at different PSS concentration (9.4, 18.2, 27.3, 40.1, 55.3, and 60.0 g/L).

2.2 Methods

2.2.1 Determination of the extracellular liquid volume by UV spectrophotometry of the PSS concentration

A known quantity of PSS was added into the cassava pulp. The high molar mass of PSS prevents it from diffusing in the cells. Thus, the PSS concentration determination in the extracellular liquid by UV spectrophotometry makes it possible to deduce the extracellular liquid volume. The spectrophotometric measurements were performed using a spectrophotometer (Shimadzu UV 2450, Japan) in Fischer FB 55923 tanks. The maximal absorption wavelength (λ_{max}) of PSS in solution is 225 nm (Dejeu, 2007) but the extracellular liquid contained in the cassava pulp also present UV absorption properties (Fig. 1). In order to maximize the UV absorption difference (ΔDO max) between the compounds naturally present in the extracellular liquid and the added PSS solution, spectrophotometric measurements were made at a wavelength of 229 nm. The PSS calibration curve was built at 229 nm, and characterized by the equation $[PSS] = 0.0303 \times \Delta DO$, where [PSS] represents the concentration of PSS (g/L) and ΔDO the optical density difference between the extracellular liquid without PSS and the extracellular liquid enriched with PSS. So this method involves, for each measurement made in the presence of PSS, to compare it to the equivalent control without PSS.

2.2.2 Validation of the method

The validation of the method involves the appreciation of the amplitude of a number of phenomena that could interfere with the measurement. Depending on their interference with the measurement, these phenomena may be neglected or not. In the case of a notable interference, they will have to be taken into account in the calculation of the extracellular volume. It is thus necessary to assess: (i) the increase in osmotic pressure generated at the extracellular level by the addition of PSS which is a strong polyelectrolyte (a significant rise could lead to plasmolysis that artificially increase the extracellular volume), (ii) the amount of PSS sorbed on plant tissues that changed its concentration in extracellular spaces, (iii) the negligible effect of centrifugation (necessary for liquid solid separation) on intracellular fluid drainage. Since this method is to be used to evaluate how fermentation and pressing operations can affect the quantity of extracellual liquid, it will be tested and validated also on the products resulting from these operations. For the study of the impact of sorption phenomenon,

149 osmotic effects and centrifugation, 200 g of freshly rasped cassava pulp were introduced into two
150 separate crystallizers. In one of them, 2 ml of PSS solution (i) at 70 g/L (for the quantification of
151 osmotic and centrifugation effects) and (ii) at different concentrations: 9.4, 18.2, 27.3, 40.1, 55.3, 60,
152 or 70 g/L (for the quantification of sorption effect) were introduced and distributed homogeneously
153 (mixed with a spatula for 2 min). After homogenization, 5 g samples of each crystallizers were collected
154 immediately for quantification of sorption effect or at different times for appreciation of osmotic
155 effects (1.5, 3, 4.5, 6, 8, 9.5, 12, 29 and 70 min). These 5 g were introduced into 50 mL polypropylene
156 centrifuge tubes. Concerning the study of centrifugation effect (iii), 2 samples of 5 g of pulp were
157 collected after homogenization from each crystallizer and introduced into two 50 mL polypropylene
158 centriguge tubes for an immediate centrifugation. Two other 5 g samples from each crystallizer were
159 also collected into 50 mL polypropylene centrifuge tubes and immediately filtered on a woven filter
160 with 150 µm mesh size (VWR, Fontenay-sous-Bois, France).
161 For all the assays, 7 mL of distilled water were added to each 50 mL polypropylene centriguge tubes in
162 order to increase the quantity of supernatant for subsequent spectrophotometric analysis. The 50 mL
163 polypropylene centrifuge tubes were vortexed and centrifuged (Eppendorf Model 5810 R centrifuge,
164 Hamburg, Germany) at 18,000 g for 30 min (excepted that collected to study the impact of
165 centrifugation that are immediately filtered). For all the assays, the liquid phase (supernatant or
166 filtrate) was collected and re-centrifuged (18,000 g for 30 min), to enable sedimentation of the
167 smallest particles. Upon centrifugation operation, the supernatants were diluted to 1/20th, and their
168 respective optical densities were measured at 229 nm. It was possible to determine the ΔDO value, (*i.e.*
169 the optical density difference measured between samples with and without PSS collected at each
170 times) and the PSS concentation (g/L) in the extracellular fluid. The calibration curve equation was
171 used to assess the PSS concentation (g/L) in the extracellular fluid after correcting the dilution factor
172 to 1/20th and considering the introduction of 7 mL of distilled water into the 50 mL polypropylene
173 centriguge tube.

