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Analytical Methods

Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E. coli* and *S. aureus*

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ABSTRACT

A rapid plate reader based method examining the antimicrobial activity of both model and food melanoidins (coffee, beer, sweet wine) is described. Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* is evaluated as area under the growth curve compared to a control. Method was settled for an aqueous melanoidin concentration of 2 mg/ml inoculated to 10^6 cfu/ml culture. All tested model and food melanoidins exerted antimicrobial activity in some extent, but inhibition was significantly higher over *S. aureus* (Gram-positive) than *E. coli* (Gram-negative). Antimicrobial activity can be further quantified by expressing it as OTEV (oxytetracyclin equivalent value, μg/l) which could serve to compare the results obtained within different laboratories, methodologies and/or compounds. Results indicate that both strains have different sensitivity against the presence of melanoidins and probably different mechanism of inhibition. Procedure can be used for a rapid screening of the potential antimicrobial properties of melanoidins, and subsequently to Maillard reaction products as well, against pathogenic strains in order to isolated substances with biological activity.

1. Introduction

An important reaction in thermally treated foods is the Maillard reaction, which takes place between the amino group of a free or protein-bound amino acid and reducing sugars (Ames, 1992, Chap. 4). Variables such as the reaction conditions (e.g., heating time and temperature), physico-chemical properties of the system (e.g., water activity or pH), chemical composition and nature of reagents can affect the kinetics of the reaction and this lead to the formation of a myriad of Maillard reaction products (MRP) with different chemical structure and, likely, different biological properties (Ames, 1992, Chap. 4; Einarsson, Snjgg, & Eriksson, 1983; Somoza, 2005). The high molecular weight compounds formed in the last stage of the Maillard reaction are called melanoidins. Melanoidins are widely distributed in foods and could exert different *in vitro* functional properties such as antioxidant (Delgado-Andrade, Rufián-Henares, & Morales, 2005; Morales & Jiménez-Pérez, 2004), antihypertensive (Rufián-Henares & Morales, 2007), metal-binding activity (Morales, Fernandez-Fraguas, & Jiménez-Pérez, 2005), antimicrobial (Rufián-Henares & Morales, 2006) and prebiotic (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004). In recent years, several studies have been mainly focused in the effect of melanoidins on the human diet and their possible nutritional, biological and health implications (Ames, Wynne, Hofmann, Plos, & Gibson, 1999; Faist & Erbersdobler, 2001).

Chemical structure of melanoidins has not been completely elucidated, but, in the last few years, more data became available. In particular, it was shown that they can have a different structure according to the different starting material and behave as anionic material: in some cases they are mainly formed by a carbohydrate skeleton with few unsaturated rings and a small nitrogen component, in other cases they can have a protein structure linked to small chromophores or phenolic residues (Bekedam, De Laat, Schols, Van Boekel, & Smit, 2007; Cämmerer & Kroh, 1995; Morales, 2002; Nunes & Coimbra, 2007).

Antimicrobial activity of MRPS has been previously studied in model systems (Stecchini et al. (1991)) and coffee (Daglia, Cuzzoni, & Dacarro, 1994) but specific studies on antimicrobial activity of melanoidins are scare. Some investigations highlight the role of melanoidins *in vivo* since melanoidins escape digestion and pass through the upper gastrointestinal tract (Faist & Erbersdobler, 2001) and then can interact with the different microbial species present in the hindgut (Finot & Magnenat, 1981). Recently Borrelli and Fogliano (2005) have shown that bread crust melanoidins can be metabolized/fermented by the human hindgut microflora and that these melanoidins selectively enhance the growth of bifidobacteria, which are desirable bacteria in the gut due to their health-promoting properties. On the other hand melanoidins have been demonstrated to develop antimicrobial activity (Rufián-Henares & Morales, 2006) which could complement the prebiotic effect

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if this inhibition of the bacterial growth could be exerted over pathogenic bacteria.

