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Optimised Procedure to Analyse Maillard Reaction-Associated Fluorescence in Cereal-Based Products

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Abstract

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Fluorescent Maillard compounds measurement provides more specific information on the extent of the Maillard reaction than other unspecific tools to monitor the reaction, and is suitable, as the first approach, to assess the nutritional quality of foods as related to protein damage. This work presents an optimised laboratory procedure for the measurement of total fluorescent intermediate compounds (FIC) associated with Maillard reaction, described and evaluated in a cereal-based product. Total FIC are evaluated using increased pronase E concentrations and different incubation times for the enzymatic hydrolysis, as well as three different sample clean-up steps after the enzymatic digestion. The effects of basic/acid media are considered for the stability of the fluorescent compounds. The standardised procedure is finally applied to breakfast cereals as a model of cereal-based products, analysing the correlation between total FIC production and fibre and protein contents. It is demonstrated that fluorescent compounds are mainly linked to the protein backbone in ready-to-eat breakfast cereals. Fluorescence measurement is presented as an inexpensive, rapid and accurate procedure to study the extent of Maillard reaction in breakfast cereals.

Keywords: Maillard reaction; fluorescent intermediate compounds (FIC); breakfast cereals

The Maillard reaction (MR), known as a non-enzymatic reaction between reducing sugars and primary amino acids, is a complex set of reactions that take place during industrial processing, domestic cooking, and storage of most foods (Friedman 1996). In the advanced stage of MR, Amadori rearrangement products undergo dehydration and fission and yield colourless reductones and fluorescent substances as well (Baisier & Labuza 1992).

Cereal-based products are usually industrially manufactured, with the application of the extrusion process (Rada-Mendoza *et al.* 2004) followed by drying and toasting steps (Huang 1998). Extrusion and drying-toasting steps, taking place at 80–95°C and higher than 150°C respec-

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tively, insure the development of Maillard reaction and the caramelisation, both contributing to the transformation of the raw material in a tasty final product (Friedman 1996).

Traditionally, the nonspecific procedures to monitor the advanced stage of MR in foods have been the measurement of colour production at 420 nm (Palombo *et al*. 1984; Kato *et al*. 1989) or by using spectrophotometric tristimulus colour measurement (Pagliarini *et al*. 1990; Rampilli & Andreini 1992). In the last decades, the determination of fluorescent compounds has been used as a slightly more specific procedure for the analysis of the total presence of Maillard reaction products (MRP). From literature, fluorescence is even frequently used in MR studies at physiological conditions with regard to glycosylation of proteins in the human body and AGEs (advanced glycation end-products) formation (Sell & Monnier 1989; Kumar *et al*. 2007; Li *et al*. 2008).

In foods, the total pool of fluorescent intermediate compounds (FIC) is composed of those free in the matrix and the linked-to-protein fraction. Free and total FIC can be determined by different analytical procedures, and then the linked-to-protein fraction is estimated by subtraction (Morales & Van Boekel 1997). Total FIC determination requires a previous enzymatic hydrolysis, usually conducted using nonspecific protease such as pronase (Morales *et al*. 1996; Ferrer *et al*. 2005).

Some authors have suggested the use of free fluorescent compounds as a heat-induced index to evaluate the heat damage in processed foods in the same way as the browning index at 420 nm (Morales *et al*. 1996). Free and total FIC have been tested in some foods such as milk and milk-resembling systems (Morales *et al*. 1996), heat-treated milk, breakfast cereals, cooked salmon, and roasted soy (Birlouez-Aragón *et al*. 2001), using different procedures. Our research group has also experience with the fluorescent determination in breakfast cereals (Delgado-Andrade *et al*. 2006), but the method employed has never been checked in relation to the optimum conditions of its performance (enzymatic hydrolysis, incubation time, and sample clean-up procedure).

