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Analytical Methods

Kinetic study on the Maillard reaction. Consideration of sugar reactivity

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ABSTRACT

The aim of the present study was to compare five reducing sugars (ribose, xylose, arabinose, glucose and fructose) with respect to their relative reactivity in the Maillard reaction (MR) (55 °C, pH 6.5) with a shrimp hydrolysate. For each system, the extent of the MR was assessed for a 24-h period by monitoring browning intensity, free amino group disappearance and sugar consumption. Results revealed the prevailing propensity of pentoses over hexoses to react in the MR, with a distinguishable behaviour for the ribose-hydrolysate system. Interestingly, pentoses were shown to mainly differ in terms of reaction rate. Complementary data on the chemical composition of the MR products (MRPs) were provided by size-exclusion chromatography, thus demonstrating the occurrence of some molecular rearrangements detected at 294 nm. The modification of protein substrates through the MR could represent a key step in the formation of new molecules and constitute a promising means to produce high value-added ingredients with biological and techno-functional properties.

1. Introduction

The monitoring of kinetic parameters of the Maillard reaction (MR) is of topical concern due to the importance attached to this famous chemical reaction, as attested by the numerous symposia recently held at a worldwide scale (Gerrard, 2006). Many research works are especially devoted to these mechanistic and kinetic aspects of the MR (Martins, Jongen, & van Boekel, 2001; van Boekel, 2001), and such fundamental studies find very concrete applications, either in the field of food science or in the medical sector. In agro-food industries, it is essential to control the MR because it is responsible for undesirable attributes (off-flavours, loss of nutritional values, generation of toxic compounds, etc.) which have to be limited, but also for desirable features (pleasant sensory characteristics, formation of biologically active molecules, etc.) which have, on the opposite, to be promoted. More generally, kinetic studies may be conducted for their predictive value on the characteristics of the products generated in the MR (Martins et al., 2001). In vivo, advanced glycation end products (AGEs) are supposed to be involved in a lot of pathologies and, more generally, in the ageing process (Thorpe & Baynes, 1996). Within this context, a better understanding of the MR progress will contribute to the development of new therapies designed to retard the formation of these reactive compounds (Booth, Khalifah, Todd, & Hudson, 1997).

The MR is one of the so-called "non-enzymatic browning reactions", which results from the condensation of an amino group and a carbonyl function. It was first discovered in 1912 (Maillard, 1912), and the main pathways of its chemistry are described in a detailed review (Hodge, 1953). Since then, new findings have continually enriched our knowledge on the MR, and a lot has still to be done.

The complexity of this chemical reaction lies in the fact that it is influenced by numerous factors, whose unique combination will each time lead to a new case study. Parameters of the MR include: nature, concentration and proportion of the reactants (amino and carbonyl compounds), water activity, heating time and temperature, pH, buffer type and concentration, presence of oxygen, light or metal ions (Ames, 1992). The regulation of all these factors is one of the means to control the MR progress.

Studying the effect of sugar type on the MR kinetics may be motivated by several reasons: (1) at first, as part of a mechanistic study, this will permit to bring new insights into the chemistry of the MR. (2) Then, such a study can be carried out for industrial applications and focus, for example, on the utilisation of rare sugars (Sun, Hayakawa, Chuamanochan, et al., 2006; Sun, Hayakawa, & Izumori, 2004; Sun, Hayakawa, Puangmanee, & Izumori, 2006). The authors revealed that D-allose and D-psicose, when reacted in the MR, could be valorised for their biological (antioxidant activity)

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or techno-functional (emulsifying ability, gelling properties, sweetening power, etc.) properties, and could represent an alternative to alimentary sugars. Investigations on the effect of sugar type can also influence the choice of the sugar to be incorporated into a food formulation. Criteria of this choice can be of two types: economic or technological. In the first case, one should attempt to replace expensive sugars by other cheaper ones without changing the resulting properties (Okazaki & Makino, 2004). In the case of technological expectations, the priority will rather be centred on the ability of a sugar to limit colour development of foods during storage (Kwak & Lim, 2004), or to promote flavour generation (Ames, Guy, & Kipping, 2001). A final argument could prompt industrialists to benefit from scientific studies on the influence of sugar: by artificially reproducing a particular food system, it is ultimately possible to establish a predictive modelling of the MR progress during storage or processing. For example, lactose can be used to simulate dairy systems (del Pilar Buera, Chirife, & Resnik, 1990) and ribose is often used as the carbonyl representative of meat (Mottram & Nobrega, 2002). (3) Finally, in the field of human health, considering the role of carbonyl compounds in the development of AGEs is of great importance because this contributes to gaining further knowledge on protein glycation in the body. Ultimately, such experimentations will help to elaborate new strategies in order to prevent from the adverse effects of the MR in vivo.

In the present study, the influence of sugar type on the extent of glycation in model MR systems was investigated. Three aldopentoses were selected (ribose as reference for its reactivity in the MR, and xylose and arabinose for comparison), as well as two hexoses (glucose and its ketose isomer fructose, as common alimentary sugar). As amino source, we chose a protein hydrolysate. Simple systems composed of a reducing sugar and an amino acid or a well-characterised protein have extensively been employed to model the MR. Conversely, reactions involving protein hydrolysates remain to be studied in detail. The second interest of using a shrimp hydrolysate is to take part in a topical challenge, that is "upgrading of seafood by-products". Indeed the shrimp hydrolysate was produced using by-products from shellfish processing. Until now, marine by-products have dramatically been underexploited, as they are mainly intended for fish meal and oil markets (Guérard, 2007). Hydrolysates constitute a novel established outlet for these remainders and their modification through the MR could additionally represent a key step in the formation of biological or functional compounds with high added value.

The sugar reactivity was assessed on the basis of classical kinetic parameters including browning development, decrease in available amino groups and sugar consumption. Moreover extra analytical data were brought through the chromatographic tool, which offers an original approach for the monitoring of the MR.

Beyond the presentation of experimental results on the MR kinetics, this paper also provides a critical analysis on the consideration of sugar reactivity in the MR, with constant regard to the practical interest of this work.

2. Materials and methods

2.1. Chemicals and reagents

The shrimp hydrolysate was supplied by Diana Naturals (Antrain, France). This brown concentrate was produced from by-products of northern shrimp (Pandalus borealis). It is characterised by a degree of hydrolysis (DH) of 55.2% (OPA assay) and its chemical composition (moisture, ash, protein, total amino acids) was determined (data not shown). D-glucose, D-fructose, D-arabinose, D-xylose and D-ribose, as well as *ortho*-phthaldialdehyde (OPA), sodium tetraborate, sodium dodecyl sulphate (SDS), β-mercaptoethanol, L-leucine, phenol and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Ethyl alcohol, sulphuric acid (96%), acetonitrile (HPLC grade), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) and di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) came from Carlo Erba (Val de Reuil, France). All chemicals and reagents used were of analytical grade.

All solutions and dilutions were performed using Milli-Q water (Millipore, Molsheim, France).

