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Flavor release by enzymatic hydrolysis of starch samples containing vanillin–amylose inclusion complexes

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ABSTRACT

Hylon VII is a high amylose corn starch which has excellent gelling properties, and its behavior as flavor encapsulant is relevant to the food industry because its ability to regulate a swelling-controlled release in aqueous media. We studied the release of vanillin, encapsulated in Hylon VII under experimental conditions in which the amylose–vanillin inclusion complex is obtained, along with the enzymatic hydrolysis of the starch carried out using salivary amylase of human origin. The flavor release into the aqueous phase was quantified spectrophotometrically and the partition into the headspace was assessed by a gas sensor array. Simultaneously, the progress of the enzymatic reaction was determined by analysis of reducing sugars. The release of flavor into the aqueous phase followed a diffusion/relaxation kinetic, whereas the headspace analysis showed an increase in the aroma intensity according to the increase of reducing sugars concentration in the solution. Furthermore, by using the Unfolded Cluster Analysis methodology it was possible to accurately discriminate the samples corresponding to each of the assayed hydrolysis times. The joint detection of the flavor in the condensed phase and in the headspace compared with the degree of starch hydrolysis provides an overall analytical painting of a daily food intake process.

1. Introduction

Starches are complex structures and their molecular features and crystalline organization depend on the chemical composition and processing conditions. As a result, such processing starches can undergo an increase in their surface area to volume ratio in solid phase, changes in crystallinity due to gelatinization and gelation processes, and/or depolymerization of amylose and amylopectin chains. All these properties will have a strong influence on the solubility of the starch and, therefore, in their digestibility by enzymatic hydrolysis.

In solution, amylose and amylopectin are rapidly hydrolyzed into a mixture of mono/oligosaccharides and branched dextrins. By contrast, it is known that in aqueous suspensions, the hydrolysis of solid starch particles is limited and kinetically slowered by factors such as the diffusion of the enzyme towards the particle, the adsorption step of the enzymes on such solid and the porosity of the substrate. In particular, the adsorption of enzymes such as α-amylase is an essential prerequisite and the first step in the process of hydrolysis. This adsorption can be inhibited by the increasing concentration of the hydrolysis products in the reaction mixture. It was also found that hydrolysis reaches a steady state level (plateau) when completing the formation of a monolayer of enzyme onto the surface of the substrate to be hydrolyzed (Foresti, Williams, Martínez-García, & Vázquez, 2014; Tawil et al., 2011).

Another factor which influences starch hydrolysis is the formation of inclusion complexes between amylose and other components either natural or added during the starch processing. Native starches contain a proportion of indigenous lipids that form inclusion complexes with the amylose component when they are gelatinized in the presence of such lipids. This was extensively studied in the pioneer work of Kugimiy, Donovan, and Wong (1980). Since then, the amylose component of numerous starches has been purposely complexed with various lipids using diverse methodologies; the obtained samples were then submitted to enzymatic hydrolysis using amylases from various sources. Two common results of numerous research studies were that, firstly, the

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inclusion complexes exhibited resistance to enzymatic hydrolysis in comparison with uncomplexed amylose and the control starches and, second, the rate of hydrolysis reached a steady state value some hours after initiation of the enzymatic reaction (Ai, Hasjim, & Jane, 2013; Gelders, Duyck, Goesaert, & Delcour, 2005; Kawai, Takato, Sasaki, & Kajiwara, 2012; Kitahara, Suganuma, & Nagahama, 1996). Also, Putseys, Lamberts, and Delcour (2010) proposed that in the presence of inclusion complexes the hydrolysis process occurs in two stages. The first stage, characterized by a high hydrolysis rate, is attributed to the enzymatic attack to amorphous regions, such as amylose residues among the helical structures; whereas the second stage is reached when the hydrolysis involves the enzyme attack to the inclusion complexes themselves, which proceeds at a slower rate.

