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Review

Chromatographic analysis of Maillard reaction products

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ABSTRACT

This paper reviews the products of the Maillard reaction (non-enzymic browning) which are most frequently analysed in food quality control. In particular, high-performance liquid chromatographic (HPLC) methods are reported for 5-(hydroxymethyl)-2-furfural and ε-N-2-furanylmethyl-L-lysine, early indicators of the Maillard reaction, ε-pyrrolelysine, an advanced indicator of the Maillard reaction, and free and oxidized N-acetylmethionine, a food additive used to overcome the degradation of amino acids. Some analytical methods are evaluated in comparison with HPLC.

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1. INTRODUCTION

There are several types of non-enzymic browning. One of them is caused by Maillard reaction [1–12], which is initiated by the combination of an amino acid with a sugar according to the following general route: sugar + amino acid → Amadori compounds → pigments [13–24]. The amino acid may be present in the food either free or as part of a protein. The sugar must contain a reactive carbonyl group.

The pigments formed are flavourful and brown or black in colour. Contrary to popular opinion, the Maillard reaction does not require high temperatures; thus sugars and amino acids, even when stored at refrigeration temperatures, can show signs of non-enzymic browning. The reaction rate, however, increases markedly with increase in temperature and with decrease in moisture level, although browning can also proceed in aqueous solution [15–30].

A second type of browning is caramelization.

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When sugars are heated at high temperatures they turn first brown and then black. This reaction involves the dehydration or removal of water from the sugar, which by various reactions results in the formation of furfural. A third type of browning is caused by ascorbic acid, which, once oxidized, forms the same type of compounds as already described.

All this can only give a very general idea of one of the most complex and most investigated phenomena of food chemistry. High-performance liquid chromatographic (HPLC) methods are reported here for determining some Maillard reaction products which are of paramount importance for the evaluation of parameters related to food processing and storage (time and temperature abuses) [31–34].

2. EARLY INDICATORS OF THE MAILLARD REACTION

2.1. *5-(Hydroxymethyl)-2-furfural (HMF)*

HMF is an intermediate formed in the early stage of non-enzymic browning. In particular, its amount in canned products has been suggested as giving an indication of the extent of damage to the product caused by excessive heat during processing or subsequent storage. Thus the HMF content may be used as a quality criterion for a wide range of products (fruit juice, tomato products, soft drinks, honey etc.) [35–39]. Two chromatographic procedures are available for HMF determination.

The sample for analysis is diluted according to its concentration [1:4 (v/v) for a fruit juice], 2 ml each of Carrez I and II reagents are then added and the volume is made up with distilled water. The Carrez clarifying agent consists of two aqueous solutions, one of 15% (w/v) potassium hexacyanoferrate (II) (Carrez I) and the other of 30% (w/v) zinc sulphate (Carrez II). The sample is centrifuged for 2 h at 40 000 *g* and 1 ml of clear supernatant is pipetted into a syringe and filtered through a 0.45-μm disc filter before injection.

The chromatographic conditions for the first method [40] can be specified as follows: column type, C_{18} (250 mm × 4 mm I.D.) with a mean particle diameter of 10 μm; eluent, water–methanol (90:10, v/v) which has been previously degassed and filtered through a 0.45-μm filter, with isocratic elution at 1.5 ml/min; detection, UV at 285 nm and injection volume, 10 μl. The limit of detection of the method is 0.1 mg/l. A typical result is presented in Fig. 1.

The second method [41] was developed to determine both furfural (another intermediate particularly important with low-pH foods) and HMF. In this version the chromatographic conditions can be specified as follows: column type, C_{18} (100 × 8 mm I.D.); eluent, acetonitrile–water (15:85, v/v) (degassed and filtered) with isocratic elution at 2.0 ml/min; detection, UV at 280 mm and injection volume, 20 μl. The results are exemplified in Fig. 2.

2.2. *ε-N-2-furanylmethyl-L-lysine (FUR) (furosine)*

Furosine forms during the acid hydrolysis of ε-deoxyfructosyllysine (ε-DFL), the most stable Amadori compound in early Maillard reaction. The level of FUR in heat-treated products is related to processing and storage conditions and may be further converted into nutritionally unavailable lysine [42–53]. Hence FUR may represent a suitable indicator of the quality of various products, in particular dairy products, dry pet foods, snack bar and dry gravies.