2.2. Preparation of sugar-hydrolysate Maillard reaction products

Maillard reaction products (MRPs) were prepared by dissolving the shrimp hydrolysate (12 mg/mL of protein, Kjeldahl equivalent) and one of the five selected sugars (30 mg/mL) in a 0.5 M phosphate buffer, pH 6.5. These parameters were selected in accordance with previous works (Sumaya-Martinez, 2004). The Maillard reaction took place at 55 °C in screw-capped glass tubes, which were placed in a temperature-controlled water bath equipped with a continuous stirring system (Julabo, Labortechnik GmbH, Seelbach, Germany). The solutions were heated for up to 24 hrs and samples were collected at 0, 6, 12, 17 and 24 h reaction. The MR was brutally stopped in an ice bath and the MRPs were allowed to stand at −4 °C until complete cooling. Aliquots were then stored at −20 °C for further uses.

Control experiments with the hydrolysate (12 mg/mL of protein) heated without any sugar, and with phosphate buffer heated alone, were also conducted.

2.3. Analyses

2.3.1. Measurement of browning

Browning intensities of MRPs samples were assessed by absorbance readings at 420 nm against water using a UV–Visible Unicam Helios β recording spectrophotometer (Unicam, Cambridge, UK).

2.3.2. Determination of free amino group content

Available amino groups were quantified by the OPA method (Church, Swaisgood, Porter, & Catignani, 1983). MRPs (50 μL, 10-fold dilution) were mixed with 1 mL of OPA reagent. After vortexing and a minimal 2 min delay in the dark at room temperature, the absorbance at 340 nm was recorded against OPA reagent.

Appropriate blanks were realised to correct for the 340 nm-absorbance specifically due to the intrinsic properties of the MRPs.

Absorbance readings were converted into free amino contents (in mM leucine equivalent) using a calibration curve obtained with L-leucine (0–5 mM) as a standard. Data were finally expressed as relative concentrations (%) in comparison with the content of non-heated samples (before MR).

2.3.3. Determination of reducing sugar

Reducing sugar content in the sample was determined according to the phenol-sulphuric acid reaction (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Briefly, to 200 μL of each sample tested (500-fold dilution), 200 μL of a phenol solution (2% w/v for arabinose and xylose, 10% for glucose and ribose, and 20% for fructose) and 1 mL of a concentrated (96%) sulphuric acid solution, were added. Mixtures were vortexed, incubated in boiling water (5 min at 100 °C) and cooled on ice for 10 min. For each sugar tested, we plotted absorbance versus wavelength (absorption spectrum) in order to determine the corresponding absorption maximum. Absorbance was read at a wavelength of 480 nm for pentoses, 485 nm for fructose and 490 nm for glucose by using a microplate reader (SpectraMax Plus^{384}, Molecular Devices, Sunnyvale, CA).

Appropriate blanks were run to correct for the absorbance of the MRPs themselves.

The absorbance value obtained for each sample test was referred to a standard curve prepared by using the specific sugar under examination (0–100 μg/mL), and was therefore converted into sugar concentrations (in mg/mL). Changes in reducing sugar were expressed as relative concentrations (%) in comparison with the original sugar content in non-heated samples.

2.3.4. Molecular weight (MW) measurement

The MW distribution profiles of the MRPs samples were assessed by fast protein size-exclusion liquid chromatography (SEC-FPLC) using a Superdex™ Peptide 10/300 GL column (Amersham Biosciences; fractionation range: 7000–100 Da) (Guérard, Dufossé, De La Broise, & Binet, 2001). The liquid chromatographic system consisted in a Waters 600 automated gradient controller pump, a Waters 717 plus autosampler and a Waters 996 photodiode array detector (Waters, Milford, MA, USA). Data acquisition and chromatographic analyses were driven by the Empower software™ program. Thirty microliters of MRPs (sterilised through 0.22 μm filters) were injected onto the column, and elution was carried out isocratically in Milli-Q water with TFA 0.1% and acetonitrile (70:30) at a flow rate of 0.5 mL/min. Nine standards were used for the calibration ($\log MW = 0.0985\ R_t + 5.7326$ with R_t = retention time, expressed in minutes): aprotinin (6511.4 Da), insulin chain B (3495.9 Da), insulin chain A (2531.6 Da), neurotensin (1672.9 Da), substance P (1347. 6 Da), luteinizing hormone releasing hormone (1182.3 Da), substance P fragment 1–7 (900 Da), leupeptin (463 Da), and glycine (75 Da). Chromatograms were recorded by UV-detection at 220 nm (detection of peptide bond rearrangements) and 294 nm (detection of intermediate MRPs), and area under the curve (AUC) was obtained for each chromatogram.

2.4. Statistical analyses

All values are the mean of at least three repetitions. Comparison (one-way analysis of variance (ANOVA) followed by Turkey's multiple range test) and correlation (simple regression) tests were performed using Statgraphics®Plus 4.0 software. Confidence levels were set at 95%, and statistical differences were considered as significant for $P < 0.05$.

3. Results and discussion

3.1. Changes in browning intensity

Brown colour development (A 420 nm) is the easiest measurable consequence of the MR as it may be estimated visually. Its intensity is often used as an indicator of the extent to which the MR took place in foods and it symbolizes the advanced stage of the MR.

Fig. 1A depicts the time course of browning as a function of heating time. When the shrimp hydrolysate was heated alone (control), no increase in browning could be detected. Concerning systems involving monosaccharides, browning gradually increased over heating time. This tendency was however less marked for glucose- and fructose-hydrolysate MRPs. Induction periods differed according to the type of sugar, and were shorter with systems containing pentoses, reflecting their higher propensity to produce the precursors of melanoidins (van Boekel, 2001). The production of coloured MRPs did not follow the same tendency as that described by other authors, who noticed a sharp increase in absorbance at 420 nm within the early phase of the reaction, accompanied with a subsequent plateau phase. In such studies, the reaction rate