The methods used for the screening of food antimicrobials may be divided into endpoint and descriptive methods (Skyttä & MattilaSandholm, 1991). The most widely used endpoint method is the agar diffusion assay although its limitations are generally recognized. Since the size of the inhibition zone is dependent upon the rate of diffusion (Davidson & Parish, 1989) misleading results may be obtained if the agent to be tested is hydrophobic and will not diffuse into the agar. Furthermore, the end point methods scarcely provide information of the effects of the compounds on the growth kinetics of the microorganisms. In descriptive tests sampling or automated recording of microbial growth is carried out at timed intervals. Consequently, one can evaluate the effects of an antimicrobial over a longer period of time owing to the kinetic recording of results. Various growth curve parameters can be used to describe the inhibitory effects. The extension of the lag phase is probably the most widely used parameter. It has been shown that in a food system, even a slight delay of a lag phase may have an important influence on its shelf life. Other parameters, such as end-absorbance (Mattila, 1987), slope, which is a gradient of the exponential growth phase (Adams & Hall, 1988), and area under the growth curve have also been used (Borrelli & Fogliano, 2005; MattilaSandholm, 1989). The latter was considered to be the best descriptor of overall inhibitory effect since it covers the entire growth period instead of referring to single points of time. Continuous monitoring offers an even more important advantage over the agar diffusion test in that the inhibition of growth can be detected as soon as the measured parameter deviates significantly from uninhibited control.

In a previous study, a first approximation to assess the antimicrobial activity of melanoidins was evaluated by a generic gram-positive thermophilic bacterium (*Geobacillus stearothermophilus var. calidolactis*) as a very efficient discriminate test (Rufian-Henares & Morales, 2007). But former approach had not a direct application to strains of interest in Food Science and Technology although is useful for a rapid evaluation of the potential antimicrobial activity. In this sense, the aim of current investigation was to study the effect of model and food melanoidins on the growth of some selected pathogenic organism (*Escherichia coli* and *Staphylococcus aureus*) by a rapid plate-based assay. Antimicrobial activity is evaluated as area under the growth curve as compared to a control. In addition, results are also expressed as Oxytetracyclin equivalent concentration.

2. Materials and methods

2.1. Chemicals

Oxytetracyclin, Penicillin G, alanine, arginine, cysteine, histidine, lysine, methionine, tyrosine, tryptophane, phenylalanine and glucose were from Sigma (St. Louis, MO, USA) and dichloromethane from Panreac (Barcelona, Spain). Bacteriological growth media, brain heart infusion broth (BHI) and brain heart infusion agar were from Oxoid (Basingstoke, UK). The bacterial strains used for the antimicrobial assay were *E. coli* American Type Culture Collection (ATCC) 11775 and *S. aureus* subsp. *aureus* (ATCC 6538P). The assay organisms were stored frozen at −80 °C in medium containing 25% glycerol.

2.2. Commercial Samples

Filtered coffee brew was produced from roasted coffee beans at two different roasting degrees: light (14.5% roasting loss) and medium (16.2% of roasting loss). Coffee brew was lyophilised and stored at –80 °C until analysis. Three widely distributed commercial brands of beer in Europe with different elaboration procedures were selected. A Pilsner-style beer from a Spanish brewery, an Abbeys-style beer from a Belgian brewery and a dry-stout beer from an Irish brewery were used. A widely consumed Spanish sweet wine, "Pedro Ximenez", was also used. This Spanish sweet wine is elaborated from dry-grapes by a process called "Soleo".

2.3. Ultrafiltration

The ultrafiltration cell was an Amicon model 8400 (Millipore, Bedford, MA, USA) equipped with a 10,000 Da nominal molecular mass cut-off membrane (YM-10, Millipore). Bacteriological growth media were autoclaved before use and melanoidins and related fractions re-dissolved in sterile water. The microplate reader (Synergy-HT multimode) used for the turbidity measurement was from BioTek Instruments (VT, USA). Sterile flat-bottomed 96-well microplates were from Greiner (Frickenhausen, Germany).

