

INDRIATI EKASARI

THE INNER QUALITY OF ORANGE JUICE AND APPLE JUICE

Assessment by the use of microbiological methods

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Promotor : dr. W. Pilnik,
hoogleraar in de levensmiddelenleer

Co-promotor : dr. W.M.F. Jongen,
universitair docent toxicologie

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INDRIATI EKASARI

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ASSESSMENT BY THE USE OF MICROBIOLOGICAL METHODS

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen
op gezag van de rector magnificus
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in het openbaar te verdedigen
op dinsdag 5 september 1989
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STELLINGEN (THEOREMS)

1. The inner quality test for fruit juices can be compared to an early pregnancy test. Both indicate an inevitable sequence of events at a time when these are not yet visible.

This thesis, chapters 5 and 7.

2. The mutagenicity of orange juices reported in this thesis is only observed towards *Salmonella typhimurium* strain TA100 after 4 hr preincubation at 37°C and pH 7.4. Therefore it does not apply to human consumption of orange juices.

This thesis, chapters 4, 5, 6, 7 and 8.

3. Automation of *Salmonella* mutagenicity test using natural medium such as orange juice can not be based on the growth of bacteria. The use of another principle must be considered.

This thesis, chapter 9.

4. The finding of antimutagenic factors in apple juice may increase the economic value of apples.

5. The concept of minimally processed food should be reevaluated in view of the formation of antimutagenic factors resulting from heat treatments.

6. The balance between mutagenic and antimutagenic foods should be considered part of dietary recommendations.

7. The significance of Maillard reactions encompasses food processing, nutrition, toxicology and physiology.

4th International Symposium on the Maillard reaction (1989).

8. The lay-public is not sufficiently aware of the difference between mutagenicity and carcinogenicity.

9. The program of food technology education should be extended to include food toxicology.

10. The philosophy of becoming a vegetarian to reduce cancer risks has no scientific basis.

*Proefschrift van Indriati Ekasari
The inner quality of orange juice and apple juice:
Assessment by the use of microbiological methods
Wageningen, 5 September 1989.*

ABSTRACT

A modified *Salmonella* mutagenicity assay and a cytotoxicity test were developed to determine the concentration of sensorially inactive Maillard Intermediate Products (MIP) in fruit juices. The formation of MIP is initiated by heat treatments necessary for pasteurization and thermal concentration. In industrial and laboratory prepared orange juices their concentration was seen to be related to the intensity of the heat treatment and to allow a shelf-life prediction in relation to non-enzymatic browning (inner quality). The microbial MIP assay(s) are therefore useful for orange juice manufactures to select concentrated juices for reconstitution and packing and to test equipment and processes. The modification of the tests involve preincubation with the tester bacteria for 4 hr at pH 7.4 and 37°C. Under natural acidic condition orange juice is not mutagenic.

It was further seen that the heat treatments did not hydrolyze orange glycosides to mutagenic aglycones which could have influenced the assay. MIP were characterized to be polar, non volatile compounds which carry no charge and have a molecular weight from 200 to 700 daltons. Automation of the mutagenicity assay by a turbidometric assay system was seen to be only possible for gel filtration and HPLC fractions of juice; in whole orange juice endogenous growth factors and cytotoxicity disturbed the assay. On the other hand measurement of cytotoxicity of whole juice could be carried out conveniently by a kinetic conductance method. Heat treated laboratory prepared apple juice also contained mutagenic fractions but the whole juice had a positive antimutagenic balance. There was a heat load and dose related reduction of the mutagenicity of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) and 4-NQO (4-nitroquinoline-N-oxide) in the presence of apple juice. These findings give new aspects to apple juice as a healthy food.

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My deep feelings of gratitude go to my mother, sisters and brothers for encouraging me to be a Food Scientist.

Wageningen, July 1989

Indri Ekasari

Dedicated to

my dear Mum, Silda, Peter,
Ridwan & Lilia,

and to

The Memory of my late Father

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1 GENERAL INTRODUCTION

The fruit juice industry is a high technology growth industry (Schobinger, 1987). Its products have to compete with numerous other beverages but seem to fulfill the modern trend in consumer expectation for natural and nutritious beverages. Thus in the USA per capita consumption of 100 percent fruit juice in 1986 was nearly 29 litres having shown a steady growth rate of 2.6 percent yearly since 1982 (Bellas, 1988). In the FRG the 1986 per capita consumption exceeds the USA figure by 10 percent having developed at a similar rate from 22 litres in 1982 (Wingenfeld, 1988).

Most of the juices are shipped and stored as chilled or frozen concentrates and reconstituted for distribution to the consumer as single strength juices (Sizer et al., 1988). Orange juice and apple juice are the market leaders.

Like all foods, (concentrated) fruit juices have a limited shelf-life. Seen their composition (sugars, amino acids, ascorbic acid) quality deterioration is mainly due to Maillard-type reactions which at non refrigerated storage result in browning and off-flavours. There is a lag period for browning in which early and intermediate Maillard products are formed which are not sensorially perceived and in which juices are still of good exterior quality (good colour and flavour). Their future deterioration due to the further proceeding of Maillard reactions, is however inevitable. This fact constitutes an intrinsic quality factor for which I have chosen the term "inner quality". It signifies the shelf-life expectation of a good exterior quality fruit juice which under equal external circumstances depends on the type and concentration of MIP, the sensorially inactive Maillard intermediary products.

For the formation of MIP the short time-high temperature treatment for thermal concentration and for pasteurisation (Fig. 1.1) play an important role. While modern technology

allows these operations to be carried out without loss of exterior quality, they do have an impact on the inner quality. A higher heat load will create more MIP. A great number of studies exist dealing with the rate of quality loss, mainly of orange juice. Methods used such as colour change measurement (Kanner et al., 1982; Mannheim & Passy, 1979; Meydev et al., 1977; Robertson & Reeves, 1981), determination of furfural (Kanner et al., 1982; Nagy & Randall, 1983) and hydroxymethylfurfural (Lee & Nagy, 1988a; Wucherpfennig & Burkardt, 1983) and determination of loss of ascorbic acid (Curl, 1949; Maeda & Mussa, 1986; Saguy et

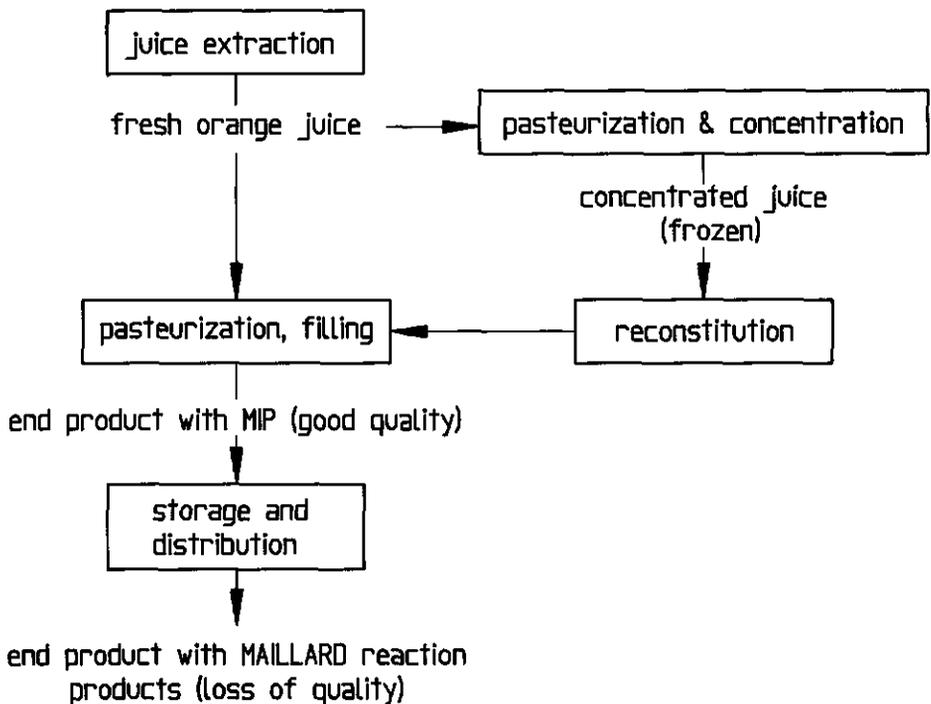


Figure 1.1 Flow diagram for processing of orange juice.

et al., 1987; Tramell et al., 1986) describe an advanced stage of Maillard reactions, at which sensory deterioration is already noticeable. Therefore they have no prognostic value.

A simple quantitative method for MIP detection would therefore be very useful to the fruit juice industry for purchasing concentrated juices and for evaluating equipment and processes.