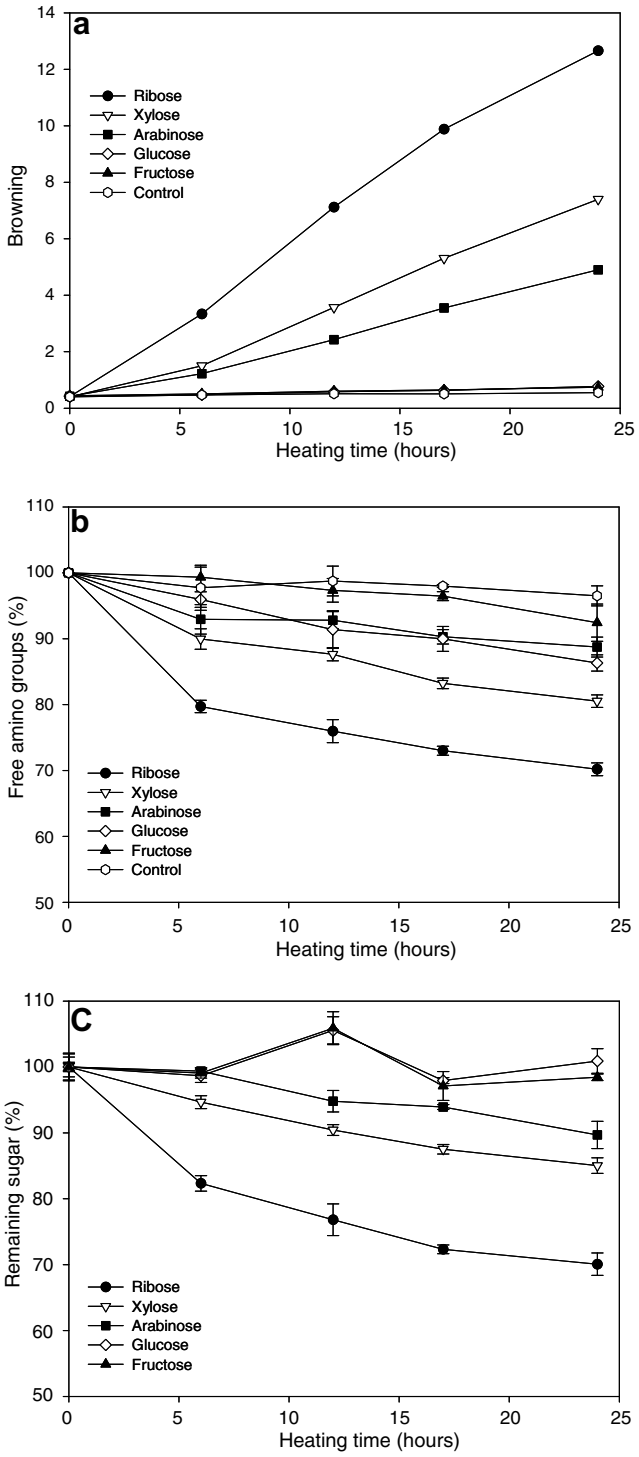


Fig. 1. Browning development (expressed as absorbance at 420 nm × dilution factor) (A) and disappearance of MR reactants on a 24-h heating period (55 °C, phosphate buffer pH 6.5): free amino group mobilisation (B) and reducing sugar consumption (C) for MRPs derived from fructose-, glucose-, arabinose-, xylose- and ribose-hydrolysate systems. The control curve corresponds to the hydrolysate heated without any sugar.

was probably accelerated with the high temperature used (100 °C at pH 8, 9, 10, 11 or 12, and for 0, 2, 4, 6 and 8 h) (Lertittikul, Benjakul, & Tanaka, 2007) or may be due to the prolonged heating time at 55 °C (up to 28 days) (Jing & Kitts, 2002).

On the whole, the browning development was favoured with pentoses (ribose, xylose and arabinose, successively) compared to

hexoses (glucose and fructose). For example, at the end of the heating period, ribose yielded MRPs with a 16-fold higher browning intensity compared to glucose. The order of reactivity according to which aldoses are more reactive than ketoses, and pentoses are more reactive than hexoses in the browning development is well stated and was supported in many studies (Jing & Kitts, 2002, 2004; Kwak & Lim, 2004; Yen, Tsai, & Lii, 1992). The relative browning degree due to fructose and glucose is however being discussed. Under the present experimental conditions, we could not significantly distinguish between their respective browning ($P > 0.05$). On the contrary, some authors found a greater contribution of fructose in the browning compared to glucose (Benjakul, Lertittikul, & Bauer, 2005; Sun et al., 2004). These discrepancies were frequently ascribed to the higher participation of ketoses to browning via caramelisation compared to aldoses (Jing & Kitts, 2002, 2004). In the present case browning indices can not be attributed to the sugar caramelisation because sugars heated alone failed to generate compounds absorbing at 420 nm (data not shown). This was not surprising given the fact that caramelisation mainly occurs at very high temperatures and at alkaline pH values (Ajandouz, Tchiakpe, Dalle Ore, Benajiba, & Puigserver, 2001) and not under the undenaturing mild conditions of this experiment (55 °C, pH 6.5).

Such kinetic studies assessing the relative contribution of sugars in the non-enzymatic browning have to be related to industrial applications. Sometimes, a sugar may be selected for its high browning ability in the MR. For instance, in the manufacturing process of chocolate, beer or bread products, brown colour is required because it influences consumer acceptability. In the cosmetic industry, the ketose dihydroxyacetone is often present as active ingredient in sunless tanning lotions due to its propensity to react with skin protein through the MR (Petersen, Wulf, Gniadecki, & Gajkowska, 2004). On the contrary, sugars such as glucose or fructose may be chosen for their weak participation in browning development, for example to prevent from the undesirable yellowing process affecting a wide range of dried milk products during storage.

3.2. Changes in free amino group content

At an early stage of the MR, terminal α-amino groups of peptides and ε-NH_2 groups of lysine react with the carbonyl function of reducing sugars present in the reaction medium. Thus, the loss of available primary amino groups is another indicator used to compare the sugar reactivity in the MR (Ajandouz et al., 2001; Benjakul et al., 2005; Brands & van Boekel, 2002; Chevalier, Chobert, Mollé, & Haertlé, 2001; Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; Lertittikul et al., 2007; Naranjo, Malec, & Vigo, 1998; Olivier, Melton, & Stanley, 2006; Sun et al., 2004; Sun, Hayakawa, Chuamanochan, et al., 2006; Sun, Hayakawa, Puangmanee, et al., 2006). To this end, the OPA method presents several advantages, especially this one to be free of sugar interferences when determined by spectrofluorimetric methods (Rufian-Henares, Guerra-Hernández, & García-Villanova, 2002).

Fig. 1B outlines the consumption of free amino groups throughout the 24 h of MR. In the case of the hydrolysate heated alone (control), the slight decrease of 3.5% is not significant. For all other systems involving sugars, the number of free amino groups continuously decreased upon heating time ($P < 0.05$). This suggested that α- and ε-amino functions brought by amino acids and peptides were progressively bound to the carbonyl moiety when the heating time increased. The strength of this linkage may be influenced by the sugar reactivity. In β-lactoglobulin-sugar systems (60 °C for 72 h), reactive sugars such as ribose and arabinose have been shown to induce sugar-dependent covalent bounds, whereas MRPs from less reactive sugars (glucose, galactose and lactose) were rather stabilised by non-covalent hydrophobic interactions and by covalent disulfide bonds (Chevalier, Chobert, Mollé, et al., 2001).

Regarding the disappearance of available amino groups, the reactivity of sugars in the MR was declining in the following order: ribose > xylose > arabinose ≈ glucose > fructose. This classification globally confirmed the results obtained with the browning evaluation previously described (Fig. 1A). When assessing the disappearance of available lysine residues, Olivier et al. (2006) showed that the sugar reactivity towards caseinate was faster with glucose, followed by fructose and lactose. The considerable influence of temperature on the disappearance rate of available lysine was also underlined. For instance, for fructose introduced at a 1:5 ratio with regard to the amino:carbonyl ratio and heated for 48 h in presence of caseinate, the remaining lysine changed from about 60% at 55 °C to 35% at 60 °C and to 10% at 65 °C. This may explain the relative inertia of fructose and glucose systems in the present study, conducted at 55 °C.

The tendency of the decreasing curve corresponding to the ribose-hydrolysate system was singularly characterised by a major amino consumption within the first 6 h of MR, subsequently followed by a steady state. This steady state can be defined as a situation where the net sugar content in the system remains constant. Conversely, less reactive sugars such as glucose and fructose displayed a progressive decrease during the entire reaction. The "no-loss period" particularly marked with ribose is traditionally explained by the propensity of amino compounds to be recycled, especially from the degradation of Amadori compounds (van Boekel, 2001).