2.4. Preparation of melanoidins from model systems

Nine water-soluble melanoidins were obtained from aqueous Maillard reaction model systems prepared from a combination of glucose and an amino acid such as alanine (A), arginine (R), cysteine (C), histidine (H), lysine (K), methionine (M), tyrosine (Y), tryptophane (T) and phenylalanine (P). Water-soluble melanoidins were isolated from model systems as described previously by ultrafiltration (Rufián-Henares & Morales, 2007). Briefly, an aliquot of each model system was subjected to ultrafiltration, using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA, USA), equipped with a 10,000 Da nominal molecular mass cut-off membrane. The retentate was filled up to 200 ml with water and washed again. Washing step (diafiltration) was repeated at least three times. The high molecular weight fraction corresponding to melanoidins was freeze-dried and stored in a dessicator at 4 °C until analysis. Two letters, first related to the sugar (G for glucose) identified melanoidins (M) isolated from model systems, and the second letter was related to the amino acid. The model named MGK referred to the melanoidin isolated from the glucose and lysine model system.

2.5. Preparation of water-soluble melanoidins from foods

Coffees: Lyophilised coffee brew (1 g) was resuspended in 100 ml of hot water (50–60 °C) and the aqueous solutions obtained were then filtered (filter paper no 40, Whatman, UK) and de-fatted with dichloromethane (2 × 200 ml). An aliquot of the coffee brews was subjected to ultrafiltration as stated above for melanoidins from model systems. Two melanoidins samples were obtained and named as MLC (light roasting conditions) and MMC (medium roasting conditions).

Beer and wine: Beer and sweet wine melanoidins were prepared in a similar way than coffee ones. In this case 100 ml of beer or sweet wine where mixed with 100 ml of water, the solutions obtained where filtered, treated with dichloromethane and finally ultrafiltered to isolate the melanoidin fraction. Four melanoidins samples were obtained; MSW (sweet wine), MLB (lager beer), MTB (Abbey-style beer), MSB (stout beer).

2.6. Antimicrobial assay

The influence of different concentrations of melanoidins on the growth of *E. coli* and *S. aureus* was determined by the microtiter plate method as described by Skyttä and Mattilasandholm (1991). Overnight suspensions of *E. coli* or *S. aureus* were growth at 37 °C in BHI until a concentration of 10^9 colony forming units

(cfu)/ml was reached. Culture of bacteria were diluted 1/1000 in fresh broth used to culture them prior to each experiment to approximately 10^6 cfu/ml. Based on their routine growth characteristics, bacterial strains were diluted to optical densities (600 nm) of 1.480 ± 0.020 and 1.150 ± 0.015 for *S. aureus* and *E. coli*, respectively. Then 250 μl of bacterial cells suspensions were inoculated into a sterile 96-well microplate. Model and food melanoidins were resuspended in sterile distilled water at a concentration of 2 mg/ml and filtered by means of a Millipore sterile 0.22 μm filter unit previously to use. Subsequently 50 μl of sample, sterile distilled water (blank assay) or 100 μg/l solution of oxytetracyclin (positive control of bacteriostatic activity) were added. Microbial growth kinetic was recorded on a Synergy-HT multidetector microplate reader driven by Gen5 reader control and data analysis software (BioTek Instruments, VT, USA). The 96-well microplates were agitated by the microplate reader for 1 min at 150 rpm and then incubated at 37 ± 1 °C for 24 h. Turbidity was measured as absorbance at 600 nm, and was taken every 5 min. The microplates were shaken for 10 s prior to the measurement to achieve homogeneous suspensions. Each experiment was performed by triplicate. Method performance was evaluated by the reproducibility of the lag-phase within days and the maximum absorbance reached at the steady-state in the blank sample. The matrix of data was transferred to a spread sheet program and the growth curves were drawn as averages of three parallel wells.

The area under the curve (AUC) was calculated according to the equation

$$AUC = Abs_0/Abs_0 + Abs_0/Abs_5 + Abs_0/Abs_{10} + \cdots + Abs_0/Abs_n$$

Being Abs_0 = absorbance at time 0, Abs_5 = absorbance at time 5 min, and so forth. However, in order to eliminate the differences in the AUC corresponding to the different absorbidities of the melanoidins, the initial absorbance of each sample was subtracted to their following readings, then obtaining the same initial lecture (equal to the absorbance of the BHI medium at 600 nm). Finally, the Net AUC was calculated by subtracting the AUC of the blank sample to the AUC of each melanoidin or positive control.