The objective of the present study is therefore the development of such a simple method to measure MIP in (concentrated) fruit juices. In the first instance orange juice has been chosen for these studies as it constitutes the largest segment of the fruit juice market. In the absence of knowledge of specific chemical structures of MIP, I have investigated the use of microbiological methods. In chapters 3, 4 and 5 the methodological aspects and development of these methods are described. The possibility of interference of some non-Maillard products with the mutagenicity method is studied in chapter 6. Application of the methods to commercial orange juices and to industrial samples is described in chapters 5 and 7. Characterisation of the compound(s) which cause mutagenicity in heat treated orange juice is described in chapter 8. The use of an automated system of the *Salmonella* mutagenicity test to assess the inner quality of orange juices is discussed in chapter 9. Apple juice was also investigated because of its increasing popularity which, in many countries, results in consumption exceeding that of orange juice (Dukel, 1988). The results are presented in chapter 10.

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2 MAILLARD-TYPE REACTIONS IN FRUIT JUICES

Introduction

Under Maillard-type reactions we understand reactions resulting in non-enzymatic browning. By such reactions the sugars in fruit juices are broken down either directly by acid catalysis or via the formation of amino acid sugar compounds, the Maillard reactions. It must be supposed that both types of reactions occur concurrently.

Direct acid break-down is observed when either models of fruit juices without amino-acids or fruit juices with amino acids removed (Varsel, 1980) are investigated. The Maillard reactions mechanism manifests itself by quicker browning when enriching juices with amino acids or protein (Roozen and Pilnik, 1974). Bland sugar syrups obtained from fruit juices by treatment with decolourizing active coal and ion-exchange resins demonstrate both mechanisms. If the treatment has removed all amino acids, browning occurs only at low pH. In the presence of amino acids browning still occurs even at low pH but is more pronounced at pH near neutrality (Lijster, 1967).

Maillard reactions

Fig. 2.1 presents the scheme of the Maillard reaction pathways proposed by Hodge in 1953. Until now, this Hodge' scheme is still widely recognized. In general the reactants are reducing sugars and amino acids and amine groups. The reactions can proceed by these compounds entering the Hodge' scheme (Fig. 2.1) at any point. It can be seen that the Maillard reactions go through three main stages, i.e. early, advanced and final (Mauron, 1981).

The early stage of Maillard reactions are initiated by reaction A followed substantially by reaction B (Fig. 2.1).

Reaction A is a condensation between a carbonyl group (as aldehyde or keton form) and a free amino group of amino acid, protein, or amine to furnish Schiff base (imine) which after following cyclization produces N-substituted glycosylamine (Fig. 2.2). The following sequence B (Fig. 2.1) is the Amadori rearrangement which produces the so-called Amadori product or ketosamine (1-amino-1-deoxy-2 ketose).

An aldosamine (2-amino-2-deoxy-aldose) can be formed via Heyn's rearrangement which is analogous to the Amadori rearrangement in the case the initial sugar reactant is a ketose sugar (Baltes, 1982; Reynolds, 1965). The early stage of Maillard reactions that proceed up to Amadori rearrangement result in compounds which are not yet sensorially active (Fennema, 1985; Mauron, 1981). These reactions are acid-catalyzed with amino acids as their own catalysts (Baltes, 1982; Hodge and Rist, 1953; Namiki, 1988; Song and Chichester, 1966). This makes it difficult to understand

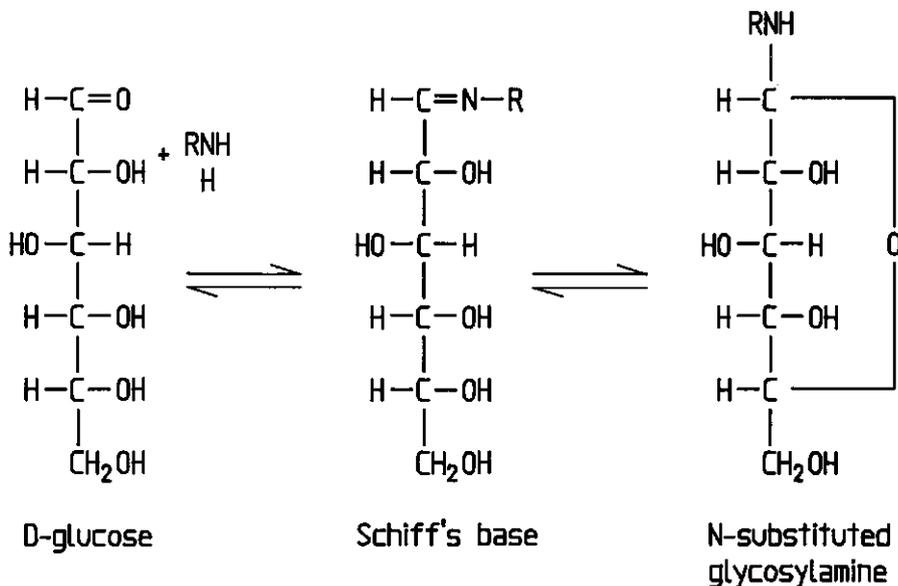


Figure 2.2 Formation of N-glycosylamine in the early stage of Maillard reactions. Cited from Namiki (1988).

why browning proceeds more quickly under neutral and alkaline conditions. Another scheme for Maillard reactions as shown in Fig. 2.3 proposed by Namiki and Hayashi (1983) may explain this phenomenon. It involves the sugar fragmentation and free radical formation during the early stage of Maillard reactions prior to Amadori rearrangement. The free radical products are \underline{N} , N' -disubstituted pyrazine cation radicals (Fig. 2.3: 1). Investigations using electron spin resonance (ESR) spectroscopy allowed them to observe the following sequential reactions: initial formation of glycosylamine, fragmentation to C2, C3 and C4 products, formation of reducing substances, free radicals, Amadori products and glycosones (Hayashi et al., 1986; Namiki, 1988; Namiki and Hayashi, 1983). The resulting fragmentation products are assumed to be glycol aldehyde, glyoxal, glycerol aldehyde, methyl glyoxal which are well known to be very reactive in Maillard browning (Namiki, 1988). It can be noted that the results lead to a similar pathway to Hodge' scheme of Fig. 2.1. Therefore it is suggested that both pathways could be considered to proceed concurrently (Danehy, 1986; Hayashi et al., 1986; Namiki, 1988; Namiki & Hayashi, 1983).

The advanced stage of Maillard reactions (Fig. 2.1: C, D and E) are dependent on the type of reactions and pH conditions. Various reactions, i.e. dehydration, sugar fragmentation, amino acids and organic acids degradations are reported to occur simultaneously. Each reaction builds up reaction products which then become reactants in another pathway (Baltes, 1982; Hodge, 1967; Namiki, 1988). Hodge (1967) described two major pathways in the advanced stage (Fig. 2.4). Under acid conditions Amadori or Heyns products enolize via 1.2 enolization resulting in the formation of 3-deoxyhexosulose intermediate, followed by dehydration to

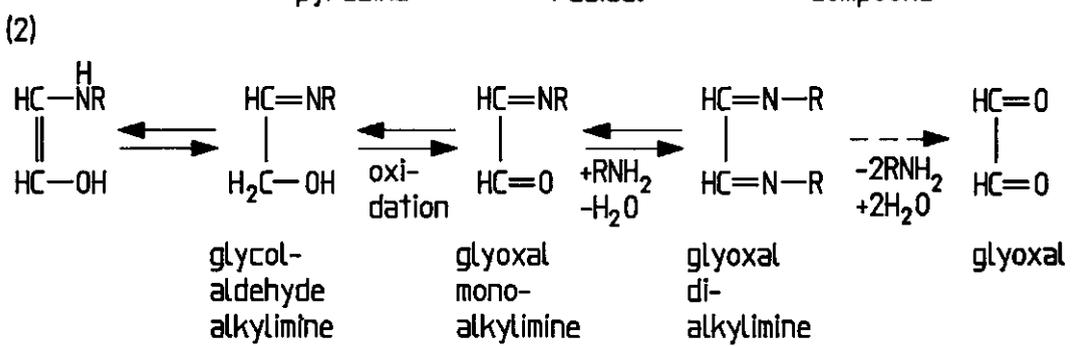
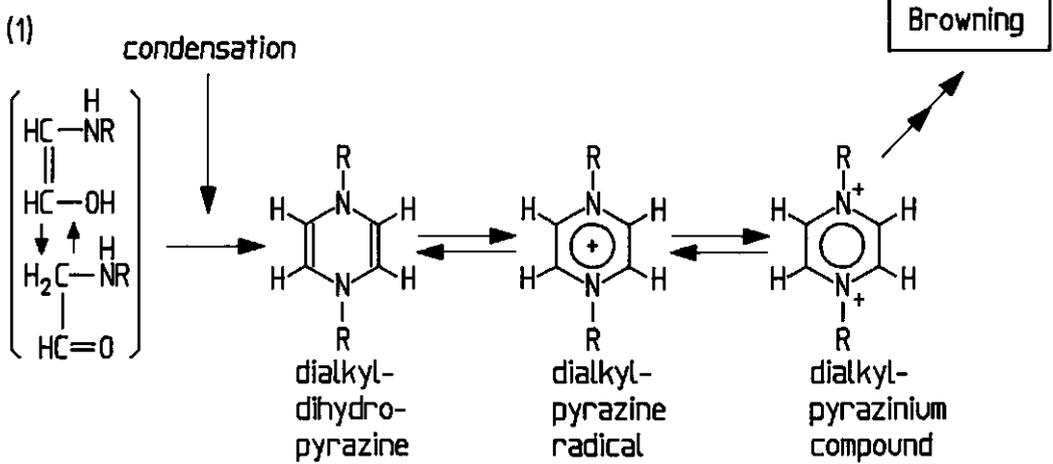
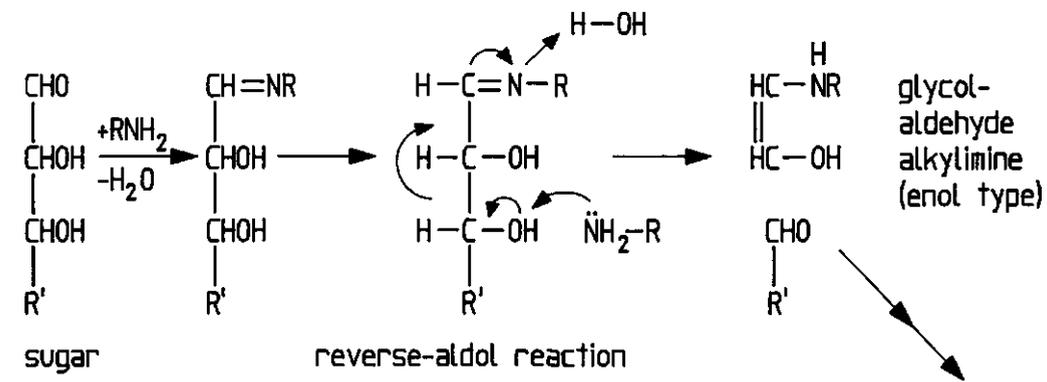