After 24 h of heat treatment, 70.2%, 80.5%, 86.3%, 88.7% and 92.4% of free amino groups remained for ribose, xylose, glucose, arabinose and fructose MR systems, respectively. It has to be stressed that only 29.8% of the initial free amino groups had at best reacted with the most reactive sugar. Similarly, such low levels were reached in some studies (Benjakul et al., 2005). On the other hand, other authors managed to reach greater extents of amino consumption (Chevalier, Chobert, Mollé, et al., 2001; Chevalier, Chobert, Popineau, et al., 2001; Lertittikul et al., 2007; Sun et al., 2004; Sun, Hayakawa, Chuamanochan, et al., 2006; Sun, Hayakawa, Puangmanee, et al., 2006), which could even go up to a complete mobilisation of amino functions (Olivier et al., 2006). The discrepancies found in the literature concerning the different levels of amino groups finally reached at the plateau phase could be explained by the diversity of conditions used to conduct the MR. The reactant losses have been shown to strongly depend on pH (Ajandouz et al., 2001; Brands & van Boekel, 2002; Lertittikul et al., 2007), heating time (Sun, Hayakawa, Chuamanochan, et al., 2006), temperature (Brands & van Boekel, 2002; Naranjo et al., 1998; Olivier et al., 2006), and moisture parameters (Olivier et al., 2006; Sun et al., 2004; Sun, Hayakawa, Chuamanochan, et al., 2006; Sun, Hayakawa, Puangmanee, et al., 2006).

In addition, the weak level of free amino group consumption observed in this study could be related to the specific use of a hydrolysate as amino source. Peptide mixes bring a lot of reactive groups, coming not only from ε-amino groups of lysyl residues, but also from the large number of α-amino groups associated with the presence of peptides. The situation is completely different when a protein source is employed. In this second case, the initial level of free amino groups is expected to be lower. As a consequence, the original pool of available amino functions is easily mobilised and decreases at a faster rate. In the present study, the large number of reactive amino functions makes the complete mobilisation more difficult, so that only 30% of mobilisation is reached at the end of the reaction.

When dealing with a hydrolysate, the DH has also to be taken into account. The same reactions conducted with a less hydrolysed sample (DH = 41.4%) provided molecules modified at a lesser

extent (data not shown). This result illustrates the interest of using hydrolysates as a substrate for the MR, as a rich source of NH_2-groups.

Chemical analyses revealed that the shrimp hydrolysate is composed of 3.40% of lysine (6.28% of the total protein content quantified by the Kjeldahl method), which ranks it as the fifth richest amino acid (data not shown). The amount of lysine has also to be considered because this amino acid is highly reactive in the MR, and may contribute to the global reactivity of this hydrolysate in the MR.

3.3. Loss of sugar

The monitoring of sugar consumption is another means to evaluate the degree of reactivity of sugars in the MR (Ajandouz et al., 2001; Brands & van Boekel, 2002; Kwak & Lim, 2004; Lertittikul et al., 2007).

According to Fig. 1C, ribose and, to a lesser extent, xylose systems underwent a progressive noticeable sugar consumption with increasing heating time ($P < 0.05$). The slight decrease observed with arabinose is not significant. It has to be stressed that for systems with hexoses, a slight increase at 12 h of heating could be perceived. These aberrant values were however not statistically significant, so that fructose and glucose amounts could be considered as constant during the course of the 24 h reaction. A control without any sugar proved that this increase could not be attributed to a possible liberation of carbonyl functions provided by the shrimp substrate (data not shown). This artefact has more probably to be linked to the methodology used.

Among all of the sugars tested, ribose was the most active. As shown when studying changes in free amino groups, most of its disappearance occurred within the first 6 h of reaction. A no-loss period was subsequently observed, reflecting that reactants were less available for the reaction with amino groups, and that the reaction rate slowed down. At the steady state, the remaining ribose proportion finally levelled 70%. Kwak and Lim (2004) revealed that a 12-h heating period led to a maximal 30% of remaining glucose when reacted with different amino acids. However, the conditions of temperature (100 °C) used in the latter study probably allowed the MR to progress to a more advanced stage. In the present study, the mild conditions did not enable the sugar to be totally consumed.

The sugar reactivity regarding the sugar consumption was in the declining order: ribose > xylose > arabinose > glucose ≈ fructose. These results further confirm the relative reactivity established when following amine mobilisation and browning development (Fig. 1A and B).

To study the relationship between amino and carbonyl disappearance, the correlation coefficient (R^2) was calculated for each sugar-hydrolysate system. The highest positive correlations were obtained with pentose-hydrolysate systems (R^2 of 0.53, 0.87 and 0.90 with arabinose, xylose and ribose MR systems, respectively). From the results, it can be assumed that both amino and carbonyl stocks were consumed at the same rate, and that there was no limiting reactant. In the literature, it is generally assumed that sugars react at a faster rate than amino compounds in the MR. This is ascribed to the fact that sugars may be used to other ends that the MR (caramelisation, sugar fragmentation), and that amino parts can on the contrary be recycled. In our case, these parallel reactions could be neglected, which could justify the similar disappearance rate for both reactants.

We initially introduced 0.20 M of pentose and 0.05 M of free amino groups (OPA assay), corresponding to a 4:1 ratio of carbonyl to amino groups. As both carbonyl and amino functions react at the same rate, as they are thought to be mainly involved in the MR (no parallel reaction), and given the 4:1 molar ratio initially incorporated, we can deduce that about four molecules of sugar reacted per NH_2 residue carried by the hydrolysate. Finally, we can presume that in the ultimate formation of melanoidins four moles of sugar were incorporated per mole of amino function. Similarly, the ideal ratio required in the MR is discussed in several studies (Cämmerer & Kroh, 1995; Olivier et al., 2006).

3.4. Evaluation of molecular rearrangements in the MRPs

According to general principles of kinetics, the extent of any reaction can be evaluated either by recording the loss of reactants, or by monitoring the formation of reaction products, as described in the following paragraph.

Evidence of molecular rearrangements is brought through the AUCs calculated from the elution profiles of MR mixtures separated by SEC-FPLC. Changes in absorbance at 294 nm were detected as a measurement of intermediate products of the MR, as reported by other authors (Ajandouz et al., 2001; Benjakul et al., 2005; Lertittikul et al., 2007) (Fig. 2A). Similarly, peptide bonds in the native and sugar-modified hydrolysate were assessed at the wavelength of 220 nm (Fig. 2B). In the field of protein hydrolysates, the use of this absorbance value is commonly admitted to detect peptide bonds (see Chevalier, Chobert, Mollé, et al., 2001, among others).