The sample for analysis is treated in the following

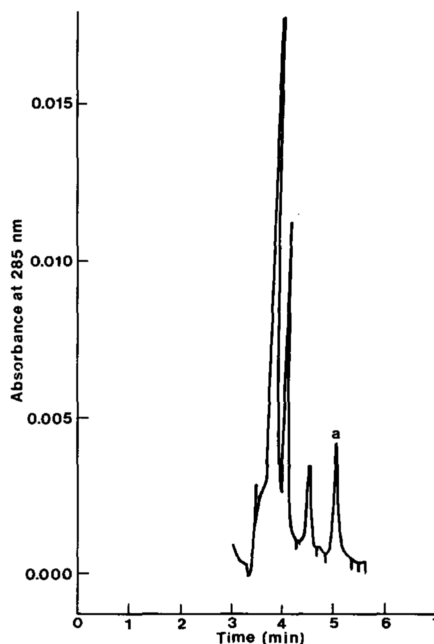

Fig. 1. HPLC profile of tomato paste on a C_{18} silica column. Peak a = HMF (5.1 min).

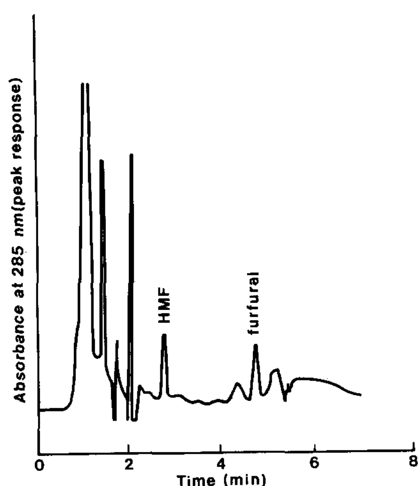

Fig. 2. HPLC profile on a C_{18} silica column of orange juice stored for 24 weeks at 40°C.

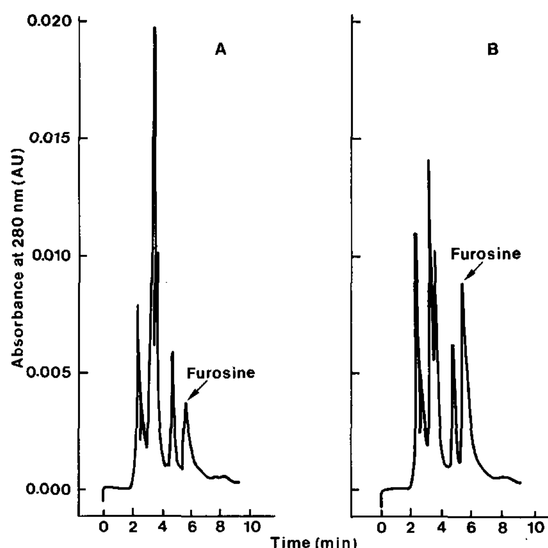

Fig. 3. HPLC profiles of acid hydrolysates of powdered meal replacer products (chocolate flavour) on a C_8 silica column. (A) Control; (B) 20 weeks of storage at 45°C.

way: 0.1–0.5 g of sample containing *ca.* 20 ml of protein is weighed into a 25-ml screw-capped vial, 10 ml of 6 *M* HCl are added and the mixture is heated in an air oven at 110°C for 20 h. Before injection, the filtered hydrolysate is diluted with 3 *M* HCl to a protein concentration of 1–2 μg/μl. The chromatographic conditions can be specified as follows [43]: column type, C_8 (250 × 4.6 mm I.D., 10 μm); eluent, 0.5% acetate buffer prepared by dissolving sodium acetate in water purified in a Milli-Q system (Millipore, Bedford, MA, USA) and adjusting the pH of the solution to 4.3 with glacial acetic acid; elution is carried out isocratically at 2 ml/min; injection volume, 50 μl and detection, UV at 280 nm. The limit of detection of the procedure is: 0.1 μmol/g. A typical chromatographic separation is shown in Fig. 3.