2.7. Statistical treatment

All of the analyses were performed at least in triplicate. The Statgraphics v. 5.1 software package was used for statistical analysis. Statistical procedures were performed at a significance level of 95%.

3. Results and discussion

3.1. Method development

The automated recording of microbial growth with microplate readers is a useful tool to study the behaviour of selected microorganisms in presence of substances with antimicrobial activity (Skyttä & MattilaSandholm, 1991; Mattilasandholm, 1989; Adams & Hall, 1988). Different curve parameters, like the extension of the lag phase, end-absorbance, slope and AUC describe that inhibitory effect. However, the latter has been considered the best descriptor of overall inhibitory effect (Skyttä & MattilaSandholm, 1991) since it covers the entire growth period instead of referring to single points of time, then minimising errors derived from inexact calculations based on only one point.

Preliminary experiments were performed in order to define the initial concentration of inoculum of both strains. Ten-fold serial dilutions with BHI from the overnight culture were made and incubated in the microplate for 24 h at 37 °C. As depicted in Fig. 1 for *S. aureus* (a), and *E. coli* (b), respectively and no growth was observed

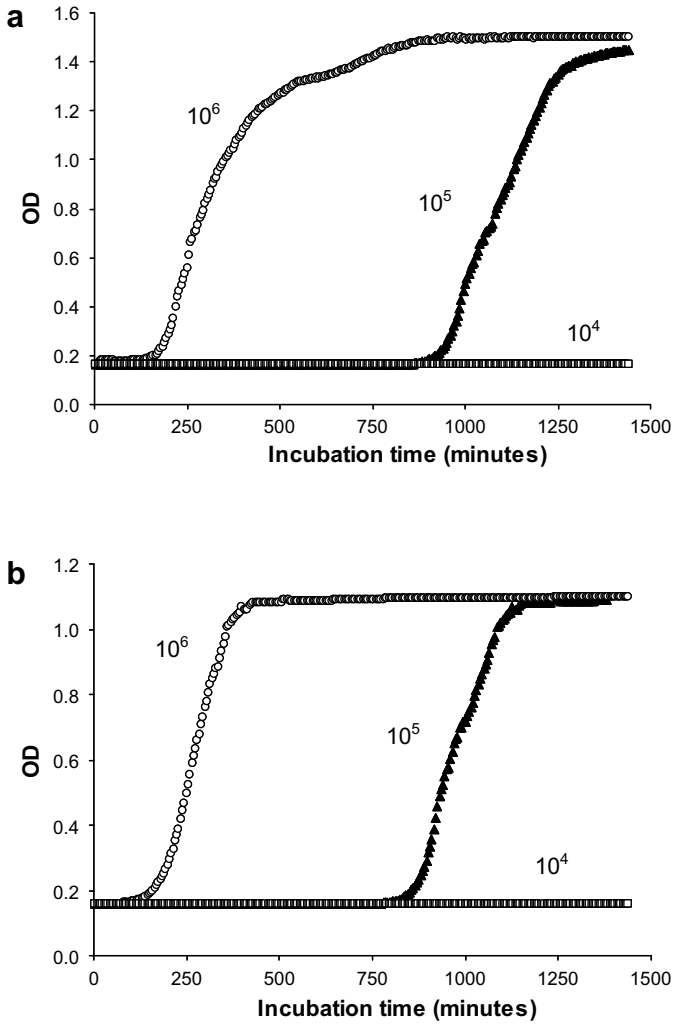

Fig. 1. Growth plot of *S. aureus* (a) and *E. coli* (b) at different inoculum concentrations and recorded at 600 nm (37 °C).

for a 10^4 cfu/ml inoculum. Optical density increase could be monitored after 850 minutes at 10^5 cfu/ml, whereas for the 10^6 inoculum only about 125 min lag phase was observed. In this sense and in order to reduce the time of analysis, to reduce side-contaminations and make it easily automatized, the 10^6 cfu/ml inoculum was selected for further studies with both Gram-negative and Gram-positive strains.