Both graphs corroborate the order of sugar reactivity previously established and again denote the prevailing reactivity of ribose, as

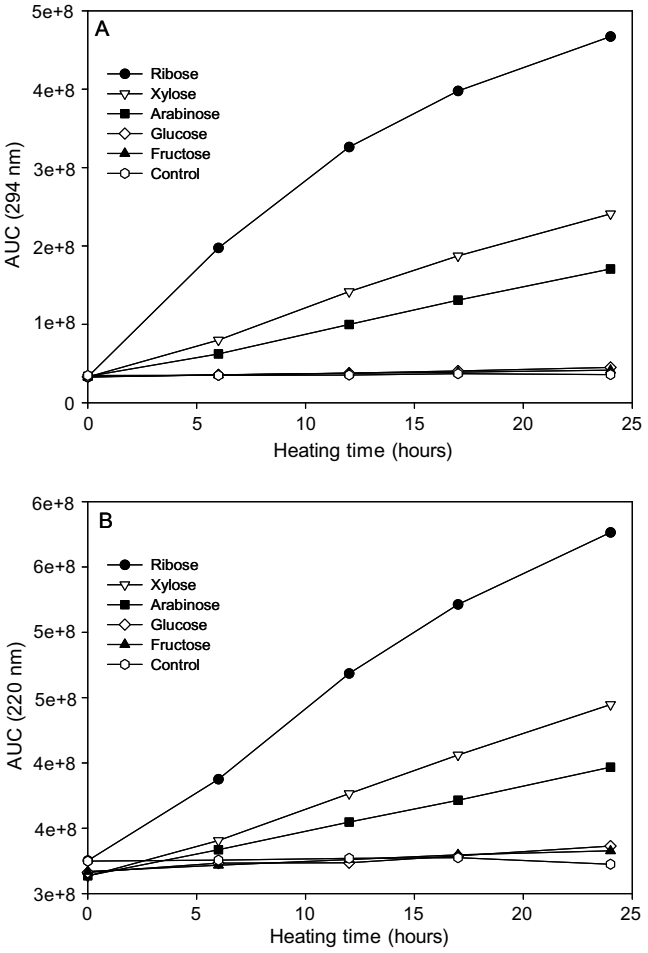


Fig. 2. Evolution of the AUCs relative to chromatograms recorded at 294 nm (A) and 220 nm (B) for the fructose-, glucose-, arabinose-, xylose- and ribose-hydrolysate MRPs heated during 24 h (55 °C, phosphate buffer pH 6.5). The control curve corresponds to the AUC of the hydrolysate heated alone.

assessed by greater increases in AUCs. Intermediate MRPs were continuously formed with increasing heating time. In the less reactive sugar systems, the amount of intermediate products followed a linear accumulation, while in the most reactive ribose system, MRPs were rather produced according to a hyperbolic tendency. The same behaviour was actually observed in other studies (Ajandouz et al., 2001; Lertittikul et al., 2007), where it was shown that at favourable pH values, absorbance at 294 nm sharply increased and finally stagnated, while at unfavourable pH values, it was less pronounced and fitted a zero-order reaction. It can be specified that the integrated rate law associated with a zero-order reaction is:

$$C = C_0 + kt$$

where C represents the concentration of the reactant of interest at a particular time and C_0 its initial concentration, k is the reaction rate coefficient, and t is time (van Boekel, 2001). More concretely and according to this equation, when absorbance readings at 294 nm are described by a zero-order reaction, this means that the change in intermediate MRPs yields a linear plot versus reaction time. Conversely, when the production of intermediate MRPs increases in the course of the MR and finally reaches a plateau phase, the reaction is called "first order" with respect to intermediate MRPs.

Changes in absorbance at 294 nm present the same pattern as browning development (Fig. 1A). From this result, it may be supposed that a large amount of intermediate UV-absorbing compounds were converted into brown products of the MR.

Intermediate products are typically characterised by a rise and fall kinetics (van Boekel 2001). However, no maximum was reached in our case. The plateau phase is the result of a decrease in the apparent rate of production of intermediate MRPs and is the sign that intermediate products were produced at the same rate as they were consumed. If the reaction had been conducted to a greater extent, the classical appearance–disappearance behaviour of intermediate products might have been observed, as shown by Ajandouz et al. (2001) when studying heated fructose solutions.

Numerous attempts have been made to further clarify the characteristics of MRPs and to discern differences stemming from the use of different sugars. The most frequently adopted method to reach this goal consists in assessing the degree of modification of the amino source. This may be carried out by means of analytical tools such as elemental analyses (Cämmerer & Kroh, 1995; Jing & Kitts, 2004), electrophoresis (Chevalier, Chobert, Mollé, et al., 2001; Olivier et al., 2006; Sun et al., 2004; Sun, Hayakawa, Chuamanochan, et al., 2006), mass spectrometry (MS) (Ames et al., 2001; Chevalier, Chobert, Mollé, et al., 2001; Jing & Kitts, 2004; Mottram & Nobrega, 2002; Sun, Hayakawa, Puangmanee, et al., 2006) and nuclear magnetic resonance (NMR) (Olivier et al., 2006).

However, comparison of chromatographic patterns obtained from different sugar-hydrolysate systems has, to our knowledge, never been used to assess the relative reactivity of different carbonyl sources in the MR. One exception could be identified (Chevalier, Chobert, Mollé, et al., 2001), but the amino substrate was β-lactoglobulin, a well-characterized protein, and not a complex mix of peptides. Furthermore, elution profiles obtained were not fully conclusive because of interferences due to the physicochemical characteristics of the compounds.

In the present study, SEC-FPLC was used to give information on the size of MRPs affected by molecular rearrangements. This is an original approach to characterise the composition of MR mixes in the course of the incubation period and to differentiate sugars in terms of reactivity in the MR. In Fig. 3, the evolution of chromatographic profiles at 294 and 220 nm is presented for the most reactive system (ribose-hydrolysate MRPs).

During the course of the MR, AUCs progressively increased but finally stagnated, expressing that the reaction rate tended to a plateau phase (as detailed above from Fig. 2). Concerning the detection at 220 nm (Fig. 3B), similar shapes were conserved and the same peaks were eluted for all of the chromatograms. This means that the complex mixes produced during the MR mainly differed in quantitative terms. However, this observation does not dismiss the hypothesis whereby new compounds detected at 220 nm can also be produced in the course of the MR (qualitative aspects). On the contrary, when comparing the relative proportion of compounds by MW in chromatograms recorded at 294 nm (Fig. 3A), one can not attest that the composition remained unchanged. As soon as the sugar-hydrolysate system was incubated at 55 °C, a major peak at 294 nm appeared (37.3 min retention time), reflecting the marked production of small aromatic intermediate MRPs. More than the three quarters of molecular rearrangements affecting intermediate MRPs occurred for compounds of MW lower than 500 Da. Conversely, rearrangements in peptide bonds also affected products with higher MW (up to 3000 Da).

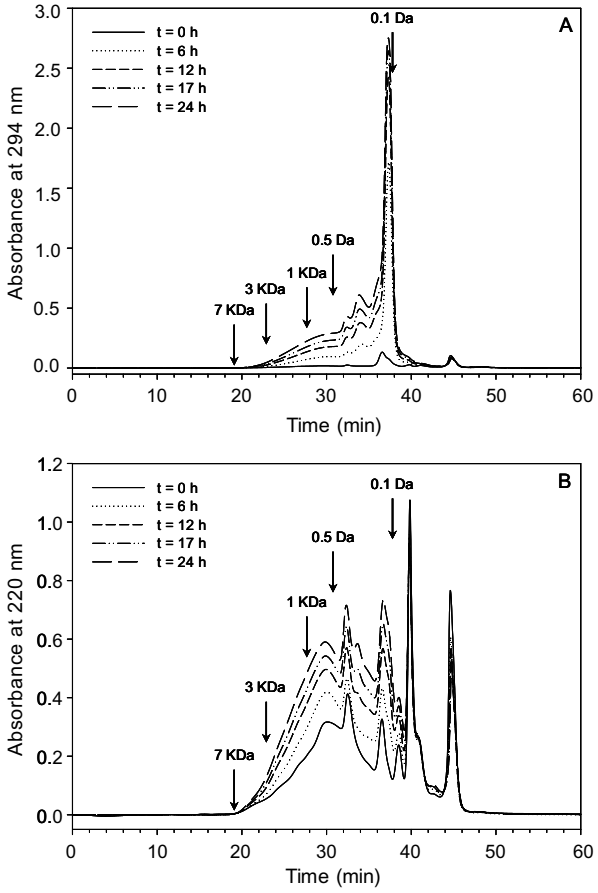


Fig. 3. Chromatograms at 294 nm (A) and 220 nm (B) for the MRPs produced from the ribose-hydrolysate system heated for 0, 6, 12, 17 and 24 h (55 °C, phosphate buffer pH 6.5).