Figure 2.3 Scheme of Maillard reaction pathways involving sugar fragmentations and free radical formations. According to Namiki and Hayashi (1983), and Namiki (1988).

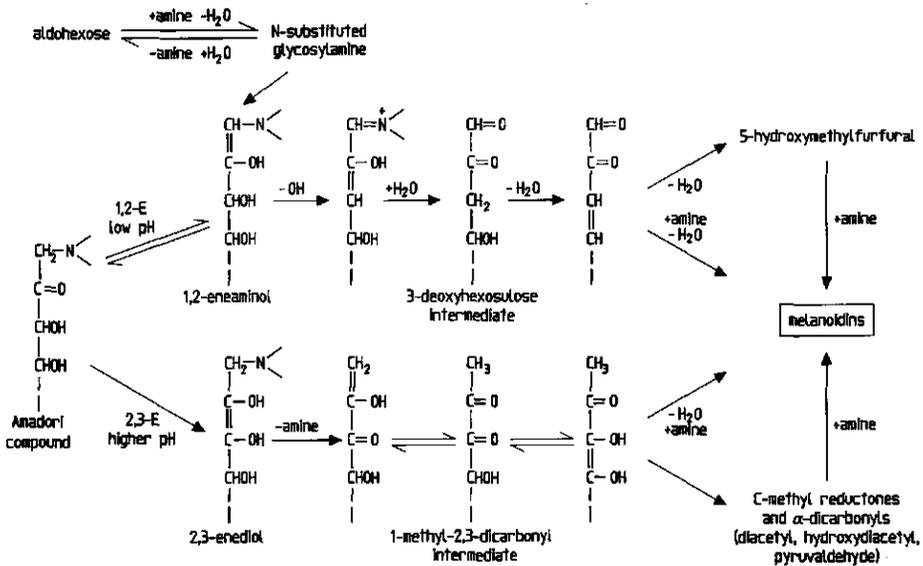


Figure 2.4 Two major Maillard reaction pathways involving 1.2 enolization and 2.3 enolization of Amadori products. According to Hodge (1967).

produce among other furfural and/or hydroxymethylfurfural. At higher pH, 2.3 enolization occurs producing methyl dicarbonyl intermediates, which further react to give fission products such as C-methyl reductones and α -dicarbonyls. These products such as acetaldehyde, pyruvaldehyde, diacetyl and acetic acid are important flavour components. Another pathway is the Strecker degradation (Fig. 2.5). Here amino acids react with α -dicarbonyl compounds to form a new Schiff base which is subsequently altered to form aldehydes, carbon dioxide and pyrazines.

The final stage of Maillard reactions (Fig. 2.1: F and G) results in the formation of brown coloured products called melanoidins with molecular weight above 1000. So far little is known on the formation of these brown pigments (Baltes, 1982; Mauron, 1981) which are not relevant to our research.

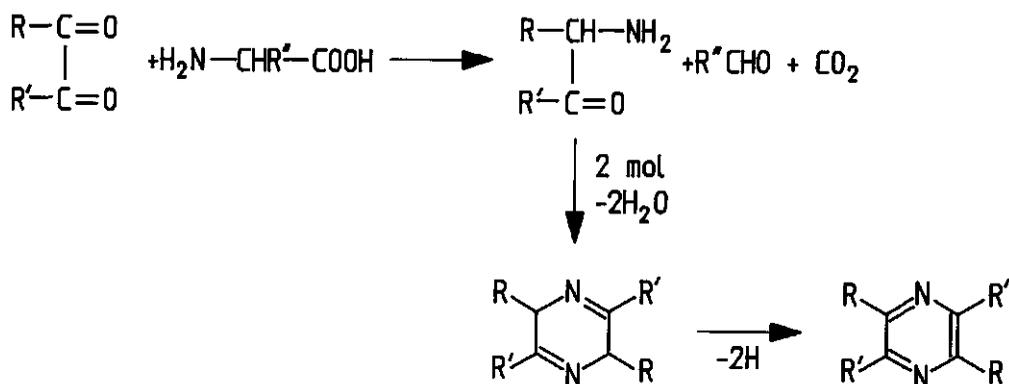


Figure 2.5 Strecker degradation pathway resulting in the formation of pyrazine. Cited from Nursten (1985).

Acid-catalyzed degradation of sugar

Reducing sugar under acidic condition enolizes to produce 1,2 enediol which after elimination of water leads to the formation of furan derivatives. The reaction pathway (Belitz and Grosch, 1987; Fennema, 1985) is similar to that of Maillard reaction shown in Fig. 2.4.

Ascorbic acid degradation

Loss of ascorbic acid in orange juice has been reported to occur in parallel with increasing browning (Marshall et al., 1986; Trammell et al., 1986).

Fig. 2.6 shows the degradation pathways of ascorbic acid, under aerobic and anaerobic conditions.

The aerobic degradation involves the participation of ascorbic acid as a reductone with enediol structure. Dehydroascorbic acid (DHA) formed can enter the Hodge' scheme to react with amino acids according to the Strecker degradation (Fig. 2.1: E; Graumlich et al., 1986; Kacem et al., 1987a and b).

The anaerobic degradation is an acid-catalyzed reaction which occurs predominantly in orange juice (Liao and Seib, 1988). This is due to the low pH and minimization of oxygen involvement during the processing (Kefford et al., 1959; Smoot and Nagy, 1980). The degradation products of both aerobic and anaerobic pathways such as hydroxyfurfural and furfural can enter the Hodge' scheme at point F and G (Fig. 2.1).

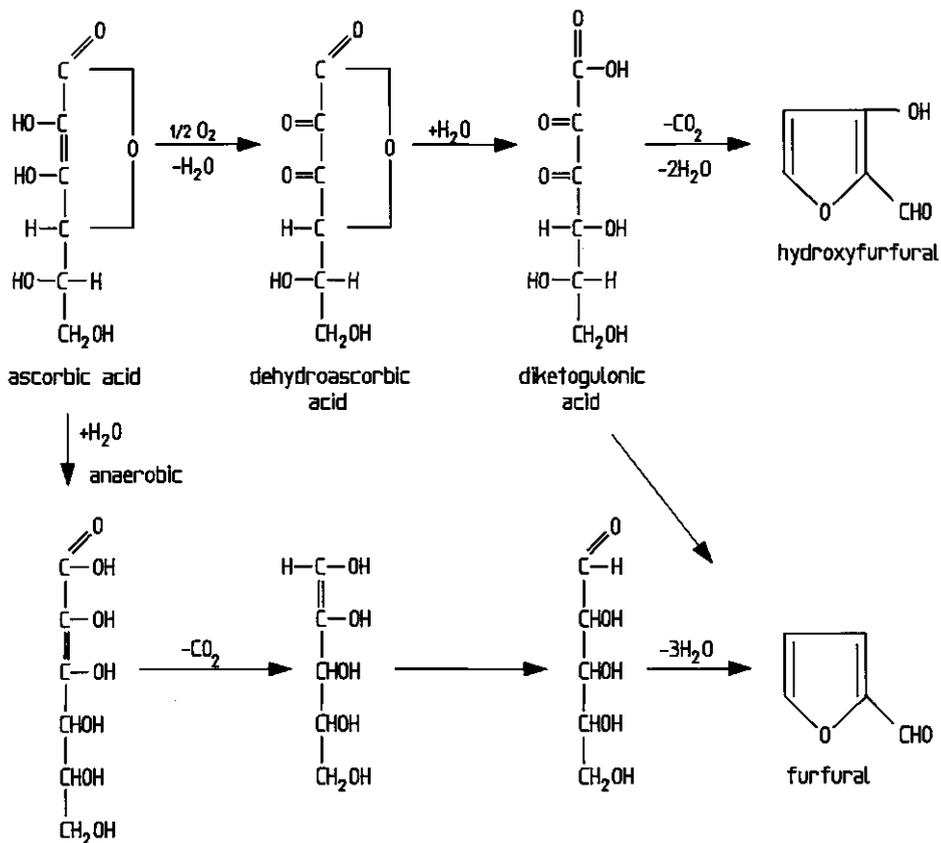


Figure 2.6 Ascorbic acid degradation pathways under aerobic and anaerobic conditions. Cited from Varsel (1980).