The background of hydrolysis studies of inclusion complexes between amylose and flavoring compounds is much less comprehensive than in the case of lipids as ligands. Nonetheless, monitoring the α-amylase catalysis during the processing of a starch is of interest either for the production of resistant starches and for knowing its effect on the release of the guests, which modulates the aroma and flavor perception during consumption of a given product (Sajilata, Singhal, & Kulkarni, 2006). In this regard, Heinemann, Zinsli, Renggli, Escher, and Conde-Petit (2005) studied the hydrolysis of inclusion complexes between amylose and various flavoring compounds such as geraniol, γ-nonalactone and δ-dodecalactone when using porcine pancreatic α-amylase, although the hydrolysis was carried out directly on the disperse systems after the heating step (without drying steps). In a latter study, Tietz, Buettner, and Conde-Petit (2008) analyzed the interaction between the flavor menthone and native tapioca starch in aqueous suspension, without performing any particular procedure intended to obtain an inclusion complex, though.

Although there may be complexes with lipids or flavors as guests, the common factor in all cases is that the release of the encapsulated molecules depends on the ability of such molecules to interact with the starch components. In previous works we studied the encapsulant capacity of different commercial starches, some of which were chemically modified for their use in processes involving a drying step, and others rich in amylose (Rodríguez, Wilderjans, Sosa, & Bernik, 2013; Rodríguez et al., 2011). Hylon VII, a natural amylose rich starch, is considered to be one of the resistant starches, and there are various studies reporting their digestibility (Autio, Vesterinen, & Stolt, 2002; Vonk et al., 2000) and its hydrolysis using α-amylase (Rendleman, 2000). The ability of such starches as encapsulants was assessed by different physicochemical techniques (Rodríguez & Bernik, 2013). In this work we focus on the study of the flavor release triggered by enzymatic hydrolysis of a dried product obtained after vanillin encapsulation in Hylon VII. The material obtained as a result of the encapsulation process contains the amylose-vanillin inclusion complex, as it was widely demonstrated in our previous work. So far, no reports have been found describing how hydrolysis processes affect the release of encapsulated flavor molecules, either in the condensed phase in which the hydrolysis occurs or in the coexisting headspace, making this work an original contribution to the study of dynamic detection of flavors during food intake.

2. Materials and methods

2.1. Materials

High amylose maize starch Hylon VII (70 g/100 g of amylose) was provided by the National Starch & Chemical Company (Bridgewater, NJ, U.S.A.). 4-hydroxy-3-methoxybenzaldehyde (vanillin, 99% purity) and the enzyme amylase (α-amylase from human origin Type XI) were purchased from Sigma–Aldrich. All the other reagents were of analytical degree and used as received.

2.2. Obtaining of Hylon VII–vanillin inclusion complex

The Hylon VII-vanillin inclusion complex was obtained as described in previous works (Rodríguez & Bernik, 2013; Rodríguez et al., 2011). Briefly, 1.0 g of Hylon VII was dispersed in 150 mL of Milli-Q water and heated at 130 °C for 90 min in a flask with a screw cap. The suspension was then left to cool down to 50 °C and at this temperature 1.2 g of vanillin dissolved in 1.5 mL of ethanol was added to the starch dispersion. The mixture was allowed to rest at room temperature for 24 h. The precipitated obtained was filtered, washed, centrifuged, and intensively dried.

2.3. Enzymatic hydrolysis of the starch samples containing amylose-vanillin inclusion complex

To carry out the starch hydrolysis of the samples containing vanillin encapsulated in Hylon VII, a procedure similar to that described by Rendleman (2000) was followed. In a glass vial, 0.20 g of the starch-vanillin solid samples (obtained as described above) were weighed and added to 12 mL of a suspension of α-amylase enzyme (52 units in buffer Tris/HCl 10 mmol/L, 1.0 mmol/L $CaCl_2$, pH = 7.2) previously thermostated at 37 °C. Ten vials were prepared from the same batch to carry out measurements by duplicate at five different hydrolysis times (0, 80, 160, 240 and 320 min). The vials were hermetically sealed with crimp caps of polytetrafluoroethylene (PTFE) and incubated on a thermostated shaker at 37 °C until each time selected for the monitoring of the flavor release.