In Fig. 4, sugar reactivity was compared on the basis of chromatographic data. After 24 h of incubation at 55 °C, glucose- and fructose-hydrolysate MRPs exhibited profiles quite similar to that of the control (heated hydrolysate without any sugar). This observation supports the weak reactivity of hexoses compared to pentoses. Concerning aldopentoses, arabinose was less reactive than xylose, which was itself less reactive than ribose. The order of reactivity between all of the sugars tested is thus confirmed by the chromatographic tool.

When analysing more precisely the behaviour of each aldopentose, it is noteworthy to see that they only differed by their reaction rate in the MR. Insets A′ and B′ in Fig. 4 evidence the perfect

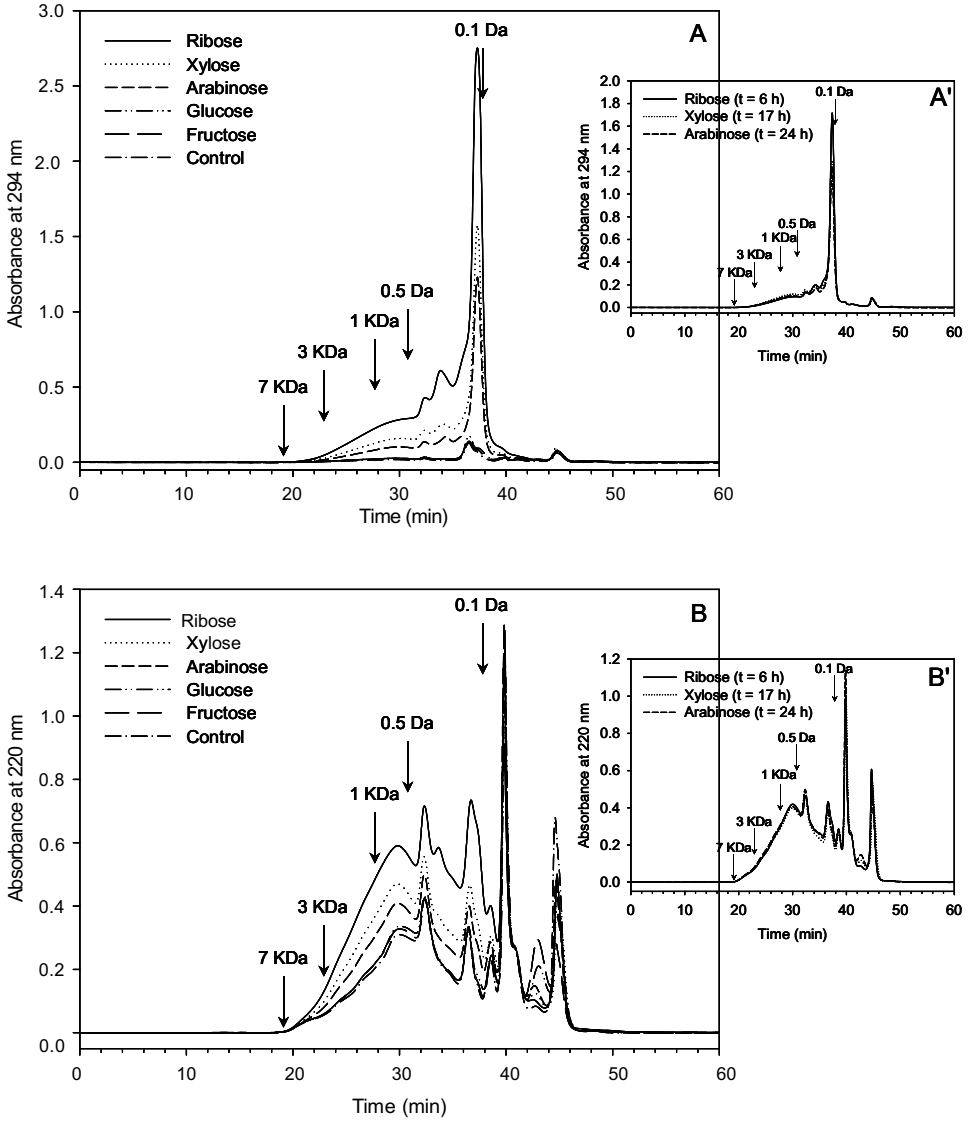


Fig. 4. Chromatograms at 294 nm (A) and 220 nm (B) for fructose-, glucose-, arabinose-, xylose- and ribose-hydrolysate MRPs after 24 h of heating (55 °C, phosphate buffer). The control chromatogram corresponds to the hydrolysate heated alone. Inserts underline the superimposition of chromatograms at 294 nm (A') and 220 nm (B') for ribose-, xylose- and arabinose-hydrolysate systems heated for 6, 17 and 24 h, respectively.

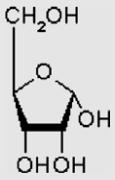
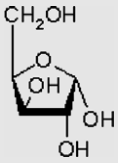
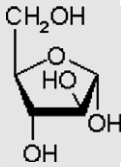
superimposition of chromatograms related to ribose-, xylose- and arabinose-hydrolysate systems heated for 6, 17 and 24 h, respectively. These interesting findings, also confirmed by data listed in Table 1, clearly reveal that the same results may be expected with different aldopentoses incubated for longer or shorter time according to their propensity in the MR. This suggests that one of these adopentoses could easily be replaced by another one (for financial reasons, for example) by adjusting the reaction time as a compensation for the difference in reactivity.

3.5. Discussion on the relative reactivity of sugars in the MR

The tendency whereby the relative reactivity of different sugars in the MR follows in the descending order pentoses > hexoses > disaccharides and aldoses > ketoses is well recognised, whatever the method used to measure the degree of reactivity (monitoring of kinetic parameters, evaluation of the biological or techno-functional properties of MRPs, polarographic and electrochemical data). Many arguments have been put forward to bring new insights into the chemical causes of the varying MR rates observed according to the sugar used. It is generally admitted that the sugar reactivity has directly to be related to the proportion of acyclic form in solution, known as the active carbonyl species in solution (Bunn & Higgins, 1981). But this argument does not justify the totality of the observed reactivity. In Table 2, a collection of arguments which have been supposed to explain the established order of reactivity among sugars, is provided. In the following part, attempts are made to interpret the different reaction rates observed with the five sugars selected in the present study.