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3 PRINCIPLES AND METHODOLOGICAL ASPECTS OF THE MICROBIOLOGICAL METHODS USED IN THE INVESTIGATIONS

Salmonella mutagenicity assay

This microbiological assay was originally developed by B.N. Ames and colleagues in the 1970's at the University of California at Berkeley, USA to measure mutagenicity of chemical compounds.

It is an in vitro bacterial test based on a reverse mutation system in *Salmonella typhimurium* TA strains, mutants which have been selected by Ames for sensitivity and specificity in being reverted from histidine (His) dependency (His auxotrophy) back to His independency (His prototrophy) following exposure to a mutagen.

The compound to be tested for mutagenicity is added to Petri dishes containing minimal agar medium together with the tester bacteria. Mutations are detected as bacterial colonies that grow in a selective medium which is poor in His (Maron and Ames, 1983).

The tester strain

Among several strains available, strain TA 100 is widely used for testing the mutagenicity of aqueous Maillard reaction products (Powrie et al., 1981; Shinohara et al., 1980). The strain TA 100, which is designed to detect base pair substitutions in the His operon, also contains other mutations, that increase its sensitivity in detecting mutagens. The *gal* and *rfa* (deep rough) mutations make the bacteria more permeable to large molecules like polycyclic aromatic hydrocarbons by elimination of the lipopolysaccharide barrier that coats the surface of the bacteria (Maron and Ames, 1983). An additional advantage of the loss of the

lipopolysaccharide barrier is that the bacteria become non-pathogenic. The *uvrB* mutation, which is a deletion of a gene coding for the DNA excision repair system extended through the biotin operon, results in an increase of sensitivity to mutations due to the lack of repair capability by this system. Mutations caused by environmental exposure will be expressed to a larger extent. Because of the involvement of the biotin operon these bacteria are biotin-dependent and therefore the presence of biotin in the medium is required for growth.

The addition of the so-called resistance factor (R-factor) implies a multi copy plasmid (pkM101) which carries a mutated His-gene increasing the sensitivity of this strain to backward mutations to prototrophy. Also the number of spontaneous and chemically induced revertants is increased by stimulation of an error-prone repair system which is normally present in these bacteria (McCann et al., 1975; Shanabruch and Walker, 1980). The presence of this plasmid can easily be checked because it also contains a tetracycline resistance gene which confers resistance to the presence of the antibiotic ampicillin in the growth medium (Ames et al., 1975; Maron and Ames, 1983). Spontaneous reversion, which occurs in the absence of added mutagens, is used to control the validity of the test. A number of spontaneous revertants ranging between 80-200 colonies/plate is considered acceptable. A deviation may indicate an alteration in genetic characteristics or contamination caused by sub-culturing.

Maintenance of stock cultures and preparation of inoculum

The tester bacteria *Salmonella typhimurium* TA100 was generously provided by B.N. Ames. Upon arrival the cultures were grown on nutrient broth for 6-8 hr at 37°C in a shaking waterbath and then tested for genotype confirmation. Details of the test procedure are described by Maron and Ames

(1983). The tests confirmed the presence of rfa-deep rough mutation (crystal violet sensitivity test), R-factor (ampicillin resistancy test), and range of the number of spontaneous revertants.

Two types of stock cultures were maintained, i.e. (1) frozen permanents to be kept at -196°C in a liquid nitrogen storage tank and (2) master plates kept at 4°C to obtain fresh inoculum for routine testing. The frozen permanent cultures were maintained in 2 ml sterile cryotubes. Each tube contained 1 ml of a fresh culture, grown in nutrient broth to which 0.09 ml DMSO was added as a cryoprotective agent. The master plate cultures were prepared by streaking out these cultures on minimal agar medium enriched with 1.5 mg His/plate. These plates can be kept for 1 month.

For every experiment, freshly grown cultures were used. These were prepared by inoculating one single colony isolated from the master plate into a 250 ml Erlenmeyer flask, containing 10 ml nutrient broth (Oxoid no. 2) enriched with L-His (10 $\mu\text{g}/\text{ml}$). The cultures were incubated at 37°C in a shaking-water bath for 6-8 hr until a density of approximately 1×10^9 cells/ml was reached, measured as optical density at 700 nm. Each plate receives 0.1 ml of this culture suspension.

Mutagenicity assay

Pour plate technique (Maron and Ames, 1983) was used in combination with a prolonged liquid preincubation step.

After the preincubation step, mixtures consisting of tester bacteria and samples, were poured onto selective agar plates containing minimal glucose agar medium and biotin (0.5 $\mu\text{g}/\text{ml}$). A trace of His is required to allow initial growth. This growth is necessary for expression of mutagenesis and results in the formation of the so-called background lawn (Ames, 1971). The resulting background lawn does not appear

as colonies but its presence can be examined under a phase-contrast microscope. It is essential to the test as an indicator of growth inhibition which may be caused by cytotoxic effects of samples. Massive cells death due to severe cytotoxicity results in a sparse background lawn of the corresponding test plates as compared to control plates. Furthermore, the surviving bacteria appear as tiny colonies because more His is available for the individual cells, allowing them to undergo more cell divisions (Ames, 1971; Ames et al., 1975; Maron and Ames, 1983).

Because the specific mutational events required are rare, it is recommended to use an inoculum size of approximately 10^8 cells/plate (Ames, 1971; De Serres and Shelby, 1979; Gøggelmann, 1981). From such amounts of cells used, depending on the mutagenicity of samples, only hundreds to thousands of bacteria can grow into visible colonies (His prototrophs) against a background lawn resulting from auxotrophic growth. Thus, the mutagenicity of the chemical can be quantified simply by counting the number of His prototrophs which appear as prominent thick white colonies after 2 days of incubation at 37°C.

All experiments were carried out at least in triplicate per data point. In all experiments the appropriate positive (diagnostic mutagen) and negative (solvent) controls were included.

Results are interpreted as positive mutagenic or negative (not mutagenic). A result is considered positive when a reproducible dose-response curve with at least a mutation ratio (induced revertant colonies per plate divided by spontaneous revertant colonies per plate) of 2.0 is obtained.

Cytotoxicity assay

Total plate count method

Total plate count method was used to determine cell

survival. As in the mutagenicity assay, pour plate technique in combination with prolonged liquid preincubation was used but plating was done on complete glucose agar medium (minimal glucose medium enriched with 1.5 mg His/plate) instead. The size of inoculum used was $\leq 5 \times 10^2$ cells/plate.

Kinetic measurement using the Malthus conductance meter

Details of the instrument are given by Baynes et al., (1983) and Eden and Eden (1985). The Malthus instrument measures bacterial growth kinetics by registering conductance changes of the growth medium. Due to metabolic activity and growth of tester bacteria, large uncharged molecules in the medium such as polysaccharide and protein are broken down into charged small molecules, i.e. sugars and amino acids, respectively. These result in an increase in electrical conductivity of the medium. In this study the criterium used for growth detection is, when three successive conductance values show an increase ≥ 1.0 microsiemens (μS). The time required to reach this point is defined as detection time (DT). Detection time is dependent on both cell concentration and growth kinetics of the tester bacteria (Eden and Eden, 1985); therefore it is assumed to be delayed by cytotoxic effect (Ekasari et al., 1988; chapter 5).

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CHAPTER 4: DETECTION OF MAILLARD INTERMEDIARY PRODUCTS IN ORANGE JUICE BY BACTERIAL MUTAGENICITY ASSAY

4 Appendix: Development of mutagenicity in heated orange juice

This chapter is based on the following publications:

1. Ekasari, I., Jongen, W.M.F. and Pilnik, W. 1986a. Use of a bacterial mutagenicity assay as a rapid method for the detection of early stage of Maillard reactions in orange juices. *Food Chem.* 21:125-131.
2. Ekasari, I., Jongen, W.M.F. and Pilnik, W. 1986b. Detection of Maillard intermediary products in orange juice by bacterial mutagenicity assay. *Proc. Int. Symp. Federation Fruit Juice Producers* 19:409-418. The Hague.