2.4. Quantification of free vanillin by spectrophotometry

Quantitative analysis of free vanillin concentration along the hydrolytic process of the starch was performed by UV absorption (PG-T60 spectrometer, PG Instruments, England) of samples with the pH adjusted to less than 5, below the pKa of the vanillin's phenolic group reported as 7.4 by Robinson and Kiang (1955). To this end, in first place a calibration curve with pure vanillin in aqueous solutions at pH 4.5 was performed for concentrations within the range of 8.0×10^{-6} mol/L and 7.5×10^{-5} mol/L, and the values at the maximum absorption band centered at 279 nm were recorded. The linear regression of the plot of the absorbance at 279 nm versus vanillin concentration yielded a slope value of 1.3×10^4 $(mol/L)^{-1}$ cm^{-1} ($R^2 = 0.997$).

To assess the free vanillin concentration in the reaction medium at different times throughout the process of starch hydrolysis by α-amylase, aliquots of each vial were filtered through a syringe filter with a pore size of 0.45 μm, and then 100 μL of each was placed and raised to a final volume of 5 mL in a volumetric flask, adjusting the pH to 4.5 with aliquots of HCl. Finally, 2.5 mL of each processed sample were poured into a quartz cuvette and the absorption spectrum was recorded within 200–400 nm. Using the calibration curve previously made, the vanillin concentration was interpolated in each case. From the concentration obtained at each hydrolysis time the amounts of vanillin were obtained considering the volume of each vial, after that the percentage of vanillin released by hydrolysis was obtained according to:

$$\text{Vanillin released by hydrolysis (\%)} = \frac{\text{g of vanillin} - \text{g of vanillin at time zero}}{\text{g of total vanillin}}$$

2.5. Quantification of reducing sugars using the Nelson–Somogyi method

Quantitative determination of reducing sugars is a widely used method which allows monitoring starch hydrolysis. Sugars with reducing property arising from the presence of an aldehyde group are released due to the cleavage of starch molecules. Samples' treatment with two reactive solutions leads to the development of a blue color which is compared with a set of standards. General procedure: from each of the samples measured with the electronic nose, aliquots of 100 μL of the supernatant of the reaction medium were taken and diluted in 1.9 mL of Milli-Q water. First, 1.0 mL of solution A was added to each of the tubes, which were heated in a boiling water bath for a period of 10 min and then allowed to cool to room temperature. Then 1.0 mL of solution B was added, homogenizing the total volume and raising to a final volume of 10 mL. Finally, the absorbance was measured at 620 nm in a PG-T60 spectrometer (PG instruments, England). For a detailed description of solutions A and B see Nelson (1944) and Somogyi (1952).

2.6. Monitoring of flavor release to the headspace by an electronic nose

An electronic nose was used in order to monitor the flavor released in the headspace of each sample at different hydrolysis time (0, 80, 160, 240 and 320 min).

The electronic nose is a device consisting of a headspace sampling which brings the aroma to a chamber fitted with a gas sensor array. The sensors signals are recorded and then analyzed by pattern recognition algorithms (Gardner & Bartett, 1999). The electronic nose (e-nose) used in this study was extensively described elsewhere (Lovino, Cardinal, Zubiri, & Bernik, 2005; Rodríguez, Monge, Olivieri, Negri, & Bernik, 2010). In the current case, seven polycrystalline tin dioxides based sensors were used. The sensor's chamber was purged with synthetic air (chromatographic grade) for 5 min prior to the start of each measurement, in order to set a baseline. Samples from incubation with the enzyme were rapidly cooled down in a water bath thermostated at 22 °C (temperature settled during the e-nose experiments) before the start of a measurement. Sensors signals were recorded over a period of 15 min for each of the samples (this measurement time was determined in preliminary experiments in order to have proper sensors recordings). The multivariate data analysis used in this work was performed using the whole set of sensors or a selection of sensors avoiding the redundant information contained in some of them.