174 ***2.2.3 Impact of fermentation or pressing on the extracellular fluid volume***

175 A quantity corresponding to 180 g of the pulp remaining in each crystallizer was then either (i) left to
176 ferment for 24 h in an oven at 37 °C, or (ii) introduced into a compression cell (IFTS, Foulayronnes,
177 France) and pressed under 300 kPa for 1 h and the drained juice was collected under the compression

cell using a test tube. This drained liquid corresponds to extracellular fluid, whose collected volume can be compared to the volume of extracellular fluid measured before pressing.

5 g of each of the fermented pulp masses (with or without PSS) were introduced into 50 mL polypropylene centriguge tubes. The methodology was the same as that described to quantify the proportion of the extracellular liquid in which the PSS was dissolved within the fermented matrix. This value is compared to that previously determined in the matrix before fermentation.

3. Results and discussion

3.1 Method validation

3.1.1 Impact of osmotic effects related to PSS introduction in the extracellular liquid

PSS is a salted polymer (sodium salt), with a high molar mass (70 kg/mol) and whose monomer unit has a molar mass of 206 g/mol. One mole of PSS contains 70000/206 = 339 monomer units. When pH is higher than 3 in solution, each sulfone function is salified and (the dissociation coefficient is 1 which is the worst case for our purposes) each monomers releases one Na^+ ion. After complete dissociation in the solution, 1 mole of PSS releases 339 moles of Na^+ and 1 mole of polymer chain (*i.e.* 340 osmoles). The PSS solution prepared in the present study to determine the extracellular volume was concentred at 70.02 g/L (*i.e.* 10^{-3} mol/L). A volume of 2 ml of this solution (containing 2.10^{-6} moles) was introduced into 200 g of cassava pulp. Assuming a dissociation coefficient of 1, the 2.10^{-6} moles released 680.10^{-6} osmoles into solution. Since the molecular mass of PSS prevents it from penetrating into the cell, it was located solely in the extracellular space. Bearing in mind that the water content of cassava is 63 g water / 100 g wet cassava, if 20% of the liquid was in the extracellular space (the worst case of those measured subsequently), the 680.10^{-6} osmoles of PSS incorporated into 200 g of wet cassava pulp should be diluted in $0.2 \times 0.63 \times 200 = 25.2$ mL. The increase in osmolarity of the extracellular space induced by the addition of PSS would then be: $C = n/V = 680 \cdot 10^{-6}/25.2 \cdot 10^{-3} = 0.027$ osmol /L. Since the osmolarity of a plant cell is in the same range of values as an animal cell (0.280 osmol/L) (Durand-Smet *et al.*, 2014), this osmolarity increase represents approximately 10 % of the osmolarity of the initial medium. The diffusive flow of liquid generated from the intracellular to the extracellular space should therefore be negligible. Fig. 2 represents the evolution of ΔDO measured experimentally between pulp samples with or without PSS over time steps ranging from 1.5 to 70 min after introduction of PSS into the medium. This represents

an experimental validation of the previous hypotheses concerning the impact of PSS introduction on osmotic effects. In the event of a diffusive flow of intracellular fluid to the extracellular space, the PSS concentration of the extracellular fluid should decrease thereby leading to a reduction in the ΔDO value. As Fig. 2 shows, the ΔDO value does not vary with time. This demonstrates the absence of diffusive flow of intracellular liquid to the extracellular space. Therefore, osmotic effects under the conditions of our study can be neglected.