3. DETERMINATION OF ε-PYRROLELYSINE IN PROCESSED FOOD AS AN INDICATOR FOR THE ADVANCED MAILLARD REACTION

During the first stage of the Maillard reaction, Amadori compounds (1-amino-1-deoxy-2-ketoses) form. The bound amino acid in these compounds is not available as a source of amino acid, which results in a decrease in the nutritional value of foods [54–57]. To date Amadori compounds have been investigated in relatively few biological products owing to the tedious procedures required for their isolation and purification. Hurrel and Carpenter [58] called the "early Maillard reaction" products those leading to the formation of Amadori compounds and "advanced Maillard reaction" products those formed in the subsequent steps. ε-Pyrrolelysine, a glucose lysine Amadori compound, has been identified in the water-soluble fraction of many processed foods. Furosine forms during early Maillard reactions and decomposes as the reactions progress. Furosine determination alone cannot be used to evaluate the extent of Maillard reaction especially in long-term stored products. The following method was applied to various stored samples such as non-dairy cream, dry dog food, instant gravy, powdered meal replacer, dried milk, hot cocoa mix and semi-moist sauce.

The measured ε-pyrrolelysine level in each product reflected the shelf-life and storage conditions closely. Moreover, the ε-pyrrolelysine level maintained its upward trend even under severe heat treatment (110°C), which suggests that it may serve as an indicator of advanced Maillard reaction. ε-Pyrrolelysine standard can be synthesized according to Nakayama *et al.* [56].

The sample preparation method is different for

different products and two examples are given here, for powdered foods and non-dairy cream.

For powdered foods 1 g of sample is weighed in a 20-ml screw-capped vial and after addition of 8 ml of warm (60–65°C) water is vortex-mixed for 1 min. The solution is then deproteinized by the addition of 1 ml of $ZnSO_4$ solution (33.4 g per 100 ml) and 1 ml of potassium hexacyanoferrate (II) solution (17.29 g per 100 ml) and centrifuged at 28 000 *g* for 15 min. The supernatant liquid is filtered through a Millipore filter (0.45 μm) and analysed by HPLC. Results are shown in Fig. 4.

With non-dairy cream samples, a small glass-wool-plugged funnel is placed on top of a 20-ml screw-capped vial, 1 g of the sample is weighed and placed in the funnel and 3 × 4 ml of $CHCl_3$ are added. With each addition, the $CHCl_3$ is allowed to drain slowly into the vial and then discarded. The sample is air-dried to remove the remaining $CHCl_3$. After addition to the sample of warm (60–65°C) water, a disposable pipette is used to push the glass-wool at the top of the funnel down into the vial and the same pipette is used to remove the remainder of the sample from the funnel. The sample is then deproteinized as described, the funnel is removed and the vial is capped. After vortex mixing, the solution is centrifuged and the supernatant is filtered

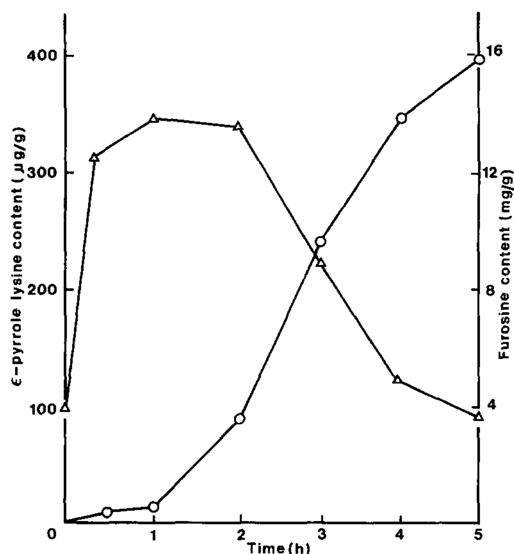

Fig. 4. (○) ε-Pyrrolelysine and (△) furosine contents of powdered meal replacer (vanilla flavour) heated at 110°C for up to 5 h.