4 DETECTION OF MAILLARD INTERMEDIARY PRODUCT IN ORANGE JUICE BY BACTERIAL MUTAGENICITY ASSAY

Indriati Ekasari¹, W.M.F. Jongen² & W. Pilnik¹

¹ Department of Food Science, ² Department of Toxicology, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT

Freshly pressed orange juice was heated up to 30 min at 93°C and assayed for mutagenicity by the Ames test with *Salmonella typhimurium* strain TA 100 modified to include a 4 hr preincubation time at 37°C and pH 7.4. It was noticed that the heat treatment induced mutagenicity which was dose related and also related to the time of heating, but which appeared only after preincubation at pH 7.4. The mutagenic response must be ascribed to Maillard intermediary products as the heat treatments were mild and not sufficient to produce brown pigments. Obviously the Maillard intermediary products are precursors for mutagenic products formed from them during the preincubation at pH 7.4. This effect may become the basis of a rapid and inexpensive method for measuring heat load and inherent quality of fruit juices.

INTRODUCTION

Fruit juices are susceptible to Maillard browning reactions because they contain reducing sugars, amino acids and in some cases ascorbid acid. Maillard reactions can occur during heat treatments, such as pasteurization and concentration and/or during storage of concentrated juice.

Hodge (1953) proposed that the whole Maillard browning reactions consist of three different stages, i.e. early, advanced and final stages. The early Maillard intermediary products (MIP) formed at the early stage neither give colour nor flavour. To a large extent the shelf-life of fruit juices depends on the content of MIP which can react further to form brown pigment and off-flavour. Methods commonly used to follow these reactions, such as the detections of hydroxy methylfurfural (HMF), loss of ascorbid acid and change of colour and taste, are reliable if the reactions enter the later stages. On the other hand other methods which are more sensitive, i.e. ultraviolet (UV), and nuclear magnetic resonance (NMR), and infra red (IR) spectrometrics, and high performance liquid chromatography (HPLC) are rather tedious and expensive. Also no chemical compounds suitable for following these reactions have been described yet. Therefore a rapid method suitable for the fruit juice technologist is desirable for a quality check on juices and concentrated juices to be reprocessed in consumer packages and also as a simple research tool to measure the heat load imparted to juices during processing.

The mutagenicity of Maillard browned common foods exposed to intensive heat treatment, e.g. crust of bread (Van der Hoeven et al., 1982), fried meat (Pariza et al., 1983) and dried fruit (Stich et al., 1981) is well known.

The purpose of our study with orange juice, was to see whether mutagenicity is also provoked by lighter heat treatment which do not cause detectable colour or flavour change.

The resulting mutagenic properties could be used as a rapid method for the detection of early Maillard intermediary products (MIP) and could help the fruit juice manufacturer in establishing the conditions of heat treatment and storage, and in the quality evaluation of concentrated juices.

EXPERIMENTAL

Preparations of samples

Laboratory prepared orange juice (code I) was obtained from Israeli Jaffa orange using an electric household juice extractor and finishing the juice by squeezing through cheese-cloth. Commercial samples were directly bottled juice (code II), juice reconstituted with distilled water according to label instruction, from canned frozen concentrated juice (code III), and juice in brick-pack made from concentrated juice (code IV).

Samples of juice I were filled in capped Kimax tubes and heated at 93°C for 0.5 min to 30 min by immersion in a boiling water-bath, and then cooled under running tap water. These heat treatments simulate industrial conditions and do not result in colour change. The commercial juices were not heat treated. Both types of samples were treated according to Fig. 4.1.

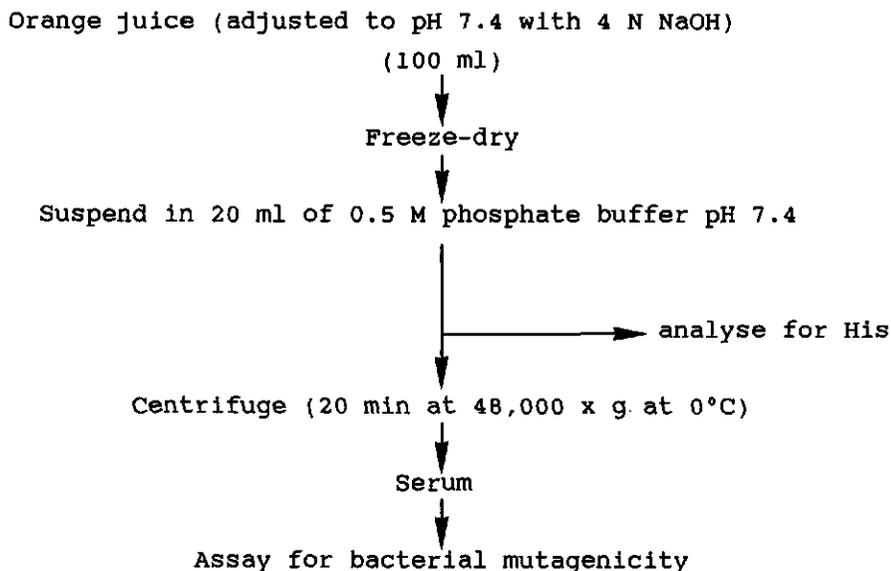


Figure 4.1 Scheme of sample preparation

Mutagenicity assay

Salmonella typhimurium strain TA 100 was used for the mutagenicity assay according to the method of Maron and Ames (1983) with minor modifications described by van der Hoeven et al. (1983). On the basis of preliminary experiments, a preincubation time of 4 hr at 37°C and pH 7.4 was applied in all tests. The procedure followed was: add 0.1 ml of 0.5 M phosphate buffer pH 7.4 in a sterile 13 x 100 mm capped culture tubes, add 0.1 ml bacterial culture and then the test samples (pH 7.4) varying from 0.1 to 1 ml, add distilled water to give a final volume of 1.2 ml. Mix gently on Vortex mixer and incubate in a shaking water-bath at 37°C for 4 hr. As negative control 1 ml distilled water and as positive control 0.1 ml DMSO containing 0.1 µg NQO (4-nitroquinoline-N-oxide) were used. After 4 hr preincubation 3 ml molten top agar (45°C) was added, mixed on a Vortex mixer and poured on-to bottom agar plates. The plates were then incubated at 37°C for 48 hr.

The number of induced revertant colonies were counted manually. The number of spontaneous revertants (93-174 revertant colonies/plate) were subtracted from the numbers of induced revertant colonies. Revertants induced by the positive control used (NQO) were $\geq 10,000$ revertant colonies/ µg. Each experiment was done at least twice and each concentration of the sample was tested at least in triplicate.

Data interpretation

A result is considered positive when a reproducible dose-response curve with at least a mutation ratio (induced revertant colonies per plate divided by spontaneous revertant colonies per plate) of 2.0 is obtained.

RESULTS AND DISCUSSIONS

There are several factors affecting the presence of mutagenicity in heated orange juice. No mutagenic effect can be observed in the Ames standard plate assay or in a preincubation assay of 20 min - 60 min which are recommended as standard methods for testing mutagens (De Serres and Shelby, 1979). Table 4.1 shows that positive mutagenic responses are only obtained on samples with pH adjusted to 7.4. The pH of orange juice is around 3.2. The addition of 0.1 ml phosphate buffer pH 7.4 to the preincubation mixtures following Ames test procedure (Maron and Ames, 1983) increased the pH to the range of 4.0 - 6.0 depending on the amount of sample assayed. At pH 6.0 the tester bacteria were able to grow but no mutagenicity was observed until the pH was adjusted to 7.4

Table 4.1. Effect of pH adjustment ^{a)} on the mutagenicity ^{b)} of orange juice heated at 93°C for 2 min.				
Amount sample (ml)	pH of preincubation mixture		Mutation ratio	
	Without pH adjustment	with pH adjustment	without pH adjustment	with pH adjustment
0.1	6.0	7.4	1.7	3.5 ^c
0.2	6.0	7.4	1.9	4.3 ^c
0.3	4.0	7.4	1.8	2.8 ^c
0.4	4.0	7.4	---	---
0.8	4.0	7.4	---	---

a) adjusted with 4N NaOH
b) after 4 hr preincubation at 37°C
c) positive mutagenic.

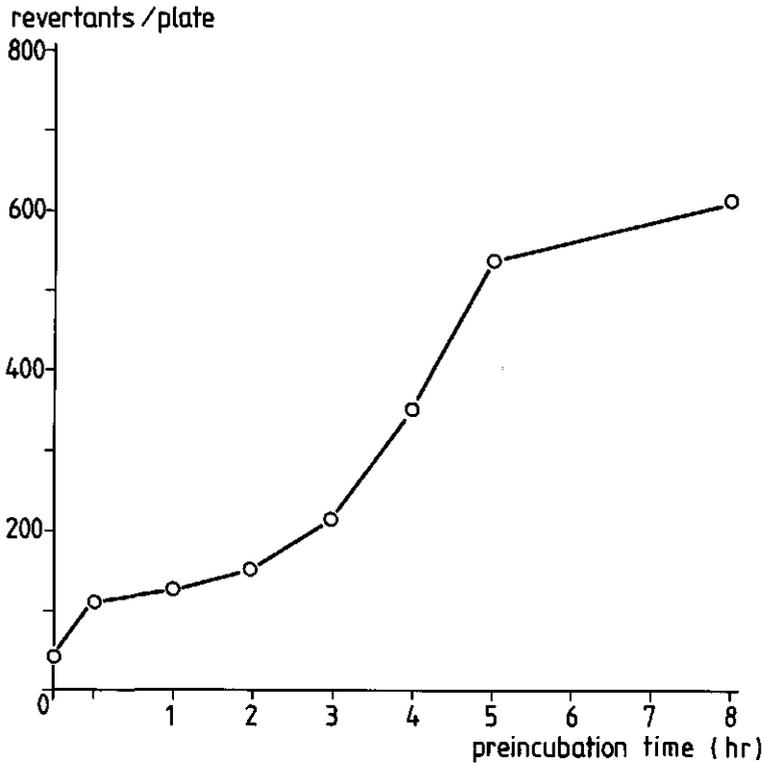


Figure 4.2 Effect of preincubation time on the mutagenicity of orange juice heated at 93°C for 2 min. Sample assayed was 0.2 ml/plate.