The greater reactivity of aldoses over ketoses is extensively justified (Table 2). In the literature, conflicting reports have however been published concerning the relative reactivity of fructose (ketohexose) and glucose (aldohexose). Whereas some authors mentioned the higher reactivity of glucose over fructose (Jing & Kitts, 2002, 2004; Kwak & Lim, 2004; Naranjo et al., 1998; Olivier et al., 2006; Sun, Hayakawa, Puangmanee, et al., 2006; Yen et al., 1992), the contrary was sometimes proved by others (Wijewickreme & Kitts, 1997). These controversial results are largely attributable to the diversity of reaction conditions chosen for the MR (reactants, temperature, pH, salts, buffer, etc.) and to the diversity

Table 1
Approximate reaction times (h) required to reach equal reactivity in browning, sugar, amino and AUC levels for each aldopentose-hydrolysate MR system (data obtained from Figs. 1 and 2)

Reducing sugar	Ribose	Xylose	Arabinose
			
Browning degree = 2	3	7	9
Residual sugar (%) = 90	3	11	21.5
Free amino groups (%) = 91	3	6	17
AUC 294 nm ~ 115 10^6 AU[a]	3	9.5	14.5
AUC 220 nm ~ 355 10^6 AU[a]	3	8.5	12.5

[a] Arbitrary unit.

of indicators monitored to evaluate the MR progress, which finally makes studies difficult to compare.

In the present study, glucose and fructose could not be significantly distinguished when considering browning development and sugar consumption ($P > 0.05$). On the other hand, amine mobilisation occurred significantly faster with the glucose MR system ($P > 0.05$). Fructose is characterised by a much less accessible carbonyl function, which is probably the main explanation why it is the less reactive sugar. Furthermore, at the temperature used in the present study, the caramelisation process, which would have led to an overestimation of browning measurements with fructose system, is not likely to occur. It is also assumed that aldoses and ketoses diverge in their reactivity because of a different reaction mechanism (Brands & van Boekel, 2002; Naranjo et al., 1998). On this basis, it could be suggested that the glucose-hydrolysate system yielded more reactive or more abundant products, leading to a faster production of MRPs compared to the fructose MR system.

Pentoses are generally recognised as more reactive than hexoses in the MR, as supported by numerous studies (Table 2). In agreement with these results, we ourselves showed that arabinose, xylose and ribose were much more reactive compared to fructose and glucose.

If differences of reactivity between aldoses and ketoses, or between pentoses and hexoses have partly been explained, far less information is available on the compared reactivity within a same class of sugars (aldopentoses, aldohexoses, ketopentoses, ketohexoses, etc.). Moreover, most of the studies designed to elucidate this question are focused on aldohexoses, and not on aldopentoses. Contrary to sugars belonging to different classes (aldoses versus ketoses, pentoses versus hexoses, monosaccharides versus disaccharides), which follow different reaction routes and whose reaction products are thought to diverge according to their nature or to their amount, sugars of the same class would rather act towards the same mechanism and differ by their reaction rate (Brands & van Boekel, 2002).

As a whole, explanations seem to be founded on the configuration of hydroxyl groups in the sugar. This structural property then determines the form adopted by the sugar (and by the resulting MRPs) in solution (open/ring form, furanose/pyranose cycle, *cis*/*trans* conformation, zig-zag conformation/gauche C–C interactions, etc.), as well as equilibrium reactions (isomerisation including aldolisation, enolisation, cyclization and ring-opening, mutarotation, etc.). More generally, the initial configuration of OH groups determines the sugar stability in solution and particularly the proportion of acyclic form, which ultimately conditions the sugar reactivity.

In our research work, the reactivity of three aldopentoses in the MR was compared. Results showed that ribose was by far the most reactive, followed by xylose and, finally, arabinose. Ribose is well recognised for its high propensity to react in the MR, and this is usually ascribed to the high proportion of its acyclic form. The acyclic form of sugars in aqueous solution at 20 °C has been estimated to reach 0.03% for D-arabinose, 0.02% for D-xylose and 0.05% for D-ribose (Hayward & Angyal, 1977). If we assume that the reaction rate only depends on the percentage of carbonyl form, then arabinose should be more reactive than xylose, which is not confirmed in the present work. However, the relative proportion of acyclic form strongly depends on temperature (Hayward & Angyal, 1977), and at 55 °C, these figures are not valid anymore.

Other criteria than the relative proportion of acyclic form have therefore to be considered. Especially, the sugar stability is of great importance. Among aldopentoses, the different reactivity between ribose and xylose has partly been explained by the relatively less stable pyranose form for ribose compared to xylose (Hayward & Angyal, 1977). As reported by Burton and McWeeny (1963), xylose was even considered to be the most stable sugar among pentoses. Thus, the higher reactivity of ribose compared to xylose, and of xylose compared to arabinose may also be the consequence of a weaker stability of the ring form, combined with a greater stability of the linear form. A lower rate of interconversion between the cyclic and the acyclic form could also be suggested for arabinose, as this constitutes a limiting factor for the MR progress.

Lastly, configuration of hydroxyl groups in the sugar has to be considered as well. Structurally, ribose only differs from xylose by its C-3 hydroxyl group (epimers about C-3), and from arabinose by its C-2 hydroxyl group (epimers about C-2) (see Haworth representations in Table 1). From this statement, it can then be deduced that the OH group about C-3 is more important than the OH group about C-2 for the sugar reactivity in the MR, xylose being more reactive than arabinose. For ribose, OH groups about C-2, C-3 and C-4 are on the same side. It can be supposed that this position creates tension within the molecule, which destabilises the cycle and forces it to open, thus favouring the reactive form of the sugar in the MR. On the other hand, C-2 and C-3 OH groups of xylose and all of the OH groups in arabinose are placed at the opposite, which increasingly favours stability in the hemiacetal ring.

In a recent study, the relative reactivity of seven aldohexoses was investigated, by reaction with ovalbumin (Sun, Hayakawa, Chuamanochan, et al., 2006). Aldohexoses could be classified in pairs, regarding the configuration of the C-2 hydroxyl group. Results showed that carbonyl analogues concerning the C-3 and C-4 positions, only differing by their C-2 hydroxyl group (called