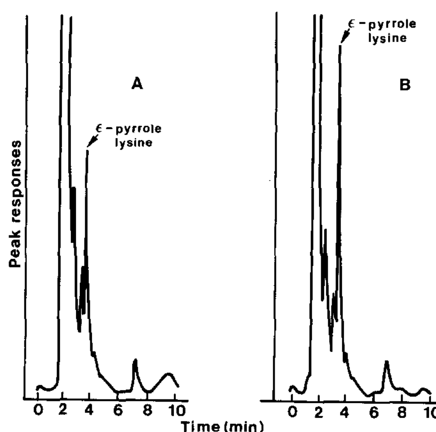

Fig. 5. HPLC of non-dairy creamers on a C_8 silica column. (A) 8 weeks of storage at 22.2°C; (B) 8 weeks of storage at 45°C.

through a Millipore filter (0.45 μm) and analysed by HPLC. Results are shown in Fig. 5.

The separation of ε-pyrrolelysine is carried out under the following conditions: column type, C_8 (250 × 4 mm I.D., 7 μm); eluent, 0.5% acetate buffer prepared by dissolving sodium acetate in Millipore-filtered water and adjusting the pH of the solution to 4.3 with glacial acetic acid; elution is carried out isocratically at 1.6 ml/min; detection, electrochemical with the guard cell voltage set at 0.7 V; applied voltage, 0.55 V.

4. SEPARATION OF BROWNING PIGMENTS

The brown pigments isolated from the model reaction between aldoses and amino acids [54,59–62] proved to be insoluble in common organic solvents; some were readily soluble in water, some slightly soluble and others insoluble. The soluble pigments were isolated by dialysis; the proportion of non-dialysable to dialysable pigment increased as the reaction proceeded. Pigments can also be isolated by passing the reaction mixture through a column of the dextran molecular sieve Sephadex G-25 Pharmacia (Uppsala, Sweden). The best separations achieved were similar to those obtained by dialysis.

As far as natural systems are concerned, a brown pigment was isolated from darkened diced apricots [63] by extracting the fruit with 50% hot acetone and after extraction with 95% and 75% ethanol. The pigment was precipitated with acid, or by addi-

TABLE 1

TERNARY SOLVENT SYSTEM FOR THE SEPARATION OF THE BROWNING PIGMENTS FORMED IN STORED FRUIT JUICE

Time	Water (%)	Acetonitrile (%)	Tetrahydrofuran (%)
0	99	0	1
5	97	0	3
38	89.5	3.5	7
78	70	5	25
98	55	5	40
103	0	90	10
108	0	90	10
111	99	0	1

tion of acetone, and was purified by dissolution in dilute alkali and precipitation with dilute acid. The yield was 5–7% of the dry mass of the fruit. The type of amounts of pigments are container dependent [64,65].

Before analysis the sample is treated in the following way: 50 ml of the sample are freeze-dried, the residue is extracted with 30 ml of methanol, filtered through Whatman (Maidstone, UK) No. 42 filter-paper and then made up to volume with methanol in a 50-ml volumetric flask. Samples are filtered through a 0.45-μm filter and stored at −10°C until analysis. The following conditions of the chromatographic separation are applied: column type, C_{18} (150 × 4 mm I.D., 3 μm) and C_{18} precolumn (50 × 4 mm I.D.); eluent, a ternary solvent system consisting of water, acetonitrile and 50% aqueous THF as specified in Table 1; flow-rate, 1.0 ml/min; all gradient rates are accomplished linearly; sample injection volume, 15 μl; detection, UV–VIS at 436 nm. The results of the chromatographic separation are presented in Figs. 6 and 7.