Fig. 4.2 shows the effect of preincubation time on the mutagenicity of orange juice heated at 93°C for 2 min. The mutagenicity increased sharply after 3 hr preincubation (pH 7.4; 37°C) and the effects became stronger as the preincubation time prolonged. For practical reasons, we preincubated the samples in every test for 4 hr.

Fig. 4.3 shows the dose-response curves of the mutagenic effects of laboratory prepared orange juice heated at 93°C for 30 sec - 30 min. It can be seen that only heated orange juice possess mutagenic activity which is dose related and also related to the duration of heat treatment.

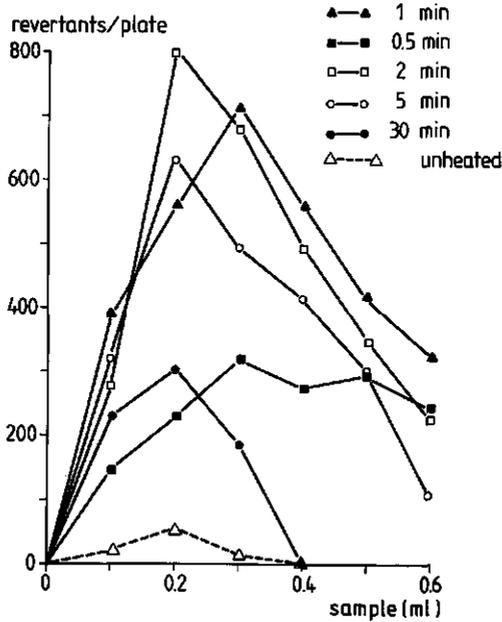


Figure 4.3 Dose-response curves of the mutagenic effect of laboratory prepared orange juice on *Salmonella typhimurium* TA 100. Samples were heated at 93°C for various times. For preparation of samples see Fig.4.1.

Fig. 4.4 shows the positive linear correlation ($r = 0.98$) between the mutagenicity and heating time which significantly increased ($p < 0.01$) up to 2 min. Moreover, this mutagenic activity was observed to decrease as follows (Fig. 4.3): 1) for samples heated for longer than 2 min when assayed at amounts larger than 0.2 ml and 2) for samples heated for less than 2 min when assayed at amounts larger than 0.3 ml. The decrease in mutagenicity of these samples was found to be related to their reduced background lawn as observed under the phase contrast microscope. This effect can be explained as a cytotoxic effect of the samples and it is not yet clear whether it was caused by the same compounds which are responsible for the mutagenicity.

EFFECT OF HEATING TIME ON THE MUTAGENIC EFFECTS

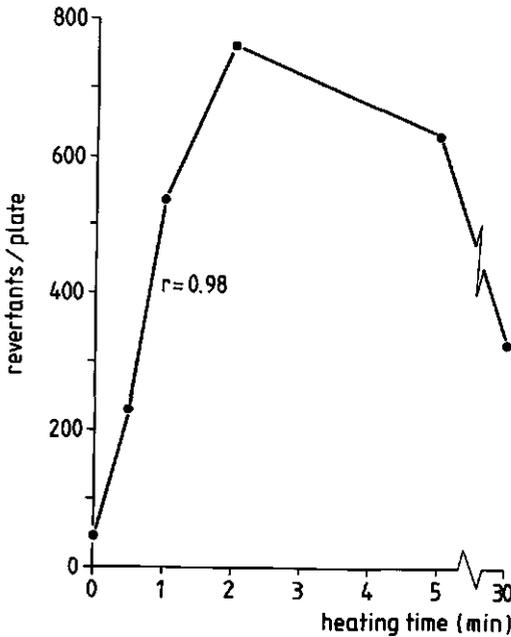


Figure 4.4 Effect of heating time on the mutagenicity of orange juice. Data were obtained from the slope of the linear portion of dose-response curves (Fig. 4.3) at a sample concentration of 0.2 ml/plate.

Table 4.2 presents the results of toxicity experiment, assayed on selective agar plate medium enriched with histidine (His; 1.5 mg/plate). It can be seen that the number of colonies on samples assayed at 0.2 ml/plate decreased as the heating time prolonged, and a complete suppression of growth is observed when higher amounts of sample (1 ml/plate) were used. However, this effect as found in the mutagenicity assay, is only observed when preincubation is used. Furthermore, it was noticed that the cells growing on the unheated samples, which were supposed to give about the same count as that of the control (0.9% NaCl in distilled water) proliferated about 5 times. His and/or other nutritional factors

Table 4.2 Comparison of two different toxicity assays using *Salmonella typhimurium* TA 100 for orange juice heated at 93°C.

	Number of colonies/plate			
	Direct plating		Preincubation ^a	
	0.2 ml	1.0 ml	0.2 ml	1.0 ml
Control ^{b)}	499	500	15	15
Orange juice heated for:				
0	420	465	78	0
1 min	414	466	70	0
2 min	411	427	60	0
5 min	408	468	36	0
30 min	468	485	30	0

a) 4 hr; pH 7.4; 37°C
b) 0.9% NaCl in distilled water.

present in orange juice may promote the growth of the tester cells (*Salmonella typhimurium* TA 100) which are His dependent (Arimoto et al., 1981; Maron and Ames, 1983). This causes difficulties in quantifying the toxicity data. In order to get more accurate results, we plan to carry out kinetic growth studies. We expect that in combination with mutagenicity data, we shall be able to draw conclusions regarding the heat load to which juices had previously been subjected during processing and/or storage.

We are aware that the extra amount of His originating from the samples can cause synergistic effect because the *Salmonella typhimurium* TA 100 used as a tester strain possess a backward mutation type in its His operon. Therefore, we investigated the His content of various oranges and assayed their juices without heat treatment for mutagenicity.

Table 4.3. Mutagenicity assay* results from freshly pressed unheated orange juices with various histidine (His) content.						
Country of origin	µg His/ml concentrate	Mutation ratio				
		Amount of sample (ml)				
		0.1	0.2	0.3	0.4	0.5
Uruguay	23.17	1.6	1.3	1.3	1.3	1.4
Spain	26.20	1.6	1.4	1.6	1.6	1.8
Brazil	33.02	1.6	1.8	1.7	1.7	1.8
Argentina	44.95	1.7	1.7	1.8	1.8	1.7

* with preincubation (4hr, 37°C, pH 7.4).

Table 4.3 shows that there was no mutagenic response observed in any of the samples tested despite the variation of His content. A slightly higher mutation ratio value was occasionally noticed on samples with higher His content either from the amount of samples or the types of oranges but this effect was not dose-related and the mutation ratio values were always less than 2.0. This confirms that the variations in concentration of His in orange juice does not significantly affect the outcome of the mutagenicity data. The mutagenicity which was observed only in the heated orange juices, was induced by the heat treatments.

Fig. 4.5 shows the mutagenicity of commercial orange juice with different technological histories. We may suppose that sample II (bottled single strength juice) has received the lightest heat treatment which is only one-time pasteurization. Sample III (canned frozen concentrated juice) received more heat treatment during concentration followed by sample IV (brick-packed reconstituted juice from concentrate) which was subjected to heat during concentration and pasteurization before packing. All samples show positive dose-related responses. At the peak points, the mutation ratios were 2.7

for sample II, 4.7 for sample III and 5.8 for sample IV. These results confirm the usefulness of the assay for quality control.

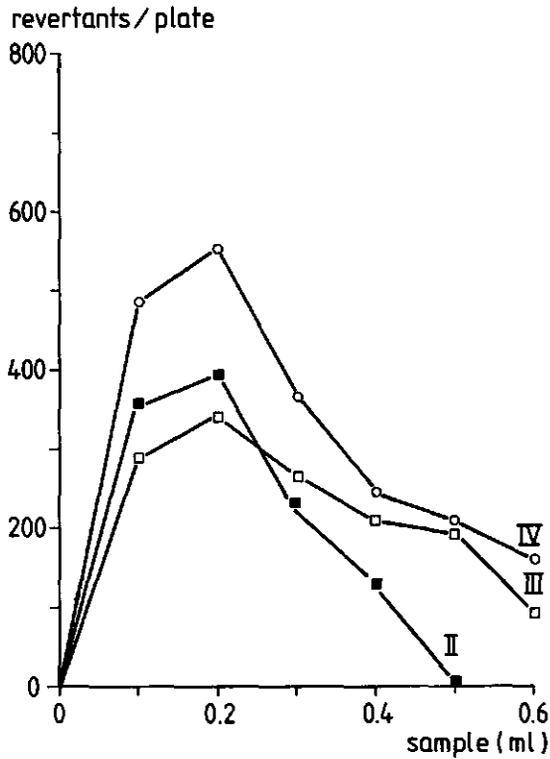


Figure 4.5 Dose-response curves of the mutagenic effect of commercial orange juices on *Salmonella typhimurium* TA 100.