2.7. Data analysis: Principal Component Analysis (PCA) and Unfolded Cluster Analysis (Unfolded-CA)

The recorded sensor's signals over time lead to a three-dimensional matrix $\underline{\mathbf{X}}$ (sample × sensor × time). Principal Component Analysis (PCA) is a well-known method for determining similarities among the objects in a two-way data set (e.g., samples by sensors). To this end, the relative locations of the samples in the two most important principal components (PC1 versus PC2, holding more than 99% of the total variance) in a bidimensional scatter plot are compared (Jolliffe, 2002). In Cluster Analysis (CA), a set of objects (in this case each recorded data from the e-nose measurements of the samples) is assigned to a number of groups (i.e., clusters), specified by the user, in such a way that objects belonging to the same cluster are much more alike to each other than objects belonging to different clusters (Johnson & Wichern, 2002). As outputs CA indicates not only by which data input are the clusters composed, but also it provides the so-called *Average Silhouette Width* (ASW) showing a perfect grouping, when ASW equals one.

Moreover, in order to apply PCA and CA methodologies, the three-way matrix $\underline{\mathbf{X}}$ is unfolded into a two-way $I \times JK$ matrix $\mathbf{X}^{\mathbf{unfold}}$ by concatenating the different (time) slices of $\underline{\mathbf{X}}$ horizontally. Then, PCA on the covariance matrix was performed, and *K-means* Cluster Analysis was applied to $\mathbf{X}^{\mathbf{unfold}}$ using the Infostat/p2011 software (Kiers,2000; Rodríguez, Barletta, Wilderjans, & Bernik, in press).

3. Results and discussion

In order to monitor the influence of hydrolysis on the release of the encapsulated vanillin in starch samples containing Hylon-VII–vanillin inclusion complexes, the amount of reducing sugars was determined by using the Nelson–Somogyi method, and also the vanillin release was assessed in the solution by spectrophotometry and in the headspace by means of an electronic nose.

Fig. 1 shows the increasing concentrations of reducing sugars in the samples due to the progress of the hydrolysis of Hylon VII molecules carried by α-amylase. The linear response range for the spectrophotometric detection of reducing sugars was set with a calibration curve using glucose solutions as standards, whose concentrations ranged from 1×10^{-5} mol/L to 5×10^{-5} mol/L. Thus, obtaining a linear regression with a slope value of 1.9×10^4 $(\text{mol/L})^{-1}\text{cm}^{-1}$ ($R^2 = 0.997$) for the absorbance at 620 nm. The curve in Fig. 1 shows a fast rise of reducing sugars concentration for the first 4 h, and a sudden decrease of hydrolysis rate at the fifth measurement time, after 5 h (320 min).

Along with the evaluation of the advance of the starch hydrolysis, the concentration of free vanillin in the reaction medium was dosed by spectrophotometry. Fig. 2 displays the absorption spectra obtained at each of the hydrolysis time points examined. It can be observed the spectra characteristic of pure vanillin in aqueous solutions, ensuring that no interferences from other molecules in the reaction media are observed in this region of UV wavelengths. The peak at 279 nm was used for quantification of vanillin as described in methods, and the increasing concentration of the free vanillin in solution with the time of starch hydrolysis is shown in Fig. 3. The trend is different from the one observed in Fig. 1 for the release of reducing sugars as products of the enzymatic hydrolysis of the

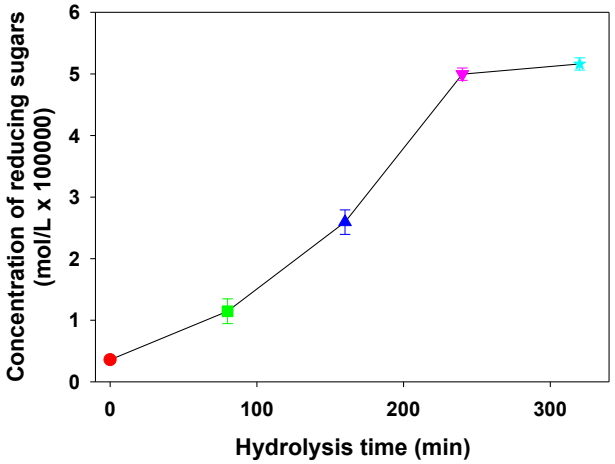

Fig. 1. Concentration of reducing sugars evaluated at different times of α-amylase hydrolysis on samples containing the Hylon VII-vanillin inclusion complex. Time (min): 0 (●); 80 (■); 160 (▲); 240 (▼); 320 (★). The recovered parameters from the linear regression of the absorbance at 620 nm vs. glucose standards molar concentration (expressed in mol/L × 10^5) for the quantification of reducing sugars by the Nelson–Somogyi method used were: slope = 1.9×10^4, $R^2 = 0.997$.