The purpose of the present work was to optimise the procedure for the measurement of total FIC associated with Maillard reaction development in breakfast cereals as a model of cereal-based products. Different factors as incubation times, enzyme concentrations, clarification steps, basic/acid media are considered to assess the stability of the fluorescent compounds formed. Moreover, free FIC and relationships between FIC formation and protein and fibre contents are evaluated.

MATERIAL AND METHODS

Chemicals. All chemical used were of analytical grade and were obtained from Merck (Darmstadt, Germany), unless mentioned otherwise. Pronase E (4 000 000 PU/g) was also purchased from this company.

Samples. Twenty-two commercial breakfast cereals from different manufactures were collected from supermarkets. The whole product contained in each package was powdered, homogenised, and stored in polyethylene containers under vacuum at 4°C until analyses were performed.

Since the aim of this study was to find out a suitable procedure for any type of breakfast cereal, randomly, four of the samples were named as A (based on corn and integral wheat flour), B (based on rice flour), C (based on wheat flour) or D (wheat flour and corn and wheat bran) and used in the optimisation of the pronase E concentration (A and B samples) and the incubation time (C and D samples) for the enzymatic hydrolysis in the total FIC determination, as well as in the selection of the sample clean-up procedure (A and B samples) employed for total FIC measurement. The rest were assigned by a number and free and total FIC formation was measured once the adequate procedures had been fixed. Breakfast cereals containing dried fruit were excluded from this study to avoid bias during data interpretation

Total protein content. Samples (0.800–1.000 g) were heated to 1050°C following AOAC 992.15 (1995) in a LECO model FP-2000 (Leco Instruments, Madrid, Spain) protein/nitrogen analyser calibrated with EDTA (Dumas method). The nitrogen-to-protein conversion factor considered was 5.70, 5.95 or 6.25% for wheat, rice, and the rest of cereals, respectively. The results were expressed as grams of protein/100 grams of the respective product.

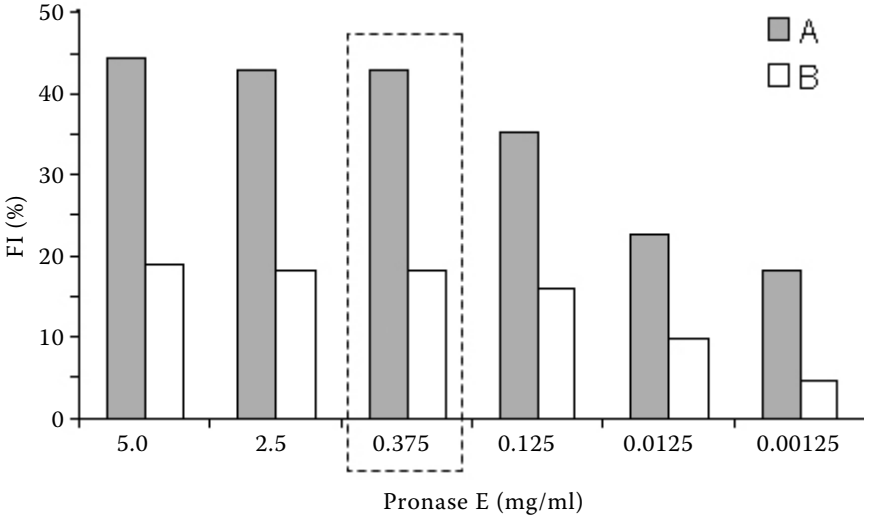
Measurement of total fluorescence intermediate compound. Total FIC (free + linked-to-protein backbone) determination was based on the procedure proposed by Morales and van Boekel (1997), involving enzymatic hydrolysis with pronase E. After different tests to study the