Later on procedure was evaluated in order to be used routinely for assessing the bioactivity of melanoidins on the growth of *E. coli* and *S. aureus* over time. Melanoidins themselves absorb slightly at 600 nm, which could be a drawback since it could reduce the range of analysis but concentrations of the studied melanoidins (0.1–2.0 mg/ml) did not show significant interferences. However a blank assay was performed for each melanoidin concentration in order to subtract any residual absorbance, if recorded.

The method was evaluated for linearity, sensitivity and precision for both strains. A standard calibration curve of oxytetracyclin was made by increasing concentrations from 1 to 15 μg/l using the previously fixed inoculum conditions (10^6 CFU, 37 °C/24 h). Fig. 2 shows a classical dose-dependent inhibition profile of oxytetracyclin of *S. aureus*. Nowadays, oxytetracyclin is a widely used antibiotic with bacteriostatic activity routinely used as control for inhibition studies (Rufián-Henares & Morales, 2006). Relationship between net AUC *vs*. oxytetracyclin concentration was plotted for both strains and a linear response in the working area was

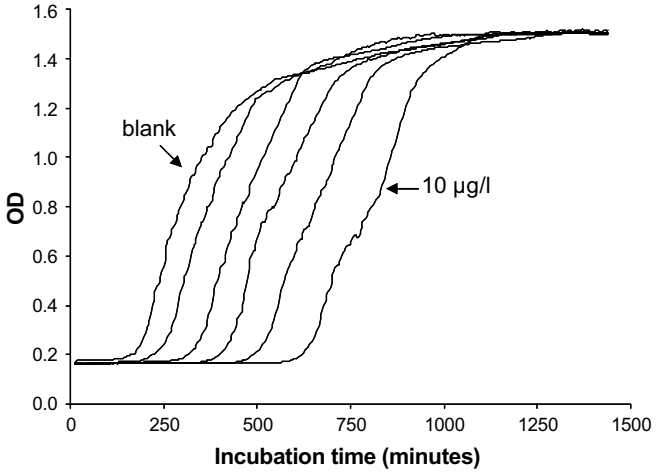

Fig. 2. Dose effect of oxytetracyclin (µg/l) on the growth of *S. aureus*.

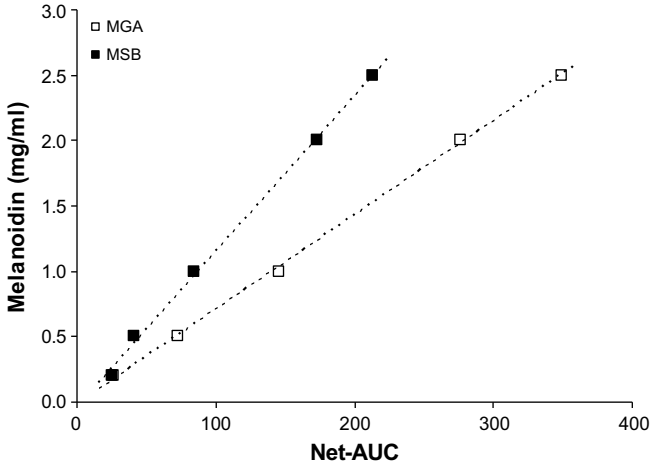

Fig. 4. Relationship between melanoidin concentration (mg/ml) and the Net-AUC for *S. aureus*. MGA (empty box), MSB (solid box).

obtained (Fig. 3). Where higher oxytetracyclin concentration in the media, later the microbial growth start. Working area, expressed as Net-AUC, was estimated up to 130 and 350 for *E. coli* and *S. aureus*, respectively. Experimental results shows that working area could be much extended for Oxytetracyclin but it is possible some side-effects at high concentrations of the unknown substance for testing. Inhibitory activity of target substances which gives Net-AUC higher than the assigned maximum values will be re-evaluated at a more diluted concentration.