3.1.2 Impact of PSS sorption

Like any element in solution in contact with a solid phase, some of the PSS introduced into the solution can exhibit a propensity for physical sorption on the solid compartment of the matrix. This sorption phenomenon may occur on the external part of the cell walls, intact or not, or on any other suspended solid elements (such as wall fragments, free starch granules, colloids). The sorbed PSS molecules cannot be measured in the extracellular fluid. The PSS sorbed on the matrix was quantified by introducing different amounts of PSS into the freshly rasped cassava pulp samples (Fig. 3). Increasing the amount of added PSS led to a linear increase in the PSS concentration measured in the extracellular fluid. The equation for the line is characterized by a non-zero intercept point at $3.16 \cdot 10^{-4}$ $g\ PSS\ /\ g\ dry\ pulp$. This value indicates the PSS content in the medium when the concentration of PSS in the extracellular fluid is zero. Therefore it correspond to the quantity of PSS sorbed on the matrix. When considering 1 ml of PSS solution at 70 g/L (*i.e.* 70 mg PSS mass), the PSS amount sorbed on 100 g of fresh pulp (whose water content was approximately 1.9 g water / g dry matter) corresponds to approximately 15 % of the PSS introduced into the medium. In view of this relatively high sorbed PSS value, the subsequent calculations of the extracellular and intracellular volume would need to consider this quantity of PSS inaccessible to experimental determination. This value is prone to increase with increasing available surface area for PSS sorption, and therefore increasing rasping time or intensity. Hence the utmost care must be taken with the rasping operation that must be performed under the same condition from one test to another (see section 2.1).

3.1.3 Impact of supernatant collection method

Cell surface damage during centrifugation at 15,000 g was demonstrated to cause significant reductions in bacteria (*E. coli*) viability compared to centrifugation at 5,000 g (Pembrey, Marshall & Schneider, 1999). Concerning plant cells (Kalanchoe leaves), centrifugation and other forms of mechanical stress can lead to cell damage and/or cell death (Pedroso & Durzan, 2000). In the present

study, a 18,000 g centrifugation step for 30 min is applied in order to separate the supernatant. It was thus fundamental to verify whether centrifugation causes lysis of the vegetal cell membrane. Actually, in the case of lysis, the centrifugation would induce the release of intracellular liquid into the medium that would artefactually modify the volume of extracellular liquid in the matrix. Solid/liquid phase separation by filtration on a 150 µm mesh size filter was tested in parallel to centrifugation, in order to compare the PSS concentration in the liquid phase. It was assumed that the filtering method did not cause any damage to the cell elements in the solid phase insofar as they are only submitted to gravitation forces. The results obtained shows that the extracellular fluid separation method (filtering *vs.* centrifugation) did not cause any significant modifications of the PSS concentration value measured in the extracellular fluid: 0.31 ± 0.04 g/L and 0.32 ± 0.03 g/L for centrifugation and filtration respectively. This demonstrates that centrifugation at 18,000 g does not cause any membrane lesions of this cell. When compared to bacteria whose viability decrease above 15,000 g, the vegetal cell resistance to centrifugation could be explained by the ability of vegetal cell wall to resist compressive forces. There is thus no artifactual salting-out effect of intracellular fluid. As the centrifugation separation method makes it possible to work at controlled temperature (20 °C) and to separate the phases in several centrifuge tubes simultaneously, this method was selected for the subsequent part of the study.

3.2 Application to measurement of extracellular liquid before and after fermentation or pressing of cassava pulp

During gari processing, cassava pulp can undergo fermentation and/or pressing steps. These unit operations could modify the liquid phase distributions (intracellular *vs.* extracellular liquid). The proportion of extracellular liquid before and after fermentation on the one hand, and before and after pressing on the other hand was assayed using PSS as an indicator compound. Fig. 4 shows that there is no significant difference in the proportion of extracellular liquid in the matrix before and after fermentation for 24 h at 37 °C. These results are in accordance with granulometric measurements made on cassava pulp before and after fermentation by Escobar *et al.* (2018): the size, and therefore the volume of intracellular liquid in pulp fragments remains constant during fermentation. The enzyme action inherent to the fermentation step (Bainbridge, Harding, French, Kaping & Westby, 1998), if acting on the cell wall, does not seem to be sufficiently effective to cause cell lysis and therefore salting-out of the intracellular liquid in the extracellular space.