Another category of naturally occurring pigments are the melanoidins from browning prune pulp [66]. Their chromatographic separation can be effected in the following way: a 4.5-g sample of pulp is cut into small pieces and ground for 3 min in an Omni mixer in 60 ml of 50% ethanol. The suspension is left to stand at room temperature for 24 h with occasional shaking and is finally centrifuged at 27 500 *g* for 20 min at 5°C. The extracts are partially purified as follows: 1 ml of 10% lead acetate and 9 ml of 2% acetic acid are added to 10 ml of prune extract and mixed thoroughly. The precipitate formed is removed by centrifugation at 2500 *g* for 5 min, an equal volume of 95% ethanol is added to the supernatant and the precipitate is removed again by centrifugation. The supernatant is concentrated to about 30 ml under vacuum at 37°C to remove the acid and diluted to 60 ml with distilled water. Successive concentration and dilution are repeated until the final pH reaches *ca.* 5.0 to eliminate

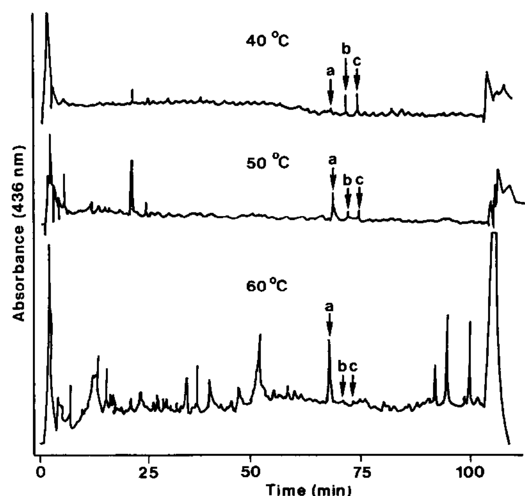

Fig. 6. HPLC on a C_{18} silica column of grapefruit juice browning pigments formed in glass bottles when stored at 40, 50 and 60°C for 12 weeks. a–c = Different browning pigment entities.

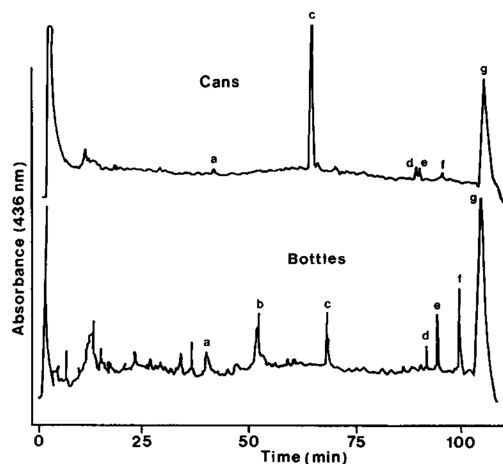

Fig. 7. HPLC on a C_{18} silica column of browning pigments formed in grapefruit juice stored in glass and cans at 60°C for 9 weeks. a–g = Different browning pigment entities.

most of the acetic acid. The pH of the extract is then adjusted to 3.0 with 2 *M* HCl in order to allow the adsorption of the phenolic compounds on Sephadex LH-20. The extracts are then passed through a Sephadex LH-20 column (40 × 2.5 cm I.D.) at a flow-rate of 1 to 2 ml/min. The non-adsorbed compounds are concentrated to 60 ml under vacuum at 37°C. The pigments adsorbed on the column are eluted with about 300 ml of acetone–water (1:1, v/v) and concentrated to 60 ml. The solution containing non-Sephadex-adsorbed compounds are passed through a Dowex 50 (H^+) ion-exchange column (15 × 2 cm I.D.) (Dow Chemical, Midland, MI, USA). After elution with distilled water, the eluate is concentrated to 60 ml. The pigments retained on the Dowex 50 (H^+) column are eluted with 300–400 ml of 1 *M* ammonia solution and the resulting eluate is concentrated to 60 ml.

5. DETERMINATION OF N-ACETYLMETHIONINE (NAM) [67–71]

The protein value of foods limited in methionine content can be improved through fortification with free methionine, but to relieve this deficiency the addition frequently makes the foods impalatable for human consumption. These flavour effects [72,73] are caused by Maillard browning and Strecker degradation reactions, which yield volatile sulphides [74]. Methional (broth-like odour) has been identified as a major product from the Strecker degradation of methionine. The nutritional losses and the flavour problems associated with methionine fortification could be overcome by using a methionine derivative, N-acetyl-L-methionine (NAM), which has been recommended [75] and approved as a food additive [76]. The protected amino group of NAM is relatively stable to Strecker degradation and offers higher sensory thresholds than methionine in amino acid-fortified foods.