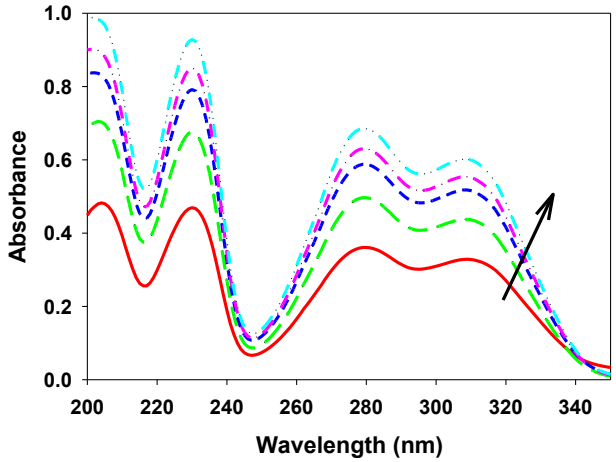

Fig. 2. UV absorption spectra of free vanillin from the supernatants of the samples treated with α-amylase, at different hydrolysis times (min): 0 (——); 80 (– –); 160 (— —); 240 (– · –); 320 (– · · –). The arrow indicates increasing hydrolysis time.

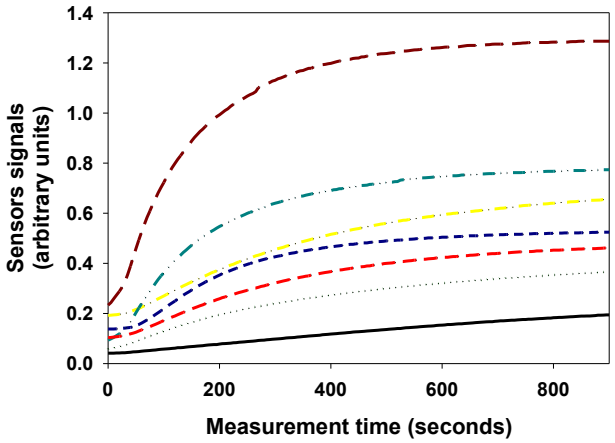

Fig. 4. Graph of an e-nose measurement "smelling" the headspace of a Hylon VII–vanillin sample after a hydrolysis time of 320 min. Each curve depicts the evolution of the signal of one sensor of the array with the measurement time (total time of each measurement: 900 s).

starch. For example, at time zero, before the hydrolysis starts, there is an initial proportion of free vanillin in solution, indicating that the encapsulation of vanillin is not 100% efficient and part of the vanillin is held back by adsorption/occlusion, being faster and more easily released when the solid starch samples are immersed in water.

The evaluation of the flavor release to the headspace throughout the enzymatic hydrolysis of the resistant starches containing amylose-flavor inclusion complexes was performed by using an electronic nose. Fig. 4 shows a characteristic plot of a measurement in which each curve corresponds to one of the seven different gas sensors of the e-nose array. The figure shows the aroma pattern of the headspace of a sample after a hydrolysis time of 320 min.

The analysis of the development of the aroma released from a system like the one under study is simple, because the aroma is originated in a flavor composed of a single molecule, the smell of vanillin is clearly detected by the e-nose and the starch in aqueous solution has practically no odor in comparison. Thus, based on previous studies regarding vanillin, we can ensure that an increase in the values of the sensor signals (expressed as conductance changes) at the plateau region represents an increase in the concentration of flavor in the headspace (Rodríguez et al., 2013). This is valid within a concentration range below the saturation of the sensors, which is the current case at the concentrations we are now using.