Conversely, Fig. 5 shows that the pressing step has an impact on intracellular liquid drainage. Firstly, measurement of PSS concentration in the extracellular liquid before pressing makes it possible to calculate the proportion of extracellular liquid in 180 g of pulp (approximatively 34 %). Then, these 180 g of pulp are introduced in compression cell (cf § 2.2.3) and pressed under 300 kPa for 1h. The drained juice collected in a test tube corresponds to a proportion of approximatively 55 % of the total water contained in the pulp. Thus, during pressing, it seems obvious that a quantity of intracellular liquid is drained to the extracellular space to top up the initial proportion of extracellular liquid. These results support those obtained by Escobar *et al.* (2018) after granulometric measurement of the pulp fragments before and after pressing. There was a clear decrease in fragment size, thereby revealing drainage of intracellular liquid.

4. Conclusion

The objective of this work was to develop a spectrophotometric based method for determining the quantity of extracellular liquid in a cell-component food matrix. This method consists in adding a known mass of PSS to the rasped vegetal matrix (cassava pulp). PSS is distributed in the extracellular liquid due to its high molar mass preventing its penetration into plant cells. After a time of contact, a solid liquid separation by centrifugation is carried out and the PSS concentration is measured in the supernatant. This concentration allows the calculation of the extracellular liquid volume. For the validation of this method, several tests are implemented: the appreciation of the quantity of PSS sorbed on the solid matrix, the appreciation of the osmotic effects and the potential effect of centrifugation. These tests show that osmotic and centrifugation effects are negligible. The use of this methodology makes it possible to show that fermentation did not cause significant transfer of intracellular liquid into the extracellular space and pressing caused drainage of the cell contents to the extracellular medium. These results are in good agreement with those of Escobar *et al.* (2018) which showed that the volume of pulp fragments and damaged cells level remains constant during fermentation, whereas the size of the pulp fragments decrease after pressing. One of the prospects of this work would be to extend it to other food matrices (*e.g.* meat matrices) in order to track the effect of enzyme complexes on salting-out of cell contents that has an impact on meat “juiciness”.

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Development of a physicochemical method to quantify the extracellular liquid volume: Application to the transformation of cassava into gari

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List of Fig.s:

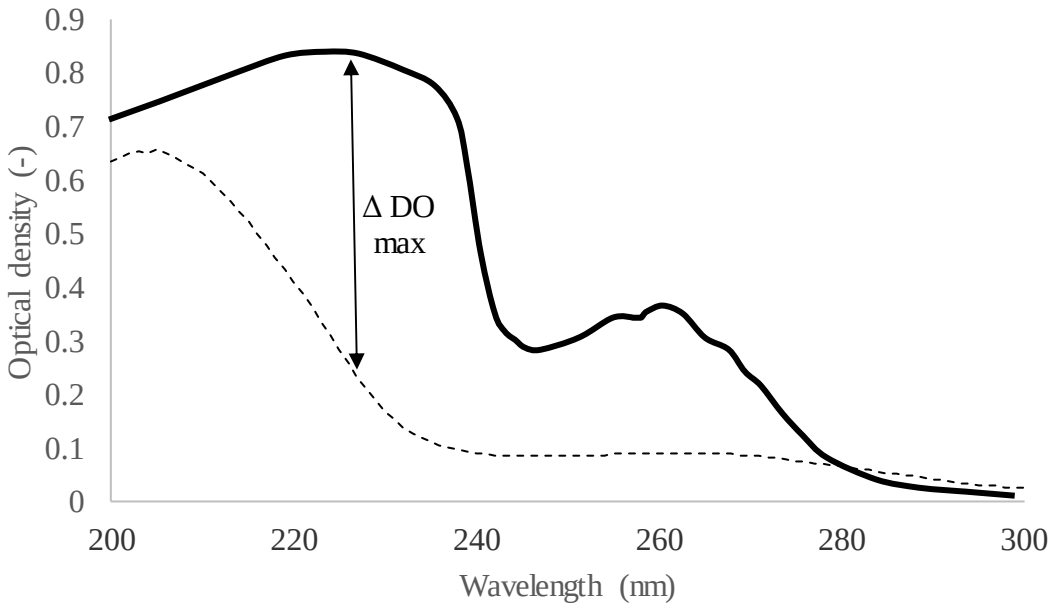

Fig. 1. UV absorption spectrum (200 to 300 nm) of the 70 g/L PSS solution (bold line) and extracellular fluid (dashed line) contained in the rasped cassava pulp.