adequate pronase E concentration (from 5 mg/ml to 1.25 µg/ml) and incubation time (from 0 h to 72 h), the procedure was finally set as follows: 100 mg of sample were digested with 3 ml of a 0.375 mg/ml pronase E solution (1500 U/ml in 1M sodium-borate solution, pH 8.2) in a stoppered test tube at 40°C for 36 h in a water bath under shaking. After cooling, the solution was centrifuged at 4500 g for 10 min at 4°C. Three different supernatant clean-up procedures were assayed (simple filtration with 0.45 µm filter; precipitation with 12% trichloroacetic acid solution (w/v) and/or with Carrez I (potassium ferrocyanide, 15% w/v) and Carrez II (zinc acetate 30% w/v solutions), and filtering (0.45 µm) was finally selected based on the efficiency of the clarification observed. Then, the samples were adequately diluted (1/50) to prevent quenching effects. The final solutions were measured at the excitation wavelength of 347 nm and emission wavelength of 415 nm. The linearity of the fluorescence response was checked with a quinine sulphate solution of 1 µg/ml dissolved in 0.1 mol/l H_2SO_4. This solution was assigned 100% of the relative fluorescence intensity (FI) and the results were expressed as percentage of the relative fluorescence in respect to the quinine sulphate solution. The data were also expressed as total FIC per mg of sample. A fluorescence spectrophotometer (SMF-25, Kontron Instruments, Milan, Italy) was used for the determination of fluorescence. Quartzglass cuvettes (QS-1.000 Suprasil, Hellma GmbH & Co, Germany) with the light path of 1 cm were used. An average of three independent readings was recorded.

The residual standard deviation for the total FIC determination in breakfast cereals was 3.32%.

Measurement of free fluorescence intermediate compounds. Free FIC were determined based on Morales and van Boekel procedure (1997). Briefly, 500 mg of the sample were suspended in 5 ml of deionised water in a 10 ml centrifuge tube. The tube was shaken vigorously for 1 min and clarified with 0.25 ml each of Carrez I and Carrez II solutions. The resulting mixture was centrifuged at 4500 g for 10 min at 4°C. The supernatant was transferred in a 10 ml volumetric flask and two further extractions were performed using 2 ml of deionised water. The supernatants were combined and the volume was made up to 10 ml with deionised water. The solution was filtered (0.45 µm) and adequately diluted (at least 1/16) to prevent quenching effects. Afterwards, the measurement of fluorescence was done following the conditions mentioned above. The results were also expressed as percentage of relative fluorescence in respect to the 1 µg/ml quinine sulphate solution and as free FIC per mg of sample.

Statistical analysis. Analyses were performed at least in triplicates. Statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by Duncan test to compare the means that showed a significant variation ($P < 0.05$).



Pronase E (mg/ml)	Total FI/mg sample	
	sample A	sample B
5.0	2182	929
2.5	2097	882
0.375	2100	886
0.125	1729	788
0.0125	1113	480
0.00125	886	230

Figure 1. Percentage of fluorescence intensity (FI) in breakfast cereals A and B after enzymatic hydrolysis at different pronase E concentrations (expressed as percentage of relative fluorescence with respect to 1 µg/ml quinine sulphate solution, assigned to 100% of relative fluorescence)

Analyses were performed using Statgraphics Plus, Version 5.1, 2001.