The applicability of the method was tested with two different melanoidins, one from a model system (MGA) and other from a food-derived (MSB). In both cases four concentrations – ranging from 0.2 to 2 mg/ml – were assayed obtaining a linear response (r^2 = 0.9989 and r^2 = 0.9958) for MGA and MSB, respectively (Fig. 4). The lowest net AUC, obtained with the 0.2 mg/ml concentration, were close to 25 AUC, which is adequate and sensitive enough for the measurement of potential inhibitory actions of melanoidins over the microbiological growth. A linear dose-response activity was obtained for both melanoidins against Gram-negative and Gram-positive microbial inhibition. As depicted in Fig. 4 a higher slope is obtained when the antimicrobial activity (stated as higher AUC values for the same melanoidin concentration) was stronger. In this sense, the 2 mg/ml concentration was selected for the rest of the analysis because of it permits a better differentiation between the antimicrobial activities of the tested melanoidins. In addition, range of concentration used for melanoidins just prolong the lag-phase but did not affect to the maximum growth rate and final concentration of bacteria was not much affected as described by Einarsson et al. (1983) and Lanciotti, Anese, Sinigaglia, Severini, and Massini (1999). Finally, the reproducibility, expressed as coefficient of variation (CV; n = 10) was assayed with the same sample MSB. The intra-day variation was 2.3% and the inter-day coefficient of variation was 3.5% for a mean net AUC value of 173.1.

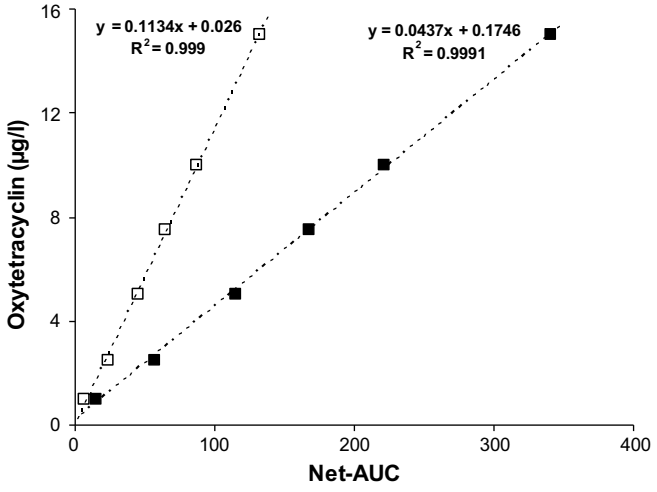

Fig. 3. Inhibition of growth for *S. aureus* (solid box) and *E. coli* (empty box) at different concentrations of Oxytetracyclin, expressed as Net-AUC.

3.2. Antimicrobial activity of melanoidins

The proposed method for measuring the antimicrobial activity was applied to different melanoidins isolated from model systems and foods. All the melanoidins investigated exerted a bacteriostatic activity over *S. aureus* and *E. coli* in somewhat extent. Similar results have been previously reported in model systems by Einarsson et al. (1983), Lanciotti et al. (1999), and by our own previous research (Rufián-Henares & Morales, 2006) by using a test for screening the non-specific antimicrobial activity (against *Geobacillus stearothermophilus var. calidolactis*) of Maillard reaction derived compounds.

Bioactivity of melanoidins can be quantified by using the Net-AUC value, inhibition time and OTEV value. Results are summarised in Table 1 for melanoidins isolated from model systems.

Table 1
Antimicrobial activity of melanoidins (2 mg/ml) obtained from model systems

Melanoidin	*S. aureus*			*E. coli*		
	IT[a]	Net AUC[b]	OTEV[c]	IT	Net AUC	OTEV
MGA	1770	277.0	31.4 ± 2.1[a]	365	52.3	6.0 ± 0.4[a]
MGC	565	74.9	8.5 ± 0.9[b]	465	69.7	7.9 ± 0.3[b]
MGH	1170	165.1	18.7 ± 1.4[c]	320	45.3	5.2 ± 0.4[a]
MGK	775	95.7	10.9 ± 1.0[d]	595	87.1	9.9 ± 0.6[c]
MGM	1705	263.4	29.9 ± 2.0[a]	485	72.6	8.3 ± 0.4[b]
MGP	1075	155.4	17.6 ± 1.6[c]	705	103.1	11.7 ± 0.6[d]
MGQ	540	67.9	7.7 ± 0.6[b]	415	59.8	6.8 ± 0.5[a]
MGR	1460	216.2	24.5 ± 2.0[e]	455	67.0	7.6 ± 0.4[b]
MGT	1265	186.2	21.1 ± 1.9[e]	1415	229.0	26.0 ± 1.1[e]
MGY	1540	231.3	26.3 ± 2.6[a,e]	355	49.5	5.6 ± 0.3[a]

Letters indicate statistically significant differences (p < 0.01).
[a] Inhibition time, expressed in minutes.
[b] Net area under the curve.
[c] Oxytetracyclin equivalent value, expressed as µg oxytetracyclin/l.