The progress of the hydrolysis detected by the e-nose through the flavor released into the headspace was in first place examined by discriminating the groups of signals corresponding to each hydrolysis time by cluster analysis using the Unfolded-CA methodology (see Section 2.7 in methods). By selecting different time windows within the measurements, the analysis leads to the correct discrimination of each group. Table 1 shows that the clustering of the samples clearly discriminates the different hydrolysis times in five groups corresponding to times 0, 80, 160, 240 and 320 min from the start of enzymatic hydrolysis for all the selected time windows chosen. Fig. 5 shows the graph of the groups in a plot of the two first principal components which hold more than 99% of the variance, shown for the particular case of the data window 650–700 s.

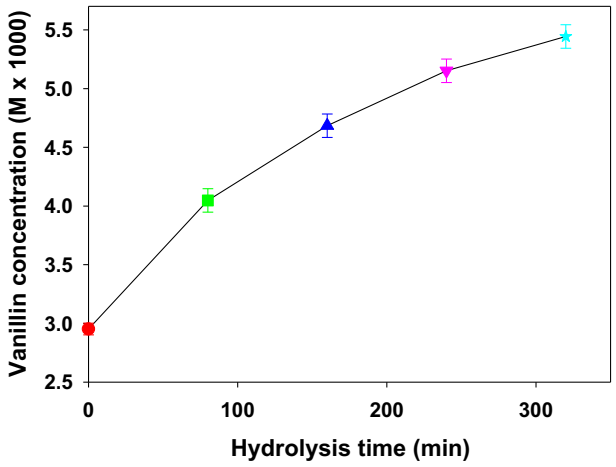

Fig. 3. Values of free vanillin concentration on the supernatants extracted from samples containing Hylon VII-vanillin inclusion complex at increasing α-amylase hydrolysis time. Time (min): 0 (●); 80 (■); 160 (▲); 240 (▼); 320 (★).

3.1. Comparison between the vanillin released in the condensed phase and in the headspace with the progress of the Hylon VII hydrolysis

The percentages of hydrolysis (dashed line) and free vanillin released in solution (solid line) versus time of hydrolysis along the traced 5.3 h are shown in Fig. 6. The curve of released reducing sugars shows an initial value near zero at time zero due to the negligible contribution of free reducing sugars from the terminal aldehyde group of the long native chains of Hylon VII, still intact. When the enzymatic attack starts, a first rapid phase of hydrolysis is observed, and it dramatically decreases its rate after 4 h, as can be seen from the little change recorded for the two last tested times

Table 1
Results obtained by Unfolded-CA for measurements with the electronic nose at each hydrolysis time (five times, five groups in Cluster Analysis) of the inclusion complex containing samples.

Initial-final time (in seconds)	Number of analyzed data	ASW[a]	Correct grouping (5 groups)
650–700	11	0.71	yes
600–900	61	0.88	yes
700–900	41	0.88	yes
800–900	21	0.88	yes

[a] ASW: Average silhouette width from cluster analysis (see Section 2.7).

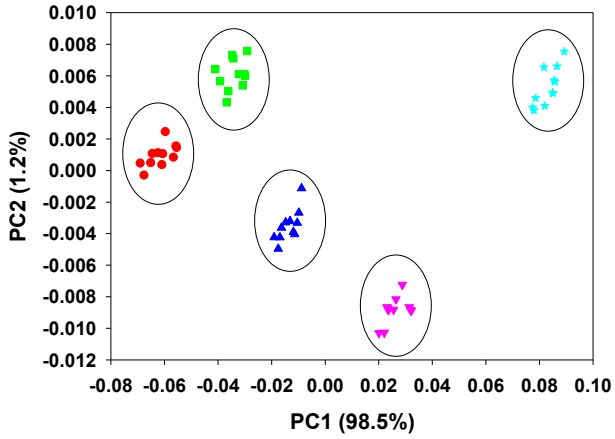

Fig. 5. Diagram of PC1 versus PC2 scores for the selected time interval of 650–700 s of the electronic nose measurements for the samples at each hydrolysis time (min): 0 (●); 80 (■); 160 (▲); 240 (▼); 320 (★).