Table 2
Reasons for the relative reactivity of sugars in the Maillard reaction

Nature of the cause	Hypothesis	Consequence	Source[a]
Aldoses > ketoses			
Structure	Terminal aldehydic function more accessible than the keto group	Less steric hindrance for aldoses, which favours the interaction between the carbonyl and amino functions	Jing & Kitts (2002)
Chemistry	Higher electrophilicity of the aldehyde carbonyl group compared to the ketone group	Facilitation of the nucleophilic attack, in the first step of the MR	Bunn & Higgins (1981), Naranjo et al. (1998), Benjakul et al. (2005)
Mechanism and energy	Ketoses not active by themselves, rather act through their degradation products	Necessity of preliminary steps before ketoses can react, whereas aldoses can immediately react	Brands & van Boekel (2002)
	Different mode of tautomerisation for aldoses and ketoses: interconversion of aldoses through their cyclic pyranose form (simple mutarotation) / interconversion of ketoses through their cyclic furanose form (more complex mechanism)	Higher activation energies required for ketoses before they can really be involved in the MR	Naranjo et al. (1998)
	Faster browning rate for Amadori compounds, produced from aldoses, compared to Heyns products, produced from ketoses	More reactive reaction products generated from aldoses	Kwak & Lim (2004)
	Different thermal fragmentation abilities of aldoses when compared to ketoses	Differences in the thermal fragmentation abilities induce differences in the pathway followed in the early stage of the MR (ionic or radical)	Cämmerer & Kroh (1995)
Particular case: fructose (ketose) > glucose (aldose)			
Form of the sugar in solution	Higher proportion of open-chain form for fructose compared to glucose	Greater proportion of active form of fructose in the MR	Naranjo et al. (1998), Hayward & Angyal (1977), Benjakul et al. (2005)
Parallel reactions	Caramelisation, one of the contributor of non-enzymatic browning, more likely to occur from ketoses than from aldoses	Possible overestimation of browning development exclusively due to the MR with ketose sugars	Jing & Kitts (2002), Ajandouz et al. (2001), Benjakul et al. (2005)
Reaction parameters	Various reaction conditions, which makes different studies difficult to compare	Diverging results concerning the relative reactivity of fructose and glucose, fructose being sometimes more reactive, depending on the reaction conditions	Jing & Kitts (2002), Naranjo et al. (1998), Benjakul et al. (2005), Brands & van Boekel (2002)
Properties and nature of the reaction products	Higher reactivity of Heyns products, resulting from the glycation with ketoses, which facilitates a faster conversion into fluorophores compared to ketonic groups present in Amadori products	More reactive and more numerous reaction products generated from ketones	Wijewickreme & Kitts (1997)
	Formation of rate-limiting products in the case of glucose and not in the case of fructose systems		Ajandouz et al. (2001)
	Multiple Heyns products formed from fructose, while only one Amadori compound generated from glucose		Ajandouz et al. (2001)
Pentoses > hexoses			
Structure	Shorter hydrocarbon chain for pentoses, bulkier skeleton for hexoses	Less steric hindrance for pentoses, so that the reaction with amino functions is promoted	Jing & Kitts (2004)
Form of the sugar in solution/ stability	Higher proportion of open-chain form for pentoses compared to hexoses	Greater proportion of active form for pentoses	Chevalier, Chobert, Mollé et al. (2001), Hayward & Angyal (1977)
	Higher proportion of furanose ring in solution for aldopentoses compared to their homomorphous aldohexoses (whose cyclization is more likely to proceed through the pyranose form)	Greater probability for pentoses to be under their open-chain form because of the weaker stability of furanose ring (higher tension within the molecule, which forces the ring to open) compared to pyranose ring	Hayward & Angyal (1977)
	Greater conformational stability of hexoses	Slower production of chromophores with hexoses	Burton & McWeeny (1963)
Differences of reactivity between sugars of the same class			
Structure	Configuration of hydroxyl groups in the sugar	The disposition of OH groups influences the stability of the molecule (including the proportion of acyclic form), affects primary steps of the MR and determines the conversion of intermediates during the course of subsequent steps of the MR, which consequently influences the global sugar reactivity	Sun, Hayakawa, Chuamanochan et al. (2006), Ames (1992), Bunn & Higgins (1981), Olivier et al. (2006), Burton & McWeeny (1963)
Form of the sugar in solution/ stability	Higher proportion of open-chain form for certain sugars compared to others	Greater reactivity for sugars whose linear form is favoured	Brands & van Boekel (2002), Sun, Hayakawa, Chuamanochan et al. (2006)
	Faster isomerisation rate of some ketoses into their aldose form	In the comparison of the relative reactivity of two ketohexoses: higher reaction rate for the sugar whose interconversion rate into its aldose form is favoured (aldoses being more reactive than ketoses)	Brands & van Boekel (2002)
	Conformation adopted by the carbonyl backbone of the sugar (itself depending on the initial configuration of the sugar)	Consequences on the resulting reactivity of the sugar	Hayward & Angyal (1977), Burton & McWeeny (1963)
	Influence of free-energies of the ring form and, to a lesser extent, of the linear form of the sugar	Higher proportion of acyclic carbonyl (and consequently higher reaction rate) for sugars whose ring form is less stable and which preferentially compete with their less stable furanose ring (compared to the pyranose form), and whose linear form is favoured (active form of the sugar)	Hayward & Angyal (1977)

(*continued on next page*)

Table 2 (*continued*)

Nature of the cause	Hypothesis	Consequence	Source[a]
Parallel reactions	Higher level of sugar degradation for certain sugars	Small carbonyl fragments are thought to be highly reactive in the MR	Brands & van Boekel (2002)
Properties and nature of the reaction products	Intermediate MRPs of various configuration and reactivity	Certain configurations *(cis/trans)* adopted by MRPs are more energetically unstable, and degrade more easily to form brown products	Ames (1992), Burton & McWeeny (1963)

[a] The authors cited in this column do not have necessarily demonstrated these statements through experimental data but they do use them as arguments in the Section 3.5.

epimers about C-2), exhibited the same behaviour. It was concluded that the OH group about C-2 had no influence on the formation of MRPs, and that the different configurations about C-3 and C-4 could explain the variability observed between aldohexoses. The order of reactivity finally established was: D-allose/D-altrose > D-galactose/D-talose > D-gulose > D-glucose/D-mannose. Interestingly, it is possible to draw analogies between these findings and our own results. In the carbonyl classification, each aldopentose can lead to two aldohexoses (two C-2 epimers), by the addition of one CHOH group. In this way, ribose is the "parent sugar" of allose and altrose, lyxose is the "parent sugar" of galactose and talose, xylose is the "parent sugar" of gulose and idose, and arabinose is the "parent sugar" of glucose and mannose. In the present study, we found that ribose > xylose > arabinose in the MR. Thus, the order of reactivity of these three sugars is kept compared to the rank adopted by the respective sugars originating from them (Sun, Hayakawa, Chuamanochan, et al., 2006).

4. Conclusion and perspectives

In the present study, converging results on the higher reactivity of pentoses over hexoses in the MR were provided, in agreement with previous published works. Both traditional kinetic parameters (measurement of browning intensity, monitoring of the disappearance of both reactants) as well as chromatographic data highlighted the following order of reactivity: fructose ≈ glucose < arabinose < xylose < ribose. Interestingly, aldopentoses could only be distinguished according to their respective reaction rate. This is of particular relevance for the food sector dealing with the MR, where a compromise between cost and efficiency has often to be found concerning the purchase of raw materials. Stoichiometry of the parallel decrease in amino and carbonyl moiety, as well as explanations for the greater propensity of some sugars in comparison to other ones, were also discussed. Finally, as an original approach, SEC-FPLC was proved to be an efficient tool to assess the relative reactivity of different sugars in the MR, even (and particularly) for complex systems composed of hydrolysates.

Sugar type is one of the numerous factors governing the MR rate. Its choice is crucial because it will notably impact the features of MRPs, either in terms of biological activities or in terms of functional properties. Numerous studies have therefore been focused on the influence of sugar type on the antioxidant capacity (Chevalier, Chobert, Genot, & Haertlé, 2001c; Sun, Hayakawa, Chuamanochan, et al., 2006), antimutagenic properties (Yen et al., 1992), gelling, foaming and emulsifying activities (Chevalier, Chobert, Popineau, et al., 2001; Sun et al., 2004) of the resulting MRPs. A sugar may also be selected for its ability to generate safe products (Wijewickreme & Kitts, 1997).

To conclude, MRPs are expected to have a great potential as active ingredients in the nutraceutical field as well as in the food additives market. In addition, by using marine hydrolysates made from by-products, a promising way to upgrade seafood waste was evidenced, thus creating value-added compounds from a low-cost and underutilised raw material. To better exploit the attractive properties emerging from the MR, mechanistic aspects have necessarily to be deepened, so that beneficial activities of MRPs can be improved, without generating toxic compounds.

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