RESULTS AND DISCUSSION

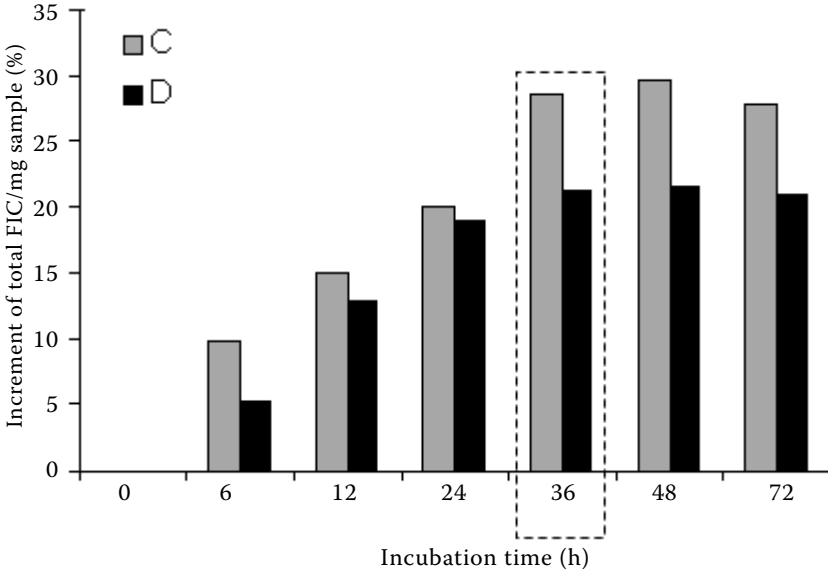
Optimisation of enzymatic hydrolysis procedure for total FIC determination

Six different pronase E concentrations were tested with the aim to reach an efficient digestion of the A and B samples ranging from 5.0 mg/ml to 1.25 µg/ml (Figure 1). The highest pronase E concentration achieved the highest release of fluorescent compounds, with maximum value of fluorescence of 2182 and 929 total FIC/mg in the sample for A and B breakfast cereal, respectively. These values were practically unmodified when pronase E concentration was decreased to 0.375 mg/ml (1500 PU/ml), since the data differed only by 3.80 and 4.64% from those recorded after digestion with 5 mg/ml pronase E solution for samples A and B, respectively. Therefore, the pronase E concentration was set at 0.375 mg/ml.

Once the assay was carried out with the pronase E concentration selected (1.12 mg pronase E/100 mg sample; 45 PU pronase E/mg sample), seven incubation periods were tested to investigate the optimal time for the enzyme action in cereal-based samples. As presented in Figure 2, total FIC per mg in samples C and D increased with the incubation time, reaching maximum after 36–48 h (1494 and 1697 FIC/mg with samples C and D, respectively), and showed a slight reduction after 72 h of incubation (1473 and 1689 FIC/mg with samples C and D, respectively). Since the values after 36 h of incubation represent more than 99% of those found after 48 h and also offered a shortened time to carry out the measurement, this incubation time was selected.

Optimisation of sample clean-up procedure for total FIC determination

Before total FIC measurement, the adequate clarification of the samples after the enzymatic hydrolysis was studied. This step was necessary to avoid possible quenching effects, apart from appropriate sample dilution. In this sense, three different procedures to improve the supernatant clean-up were tested with A and B samples: the addition to the supernatant after the enzymatic digestion of 12% trichloroacetic acid solution (w/v), of Carrez I and II, and filtration using 0.45 µm nylon filters. After the clean-up, the samples were centrifuged at 4500 g and 4°C for 5 min and the measurements were performed. The highest total FIC values for samples A and B were shown by the application of a single filtration procedure (2100 and 886 total FIC/mg in the sample, respectively) (Table 1). After the first and second clarifying steps, significant losses in total FIC detection were manifested, ranging from 19.0% to 71.8%



Incubation time (h)	Total FIC/mg sample	
	sample C	sample D
0	1152	1396
6	1266	1469
12	1325	1573
24	1383	1660
36	1481	1693
48	1494	1697

Figure 2. Percentage of increment of total FIC/mg in C and D breakfast cereals at different incubation periods used for enzymatic hydrolysis (0.375 mg/ml pronase E)

Table 1. Total FIC values after different clarifying procedures on samples A and B. Percentage of loss of fluorescent compounds

Procedure	Sample A		Sample B	
	total FIC[1]	loss FIC[2]	total FIC[1]	loss FIC[2]
Filtration (0.45 μm)	2100	–	886	–
Precipitation (12% TCA)	1273	39	718	19
Precipitation (Carrez I and II)	592	72	333	62

[1]Expressed as total FIC/mg of breakfast cereal; [2]expressed as % of total FIC after filtration procedure, assigned 100%

of the total amount determined after filtration. Therefore, only the filtration procedure of the supernatants can be applied to clarify the samples since the addition of the precipitant agents seems to induce the inactivation or destruction of the fluorescent structure.