(240 and 320 min). The initial rise probably arises from the fast enzymatic cleavage of the available amorphous regions of the starch particles. The subsequent deceleration would indicate that the enzyme starts attacking the crystalline regions containing the amylose-vanillin inclusion complex, characterized by being less susceptible to hydrolysis. Thus, during the first 5.3 h the hydrolysis degree reaches about five percent of the total starch present in the sample.

The curve of percentage of vanillin released with respect to the total amount of vanillin encapsulated in the samples submitted to hydrolysis is also shown in Fig. 6. This curve was obtained from the data plotted in Fig. 3 subtracting the initial free vanillin at time zero in order to consider only the vanillin released during the hydrolysis process. Overall, the release profile of vanillin shows a positive slope that decreases steadily with time, releasing about a 5% of the vanillin trapped, thus matching the percentage of the starch hydrolyzed in the same period.

In order to see whether the vanillin is released by simple diffusion, the curve of vanillin can be adjusted to a diffusional Fickian's dependence by means of Equation (1),

$$C(t) = A\sqrt{t} + C(t=0) \tag{1}$$

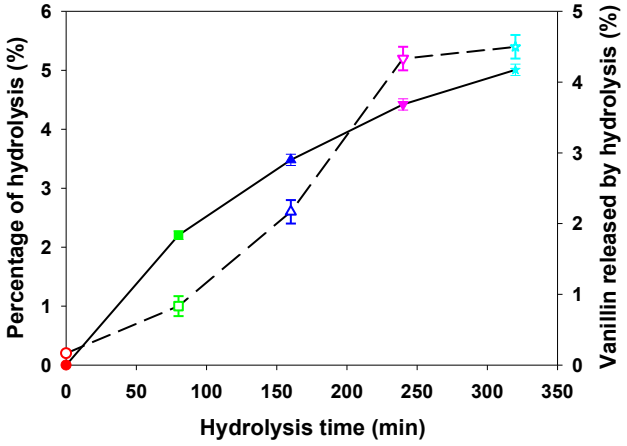

Fig. 6. Scatter plots of the percentage of starch hydrolysis (hollowed symbol, dashed line) and percentage of vanillin released to the reaction supernatant (filled symbol, solid line) as a function of hydrolysis time (min): 0 (○;●); 80 (□;■); 160 (△;▲); 240 (▽;▼); 320 (☆;★).

However, if the release kinetics involves additional features related to solute–matrix interactions, there may be probably other models which consider non-Fickian release mechanisms that would adjust the experimental data more properly. One possibility is to use the well-known exponential model developed by Ritger and Peppas for swellable drug delivery systems (1987) whose general equation is (2),

$$C(t) = kt^n + C(t=0) \tag{2}$$

where the variables k and n are related to diffusion/mass transport mechanisms and also to the geometry of the release matrix. Thus, for a radial release from spheres driven only by diffusion the exponent n should be equal to 0.43.

Using this equation the obtained value for the exponent was $n = 0.59 \pm 0.04$ ($R^2 = 0.993$), indicating that the vanillin release process has a contribution of Fickian diffusion and also of polymer relaxation/swelling-controlled mechanisms. Moreover, the exponential function (2) can be split into two components which represent the relative contributions of Fickian diffusion and swelling/relaxation release mechanisms, as shown in Equation (3),

$$C(t) - C(t=0) = k_d t^{0.43} + k_r t^{0.85} \tag{3}$$

where k_d and k_r are obtained from non linear regression curve fitting of the release data. When k_d is larger than k_r the release is mainly controlled by diffusion, and when k_r is larger than k_d the release is mostly due to matrix swelling (Peppas & Shalin, 1989).

Fig. 7 shows that this last equation renders a good fit to the experimental data (full line) in comparison with the pure Fickian model (Equation (1), dashed line), and similar to the single exponential model (inset Fig. 7). The relative values of the k_d and k_r parameters obtained ($1.3 \pm 0.2 \times 10^{-4}$ and $7.7 \pm 1.9 \times 10^{-6}$, respectively, $R^2 = 0.993$) indicates, however, that a diffusion mechanism is the main contributor to the vanillin release.