The following conditions were developed to determine NAM in model systems: column type, C_8 (25 × 4.6 mm I.D., 10 μm); eluent, phosphate buffer (0.01 *M*, pH 7.0)–acetonitrile (70:30, v/v) with isocratic elution at 2.0 ml/min (retention time *ca.* 4 min); detection, UV–VIS at 254 nm; injection volume, 10 μl. The results shown in Fig. 8 indicate that NAM offers much greater stability than methionine to Maillard browning.

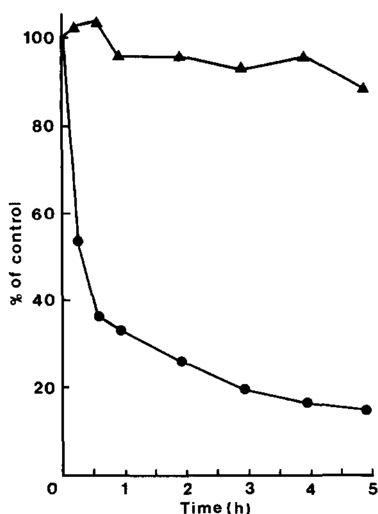

Fig. 8. Recovery of (▲) N-acetylmethionine (NAM) and (●) methionine in a model system containing glucose heated at 95°C.

6. COMPARISON BETWEEN OPTICAL AND CHROMATOGRAPHIC METHODS IN THE DETERMINATION OF SOME INTERMEDIATES OF THE MAILLARD REACTION

6.1. *Hydroxymethylfurfural [40]*

In the determination of HMF, the commonly employed spectrophotometric method is that of Winkler [77], which is based on the use of particularly toxic reagents such as barbituric acid and *p*-toluidine solutions, to be prepared daily. With this method, the recoveries are highly variable and in any case do not exceed 83%. The first important limitation to the Winkler method is the difficulty in following the colour reaction accurately over time because maximum colour intensity is obtained within an interval of 1–4 min, so its optimum recording is not always possible when large amounts of samples are to be analysed.

The linear regression equation ppm HMF (Winkler) = $-3.30 + 0.96$ ppm HMF (HPLC) (R = 0.99) can be used to convert data correctly from one method to the other.

Typical results are shown in Figs. 9 and 10.

6.2. *Absorbance measurement at 420 nm*

Generally, the intensity of browning based on absorbance measurement at 420 nm is largely in agreement with the visual observation of the products