Net AUC ranged from 67.9 to 277.0 and from 49.5 to 229.0 for *S. aureus* and *E. coli*, respectively. It is important to highlight that the melanoidins with the lowest and the highest activities are not the same for *S. aureus* and *E. coli*, indicating that the type of amino acid plays an important role in the melanoidin formed and on its antimicrobial activity. In addition, the results shows that melanoidins exerts a higher antimicrobial activity over *S. aureus*, probably because of this is a Gram-positive bacteria whereas *E. coli* is a Gram-negative one, which are known to be more resistant to the action of antibiotics in a general way. These results agree with those reported by Daglia et al. (1994) and Lanciotti et al. (1999) for the antimicrobial activity of MRPs from coffee against different strains as. However different results were obtained by Del Castillo et al. (2007) for studying the antimicrobial activity of MRPs obtained from a gluten-glucose model systems being Gram-negative (*E. coli* ATCC 25922) more susceptible of inhibition than *S. aureus* ATCC 25923. However, high molecular weight fraction, which is related to melanoidins, was clearly responsible for the bacterial growth inhibition (Skyttä & MattilaSandholm, 1991).

The results are also expressed as oxytetracyclin equivalent value (OTEV) which is obtained by substituting the Net AUC of the melanoidin into the standard curve of oxytetracyclin, and then expressed in μg of oxytetracyclin per liter. This value gives information of the activity of every melanoidin at a standardised concentration compared with a reference bacteriostatic antibiotic. OTEV value is useful for interlaboratory comparison for the same methodology and if different approaches are being used. For example, the OTEV value of 31.4 obtained for MGA against *S. aureus* means that this melanoidin exerts an antimicrobial activity similar to the one of a 31.4 μg/l solution of oxytetracyclin. An average OTEV of 21.3 and 8.3 were obtained for *S aureus* and *E. coli*, respectively. In the case of *S. aureus* the most intense activity was for MGA and MGM, followed by tyrosine, phenylalanine and tryptophan (all of them with an OTEV value higher than 20). In contrast, in the case of *E. coli* only MGT had a value higher than 20 μg/l whereas the rest of the values ranged from 5 to 10 μg/l. Finally, the mean inhibition time (IT), which is the time where the melanoidins are able to delay the microbial growth, was also reported. It is clear the relationship between OTEV and IT: the higher the OTEV value, the longer the inhibition time (up to 1770 and 1415 minutes for *S. aureus* and *E. coli*, respectively).

Because of the successfully application of the method to model melanoidins, the method was applied to food-derived melanoidins. The results obtained are shown in Table 2. In the case of *S. aureus* the net AUC values ranged from 69.4 to 1342.0 for MLB and MMC, respectively. For this assay the OTEV values ranged from 7.9 to 152.3 μg/l, with a median value of 17.3. Although this value is close to the one obtained for model systems (21.1) in fact the mean antimicrobial activity of food derived melanoidins is lower because of the activity in MMC is so higher (153.2 μg/l) that produce a bias in the median value (which is of 15.5 is the MMC value is omitted). In the case of *E. coli* the values, of both net AUC and OTEV, where lower than those obtained for *S. aureus* as stated previously for model systems. The OTEV values ranged from 8.9 to 5.8 with a median value of 7.4 μg/l, which was very close to the results of model systems derived melanoidins. In the case of the inhibition time, it was clearly lower for *E. coli* (a mean IT of 500 min) whereas for *S. aureus* was longer for many food melanoidins. It is noteworthy to underline that MMC melanoidin was able to delay the *S. aureus* growth during the fixed time for incubation, and then a prolonged period was programmed in the microplate reader a serial dilution was applied to MMC. In this sense it was found that this melanoidin could delay the microbial growth up to 8315 min, which was the highest time found in the melanoidins studied.