The release of vanillin to the headspace is connected to the concentration of free vanillin in solution through the solution-headspace partition of the flavor. In a previous work it was demonstrated that, when sensors signals of different samples with flavor increasing concentrations are analized by Principal Component Analysis (PCA), the recovered values of the main component PC1 are directly related to the intensity of aroma perceived by the sensors (Rodríguez et al., 2010). Following this procedure, the plot

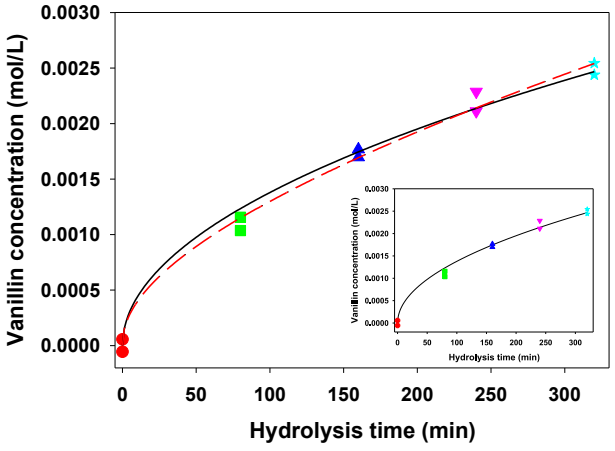

Fig. 7. Fitting curves of free vanillin concentration in solution released by enzymatic hydrolysis by a diffusional Fickian's dependence $C(t) = At + C(t=0)$(solid curve), or to a release kinetic involving solute–matrix interactions: $C(t) - C(t=0) = k_d\, t^{0.43} + k_r\, t^{0.85}$ (dashed curve). Inset: fit by the exponential model: $C(t) = kt^n + C(t=0)$. For further details of the parameters recovered see Section 3.1.

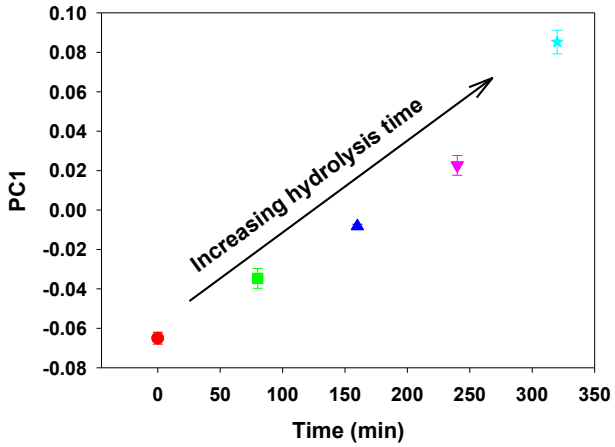

Fig. 8. Plot of the mean values of PC1 obtained by PCA on the time window 850–900 s for each e-nose measurement at each starch hydrolysis time (min): 0 (●); 80 (■); 160 (▲); 240 (▼); 320 (★).

of PC1 versus the hydrolysis time yields a curve shown in Fig. 8, which represents the development of the aroma in the headspace achieved through the expected vanillin solution-headspace partition equilibrium. The curve resembles the one developed for headspace concentration of isoamyl acetate in solutions of increasing sucrose concentrations (Friel, Linforth, & Taylor, 2000), suggesting the influence of sugars released by the starch hydrolysis in the partition profile of the flavor (Rabe, Krings, & Berger, 2003).

4. Conclusions

This study showed experimental evidence linking the hydrolysis of starch-vanillin samples containing amylose-vanillin inclusion complexes with the increasing concentration of free vanillin in solution and its release to the headspace. The change in the speed of enzymatic hydrolysis, from a fast to a slow process in less than 6 h, constitutes additional evidence of the formation of vanillin-amylose inclusion complexes. The vanillin release in solution was consistent with a coupling of diffusion and relaxation mechanism, whereas the release of the flavor to the headspace was influenced by the increasing concentration of sugar in solution due to the starch hydrolysis.

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