This study about the enzymatic hydrolysis conditions as well as the sample clean-up procedure ratify the method used in the previous works by our research group, designed just on the base of related literature (Delgado-Andrade *et al*. 2006).

Effect of a basic/acid media on the stability of free FIC

Additionally, seven different basic/acidic media (pH between 1.5–12.2) were tested on two randomly selected samples after free FIC de-

Table 2. Content of free and total FIC in commercial breakfast cereals (mean ± SD)

Breakfast cereal	Flour	Free FIC/mg	Total FIC/mg	Protein (%)	Fibre (%)[1]
1	mixture	697 ± 18	1925 ± 64	11.54	31.3
2		198 ± 10	1886 ± 11	11.41	20.2
3		92 ± 8	550 ± 7	7.19	7.8
4		120 ± 8	1230 ± 25	8.08	6.5
5	rice	30 ± 0	657 ± 30	5.96	1.0
6		46 ± 3	780 ± 6	4.70	2.5
7		115 ± 8	1042 ± 6	5.29	–
8	corn	69 ± 0	624 ± 35	5.17	2.0
9		84 ± 2	798 ± 8	4.30	1.5
10		96 ± 4	1102 ± 2	7.36	3.0
11		137 ± 5	716 ± 12	5.51	2.0
12		40 ± 1	381 ± 2	5.74	3.0
13	wheat	231 ± 11	1657 ± 37	12.75	28.0
14		303 ± 1	2214 ± 33	9.14	15.0
15		77 ± 5	1229 ± 42	8.74	10.0
16		212 ± 9	1569 ± 4	11.69	22.0
17		301 ± 1	2409 ± 72	14.50	28.0
18		98 ± 4	1886 ± 92	13.96	35.0

[1]Values declared by industry. Hyphen indicates no declared data

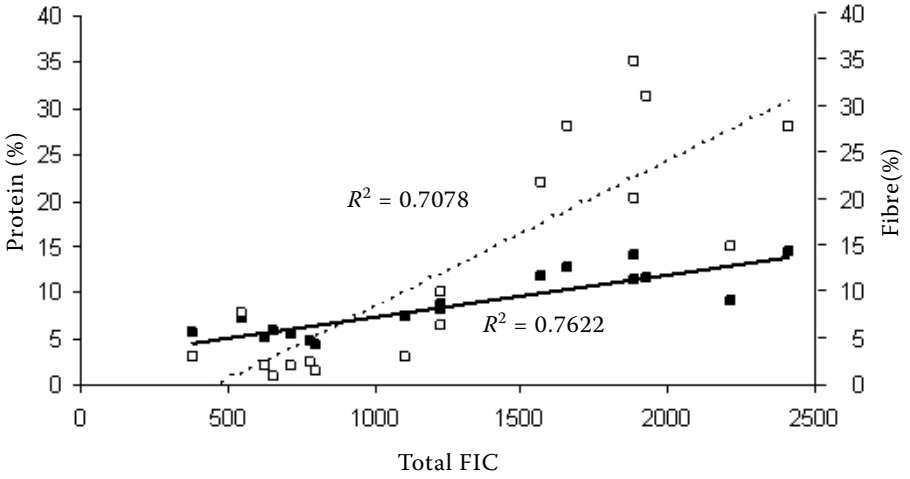


Figure 3. Relationship between total FIC per mg sample and protein (%) (right) or fibre content (%) (left) in the commercial breakfast cereals assayed

termination in order to evaluate the stability of FIC after 1 h and 24 h at room temperature. Incubation with sodium-borate (pH 8.2 and 9.5), sodium-phosphate (pH 7.5) or sodium-acetate (pH 3.5 and 5.0) buffers (0.05M) did not exert a significant reduction of the parent fluorescence (data not shown). However, incubation with sodium hydroxide (0.1N) or hydrochloric acid (0.1N) showed a reduction in the fluorescence measurement of up to 20%.