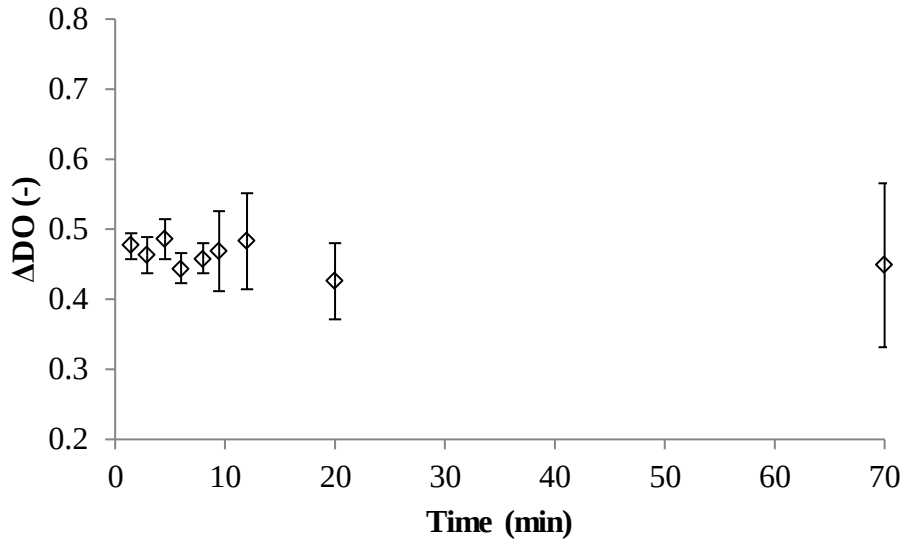

Fig. 2. Evolution of the ΔDO value as a function of the PSS residence time in the cassava pulp extracellular fluid. Each point is the result of a triplicate.

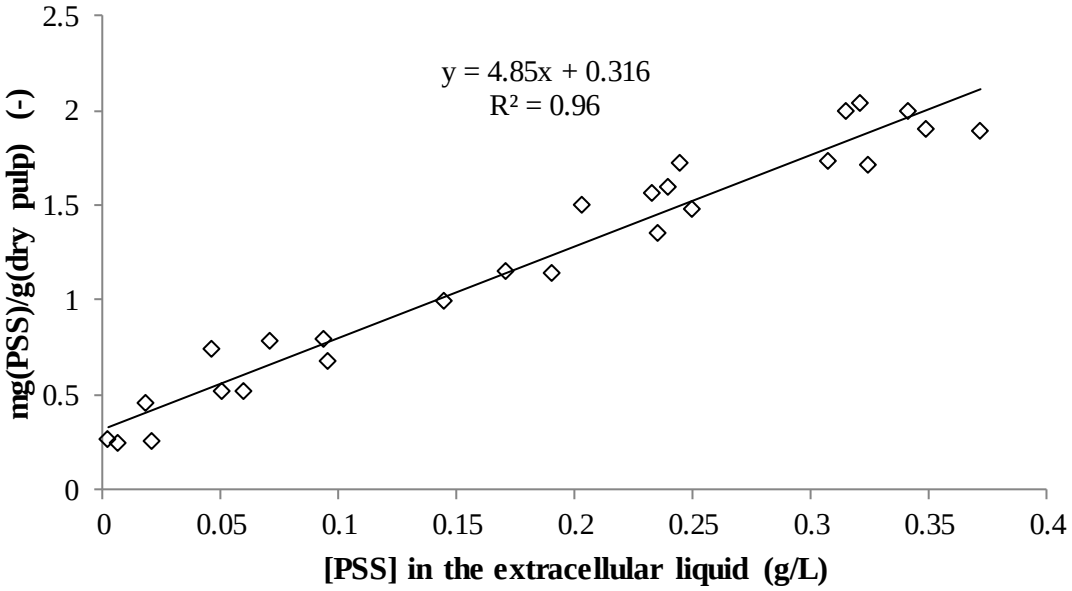

Fig. 3. Quantification of PSS sorbed on the solid matrix (intercept point) (in g (PSS)/g (dry pulp))