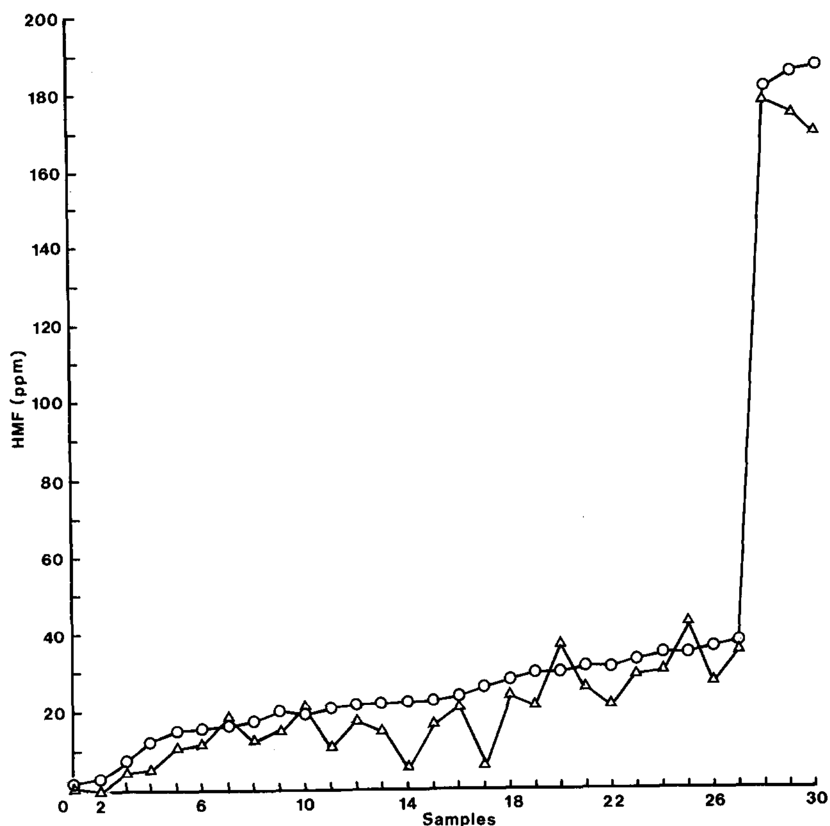

Fig. 9. HMF contents (mg kg^{-1}) obtained by two methods (△ = Winkler spectrophotometric method and ○ = HPLC on a C_{18} silica column) in tomato paste samples of different concentrations.

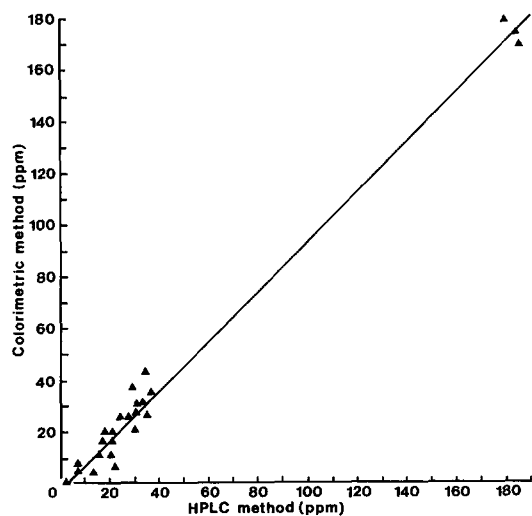

Fig. 10. Correlation between the spectrophotometric (Winkler) and HPLC (on a C_{18} silica column) methods.

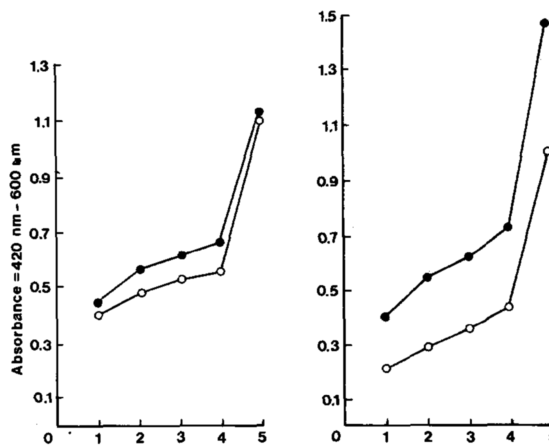

Fig. 11. Correlation between the visual colour tone index (sensorial) and the absorbance of dried apricots. Colour (abscissa): 1 = yellow-golden; 2 = yellow-amber; 3 = light brown; 4 = brown; 5 = dark brown.

(fruit in particular). For fruit juice, extraction is simply carried out by diluting the samples to 2.5°Brix and filtering, whereas with solids, *e.g.*, dried apricots, extraction is carried out on cut samples using 50% ethanol and ultracentrifuging at 30 000 *g* for 20 min at 5°C. Sometimes the absorbance at 420 nm is corrected for turbidity by subtracting the absorbance at 600 nm.

Fig. 11 shows the correlations between visual colour tone (sensorial) index and absorbance (420 and 600 nm).

7. CONCLUSIONS

Further investigations on non-enzymic browning (Maillard reaction) are still required above all to elucidate the numerous and complex reaction mechanisms and the nature and composition of the compounds formed. Studies in progress mainly concern the determination of volatile compounds from interactions in the Maillard reaction and these results will provide a better understanding of some of the chemistry taking place during the cooking of food. Another step to be taken should be towards the development of simple multi-component techniques so as to give a greater number of food manufacturers the possibility of controlling the Maillard reaction by using up-to-date methods. However, there are some chemical parameters that are well suited to the requirements of quality control laboratories as regards the identification of damage caused to food products by the Maillard reaction, and this paper has dealt with the determination of some of these parameters.

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