Application of the optimised procedure of total FIC analysis to breakfast cereals

Free FIC determination. Free and total FIC were analysed in eighteen commercial ready-to-eat cereals. The results are expressed as free or total FIC/mg of the sample in Table 2. The mean FIC values both for free and total fluorescent compounds in the samples assayed were 163 ± 157 and 1258 ± 622, respectively (mean ± SD), ranging from 30 to 697 in the case of free, and from 381 to 2409 in the case of total FIC. According to the type of flour used in the manufacture, the breakfast cereals made from wheat showed the highest total FIC (mean value 1827), followed by those made from a mixture of flours (mean value 1398). Wheat is the cereal with the highest protein content (the mean value of protein among the breakfast cereals made from wheat was 11.8%), which supports the finding that the increased production of fluorescent compounds is linked to the protein content in the formula. In this line, a high correlation was found between the protein content and total FIC (Figure 3) (n = 18; R = 0.8730; P < 0.001). Fluorescence associated with MRPs is clearly distinguishable from that associated with tryptophan (excitation 290; emission 336 nm). Another surprising relationship was shown between total FIC and the fibre content (Figure 3) (n =18; R = 0.8413; P < 0.001), and a little weaker for free FIC (n =18; R = 0.7209; P < 0.01). Therefore, the fibre enrichment of breakfast cereals which leads to an increased protein fraction enhances the rate of Maillard reaction. In this sense, it was observed that the samples with high fibre contents corresponded to the highest protein contents (1, 2, 13, 16, 17, and 18) and presented the highest total and free FIC values.

The study of fluorescence associated with the Maillard reaction development is a topic of growing interest for the scientific community, since the fluorescence measurement is considered a potential tool for addressing the key problems of food deterioration as well as an early marker or index of the damage to biomolecules (Matiacevich *et al.* 2005). In this sense, the determination of several fluorescent AGEs have been employed in medical sciences, such as pentosidine, crossline, pentodilysine, pyrropyridine, and 2-(2-furoyl)4(5)-(2-furanyl)-1H-imidazole (FFI). Related to foodstuffs, Rufián-Henraes *et al.* (2002) performed fluorimetric measurements using the supernatant obtained after protein precipitation with trichloroacetic acid on enteral formula and assigned high fluorescence values to UHT and sterilised formulas. Ferrer *et al.* (2005) employed the fluorescent measurement, among other indexes, to monitor the Maillard reaction development during the storage of infant formulas. Similarly, Morales *et al.* (1996) studied fluorescence associated with Maillard reaction in milk and milk

resembling systems, exhibiting free FIC values lower than those found in this work, probably due to the intense processing conditions applied during the manufacture of breakfast cereals. Birlouez-Aragón *et al*. (2001) also investigated the fluorescence production in ready-to-eat cereals, but due to the application of the FAST method, based on front face fluorescence, their results are not comparable with those in the present study.

CONCLUSIONS

This paper presents an optimised and checked method to determine total FIC, evaluating non-specifically the appearance of advanced and final MRP. The adequate ratio between pronase E-sample and the incubation time for the enzymatic hydrolysis has been established for breakfast cereals as a model of cereal-based products, as well an adequate procedure for the sample clean-up and clarification. After the successful application of the standardised method, a high correlation is manifested between the protein and fibre contents in the formulation of breakfast cereals and the total FIC production.

Although Maillard reaction is often desired during the breakfast cereal manufacture, the method can be suitable for manufacturers in order to maintain the adequate rate of Maillard reaction and control the thermal damage throughout the different steps of the breakfast cereals production, as well as in the final products. Moreover, it represents a fast and accurate procedure for the study of the extent of Maillard reaction in this foodstuff.

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