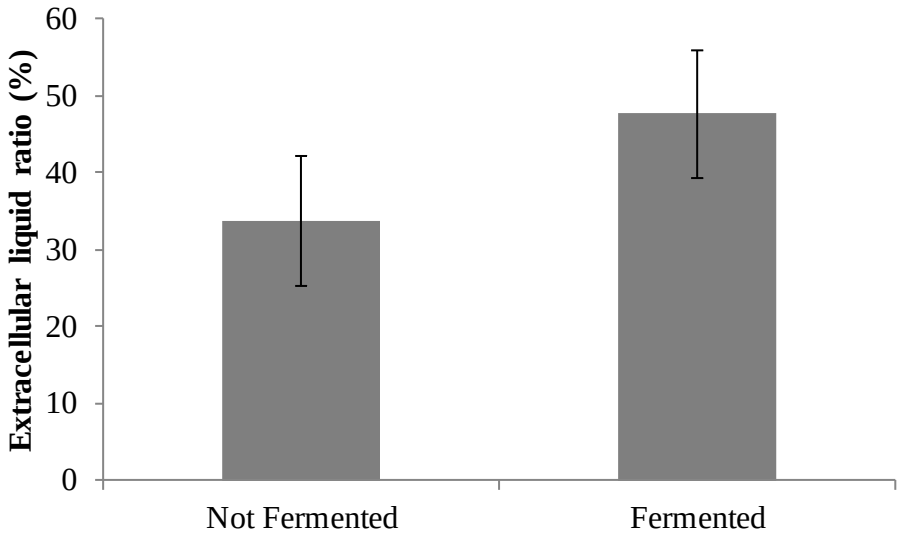

Fig. 4. Proportion of extracellular liquid in the matrix before pressing: comparison of not fermented *vs.* fermented product. Results from triplicate measurements.

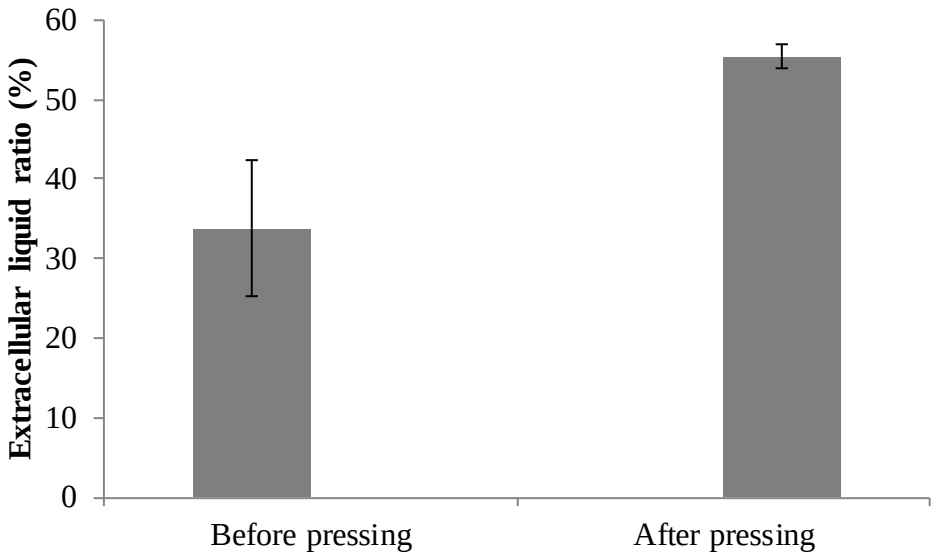

Fig. 5. Comparison between the proportion of initial extracellular liquid in the pulp measured by assaying the PSS concentration before pressing and the proportion of liquid collected in the test tube after pressing. Results from triplicate measurements.

Highlights

- A new method for extracellular liquid quantification has been developed and validated.
- Adding PSS to the extracellular liquid did not generate osmotic flows.
- The sorption phenomenon of the polymer on the solid surfaces is significant.
- The extracellular fluid extraction method by centrifugation does not alter PSS concentration.
- This method applied to cassava pulp gave results coherent to that obtained with other methods.