Table 2
Antimicrobial activity of melanoidins (2 mg/ml) obtained from food

Melanoidin	*S. aureus*			*E. coli*		
	IT[a]	Net AUC[b]	OTEV[c]	IT	Net AUC	OTEV
MSW	890	136.5	15.5	455	63.7	7.3
MLB	475	69.4	7.9 ± 0.2[a]	365	50.8	5.8 ± 0.3[a]
MTB	520	79.7	9.1 ± 0.8[b]	475	66.3	7.5 ± 0.3[b]
MSB	1100	173.1	19.7 ± 1.3[c]	565	78.6	8.9 ± 0.5[c]
MMC	8315	1343.0	152.3 ± 2.6[a]	540	74.5	8.5 ± 0.5[a]
MLC	525	79.7	9.1 ± 1.0[b]	425	59.0	6.7 ± 0.3[b]

Letters indicate statistically significant differences ($p < 0.01$) for different types of beer and coffee.
[a] Inhibition time, expressed in minutes.
[b] Net area under the curve.
[c] Oxytetracyclin equivalent value, expressed as μg oxytetracyclin/l.

There were statistically significant differences ($p < 0.01$) between the different melanoidins of the same type of food in the case of both *S. aureus* and *E. coli*. In addition, as reported previously (Rufián-Henares & Morales, 2006) melanoidins isolated from more severely treated samples, such as MMC and MSB, exerted higher inhibitory bacterial growing activity for both *S. aureus* and *E. coli*. Results are in line with Daglia et al. (1994) which reported that the antibacterial activity of coffee depends on the degree of roasting and not related to the procedure to obtain the coffee beverage. Murata, Nakajima, and Homma (1995) also reported on the inhibition of *S. mutans* and *S. sobrinus* by dark beer.

In our samples, there was a very important increase in the antimicrobial activity with the roasting degree of coffees, from an OTEV of 9.1 for light roasting to 152.3 for medium roasting, in the case of *S. aureus* whereas in the case of *E. coli* that increase was lower, although statistically significant. A similar behaviour was observed for beer melanoidins, showing the black beer a higher activity (OTEV of 19.7) than that obtained for lager beer (OTEV of 7.9). The antimicrobial activity of MSW, MTB, MSB melanoidins was much lower than MMC melanoidin for *S. aureus* but, in the case of *E. coli* these differences where quite lower resulting in similar values for MSB and MMC.

A number of studies have shown that MRPs themselves are inhibitory to the growth of micro-organisms (Einarsson et al., 1983; Rufián-Henares & Morales, 2006; Del Castillo et al., 2007; Lanciotti et al., 1999; Mattilasandholm, 1989). Einarsson et al. 1983 measured the antimicrobial activity of arginine–xylose and histidine–glucose Maillard reaction mixtures. They fractionated the model systems by dialysis (molecular cut-off at 1000 Da) and found that the high molecular weight fraction exerted a higher antimicrobial activity than the low molecular weight one. This authors stated that the high molecular weight fraction (melanoidins principally) could develop its antimicrobial action by binding essential metals like iron, which is essential for growth and survival of pathogenic bacteria. In addition other authors referred the antimicrobial activity of Maillard reaction compounds to interference with the uptake of serine, glucose, and oxygen (Einarsson, 1987), to inhibit the sugar catabolising enzymes of microorganisms (Lanciotti et al., 1999) or their potential antioxidant activity (Mattilasandholm, 1989). However, the exact mechanism by which melanoidins or MRPs affect the bacteria growth is not know and this investigation will provide of a reliable and fast approach for screening of bioactivity of MRPs towards different target microbial.

In conclusion, considering that *S. aureus* and *E. coli* are food-degradative microorganisms and pathogens for human beings, the results reported here are of particular relevance to food. It is difficult to correlate the antimicrobial activity of melanoidins against pathogenic microorganisms to their structure because it is unknown. However, these results are important to describe the antimicrobial activity expected by melanoidins in a food or model system by a single and easily automatised test.

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