II = bottled single strength juice

III = canned frozen concentrated juice

IV = brick-packed reconstituted juice from concentrate

CONCLUSIONS

- 1 Freshly pressed unheated orange juice has no mutagenic properties.
- 2 Mutagenicity and cytotoxicity observed in heated orange juice appeared only under the specific preincubation conditions described (pH 7.4, 4hr, 37°C). These effects are not seen in the Ames standard plate assay which is the currently recognized method for testing mutagens.
- 3 The mutagenic response must be ascribed to MIP (Maillard intermediary products) as the heat treatments were mild and not sufficient to produce brown pigment.
- 4 The mutagenicity of MIP is dose-related and related to the duration of heat treatment and to the presumed heat load of commercial orange juices.
- 5 Mutagenicity of MIP may become the basis for a rapid, simple, and inexpensive method for measuring heat load and inherent quality of fruit juices.

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4 APPENDIX.

DEVELOPMENT OF MUTAGENICITY IN HEATED ORANGE JUICE

The results of chapter 4 show the necessity of preincubation at pH 7.4 at 37°C. They do not show whether this preincubation is necessary for the further development of mutagenic MIP from precursors formed during heating or whether preincubation is necessary because of the weak mutagenicity of MIP. We have therefore carried out experiments in which the effect of preincubation in the presence and in the absence of tester bacteria was investigated. The results are given in Table 4.A1 below.

Amount of sample (ml)	Mutation ratio	
	Preincubation with bacteria	Preincubation without bacteria
0.1	3.5 ^b	1.4
0.2	4.3 ^b	1.4

a) 4 hr preincubation at 37°C and pH 7.4
b) positive mutagenic response (mutation ratio > 2.0).

It is clearly seen that the presence of the bacteria during the preincubation period is necessary. Taking further into consideration that pH adjustment to 6.0 has no effect and adjustment to pH 7.4 is necessary (Table 4.1 in chapter 4) the results of Table 4.A1 seem to allow the conclusions (1) that mutagenic MIP are formed during the preincubation period from precursors in the heated orange juice and (2) that due to their weak mutagenicity these must be preincubated

together with the tester bacteria. The shape of the curve obtained for mutagenicity with increasing preincubation times at pH 7.4 and 37°C (Fig. 4.2 in chapter 4) also supports conclusion (1). For conclusion (2) alone the increase with time would be expected to be linear. Further reactions of mutagenic compounds must also be taken into consideration. A full explanation for the phenomena observed will only be possible when more is known about their chemistry. Our main aim is the elaboration of a reliable method for measuring heat load of fruit juices.

CHAPTER 5: MEASUREMENT OF HEAT LOAD IN ORANGE JUICES: USE OF MICROBIOLOGICAL METHODS

Indriati Ekasari¹, W.M.F. Jongen², A.E.M. Vermunt³ &
W. Pilnik¹

¹Department of Food Science, ²Department of Toxicology,
Agricultural University, Bomenweg 2, 6703 HD Wageningen.

³State Institute for Quality Control of Agricultural
Products, 6700 AE Wageningen, The Netherlands.

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5 MEASUREMENT OF HEAT LOAD IN ORANGE JUICES: USE OF MICROBIOLOGICAL METHODS

Mutagenic and cytotoxic properties resulting under specific test conditions can aid in evaluating the inner quality of concentrated orange juices

Orange juice is very sensitive to the heat load it receives during the processing. This sensitivity is mainly due to chemical reactions of the Maillard type (Varsel, 1980). When Maillard intermediary products (MIP) are first formed, they are not perceived sensorially, but they react further to form compounds causing off-color and off-flavor (Baltes, 1982; Clegg and Morton, 1965; Fox et al., 1983; Saguy et al., 1978) The concentration of MIP in a juice or concentrated juice will therefore determine the shelf-life of the (reconstituted) juice.

A simple method which can quantify the presence of MIP will therefore be very useful to orange juice processors. As the chemical constitution of MIP responsible for these phenomena are not yet sufficiently known, we have applied microbiological methods for their detection.

The purpose of this article is to discuss certain aspects of the use of a bacterial mutagenicity assay previously reported by the authors (1986a; chapter 4) and to present further data on a correlation between heat load and mutagenicity. The extension of the mutagenicity to a cytotoxicity assay is also described.

Mutagenicity in Heated Orange Juice

We have shown (Ekasari et al., 1986a) that orange juice heated at 93°C from 30 sec to 30 min induced mutagenicity

towards *Salmonella typhimurium* TA 100, a strain used to test the mutagenicity of Maillard browning mixtures by other workers (Powrie et al., 1981; Shinohara et al., 1980). We presume that the mutagenicity in the heated orange juice samples must be ascribed to MIP as: (1) the heat treatments were not sufficient to cause browning; (2) the mutagenicity was dose and heat load related; and (3) freshly pressed unheated orange juice had no mutagenic effect.

Two important factors were observed to strongly affect the expression of mutagenicity: pH adjustment of the sample to 7.4 and 4 hr preincubation at 37°C and pH 7.4.

The shapes of the dose-response curves for various heat loads are shown in Figure 5.1. A positive linear correlation between the mutagenicity and heating time was observed up to 2 min (Fig. 5.2). Longer heating times resulted in a decrease in mutagenicity. This was due to cytotoxic effects of the samples as observed by examination of the background lawn on the plate under the stereo phase-contrast microscope.

Histidine (His) concentrations have to be considered in order to avoid misinterpretation of mutagenicity data (Aeschbacher, 1981; Stolz et al., 1982; Van der Hoeven et al., 1983). We have reported (Ekasari et al., 1986b) that in no case did unheated orange juice from various types of oranges with different His contents show any mutagenic response.

Table 5.1 shows the effect of addition of free L-His to the preincubation mixture without orange juice. Only a slight increase of the number of spontaneous revertants was observed.

We also investigated the influence of different His concentrations on the mutagenicity of heated orange juice. Samples with extra His, added after the heat treatment, did

Table 5.1 — Effect of Free Histidine (HIS) in the Mutagenicity Assay on the number of spontaneous revertants at various preincubation times

Amount of HIS added ^a (µg)	No. of spontaneous revertants/plate at preincubation time of:							
	0	20 min	1 hr	2 hr	3 hr	4 hr	5 hr	8 hr
0	157	166	183	189	181	187	188	180
3.4	175	201	200	191	202	198	201	165
13.3	211	216	249	225	226	252	262	215
26.6	260	245	285	252	308	273	281	231

^aHIS was added to the preincubation mixtures without orange juice sample. The concentrations correspond to the minimum (3.4), maximum (13.3), and twice the maximum (26.6) concentration of naturally occurring HIS per mL of single-strength orange juice

Table 5.1

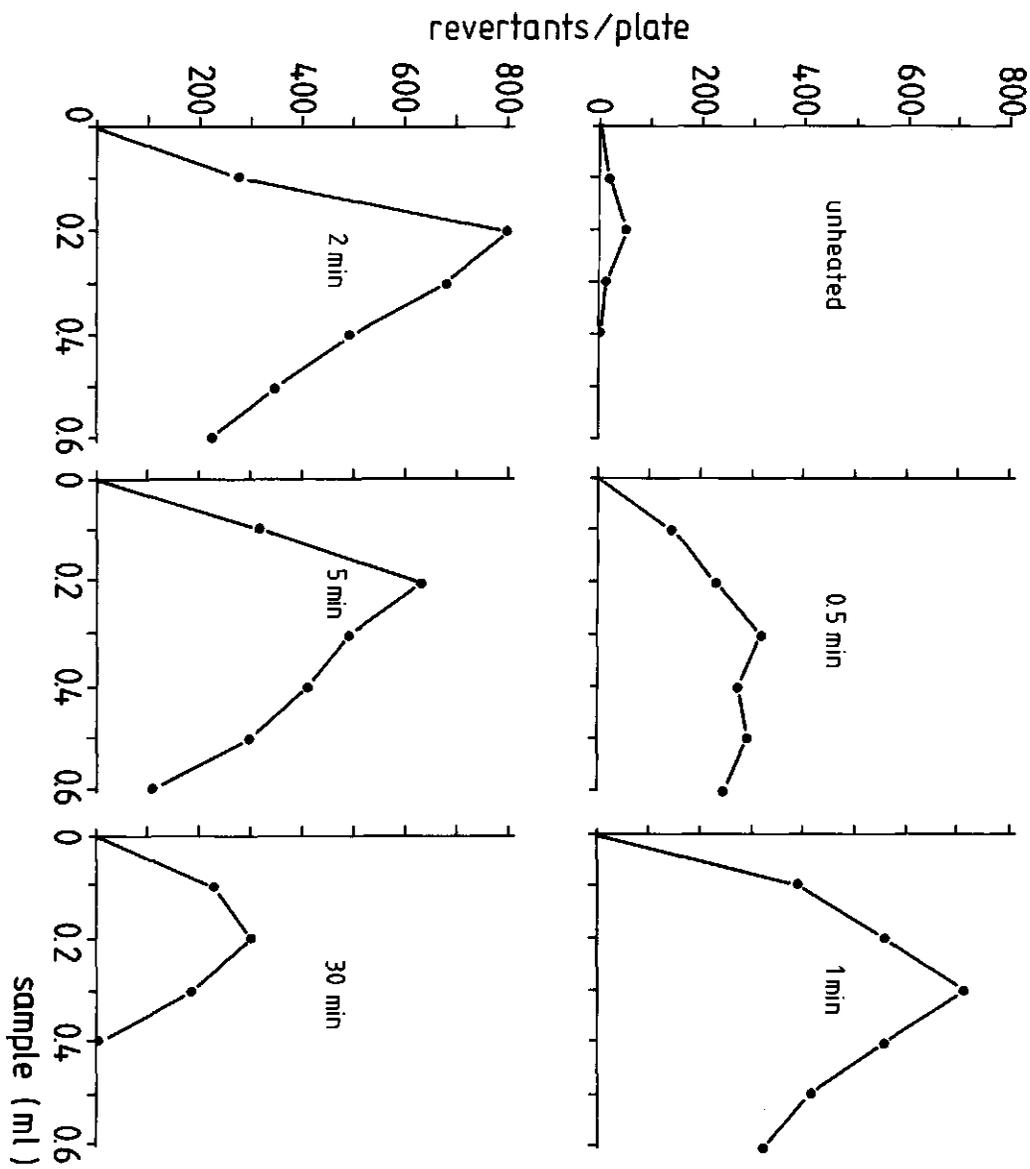


Figure 5.1 Shape of dose-response curves of the mutagenicity of heated orange juices. Samples were laboratory prepared orange juice heated at 93°C (30 sec - 30 min) and submitted to the modified mutagenicity assay (4 hr preincubation at pH 7.4 and 37°C with *Salmonella typhimurium* TA 100). Data used were taken from Ekasari et al., 1986a.

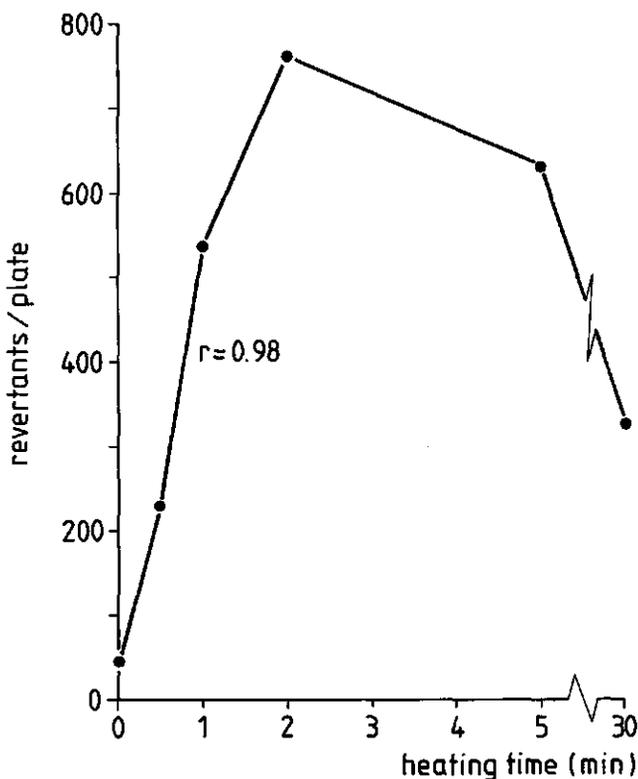


Figure 5.2 Effect of heating time on the mutagenicity of heated orange juices. Data were obtained from the slope of the linear portion of dose-response curves (Fig. 5.1) at a sample concentration of 0.2 ml/plate (from Ekasari et al., 1986a).

result in a higher number of revertants when heated longer than 2 min. However, the shape of the dose response curve (Fig. 5.3) did not change. This indicates that within limits the shape of the dose-effect curve, which is heat load associated, is not influenced by the His content although its presence at high concentrations may cause a synergistic effect. Again we observed a decrease in mutagenicity upon heating times longer than 2 min on both samples, which was caused by cytotoxic effects.

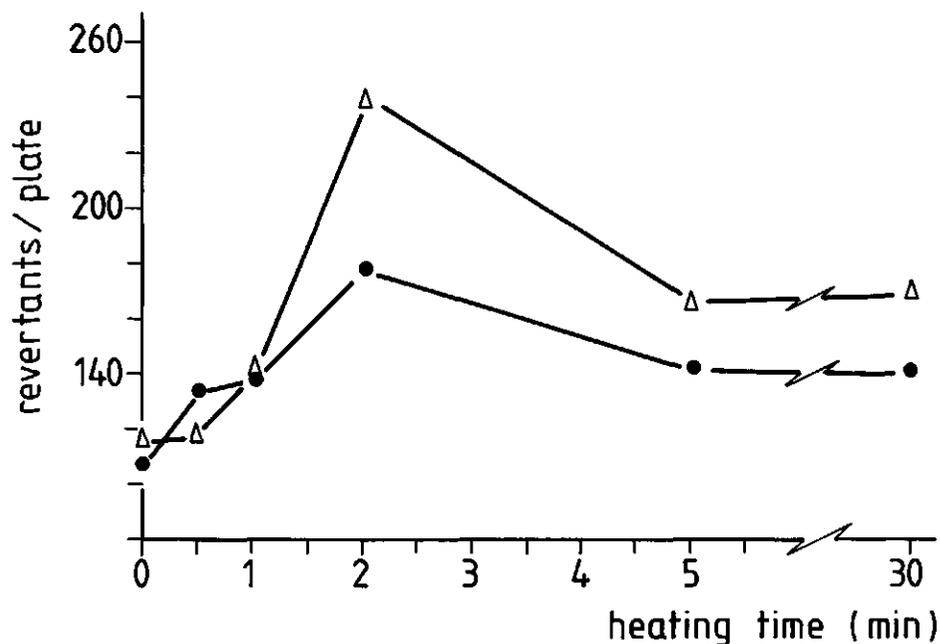


Figure 5.3 Influence of histidine (His) concentration on the mutagenic effect of heated orange juices. Samples were laboratory prepared orange juice heated at 93°C (30 sec - 30 min) and submitted to the modified *Salmonella* mutagenicity assay (4 hr preincubation at 37°C and pH 7.4) with *Salmonella typhimurium* TA100 using 0.1 ml sample/plate.

Symbols: ● and Δ refer respectively to sample A with His content of 10 μg/ml single strength juice and sample B which was sample A with 6 μg L-His/ml juice added after heat treatment.

Methods used for studying cytotoxic effects

We conducted our study on cytotoxicity by using the following methods:

Determination of cell survival by total plate count (TPC)

An earlier study (Ekasari et al., 1986b) using selective agar plates (Maron and Ames, 1983) enriched with His (1.5 mg/plate) showed that no cytotoxic effects were observed when a direct plating method was used. The cytotoxic effects could be observed only when the samples were preincubated with the tester inoculum under our specific test conditions (4 hr preincubation at pH 7.4 and 37°C) before plating.

In a previous experiment (Ekasari et al., 1986b), we noticed a proliferation of the cells of ca 5 times when unheated orange juice was tested. In the present study, we found a proliferation of about 40 times by using an inoculum of 50 cells/plate. An explanation for this proliferation is that His became available in a higher quantity for the inoculated cells as a less number of cells were used than in the mutagenicity assay in which a titre of ca 1×10^8 cells/plate is required. This condition allows the cell inoculum to undergo more cell divisions. Nevertheless it is seen (Fig. 5.4) that the number of survivors in heated orange juices decreased with longer heating times. Obviously, this strong reduction of survivors contributed to the decrease of mutagenicity as shown in Figures 5.2 and 5.3. In view of the effect of His concentration on the TPC method, which may cause difficulties when screening unknown samples, we investigated the cytotoxic effects with a kinetic method.

Measuring conductance

Conductance changes associated with *Salmonella typhimurium* TA100 metabolism on orange juices were detected by a Malthus microbiological growth analyzer, model 128 channel (Baynes

et al., 1983; Eden and Eden, 1985). It was programmed to measure conductance changes in intervals of 6 min during 24 hr. The time required to reach the point where three successive conductance values showed an increase ≥ 1.0 microsiemens (μS) was defined as detection time (DT). DT in hours is indicative of the metabolic activity of the tester organism (Oker-Blom, 1912). We assume that it is influenced by the cytotoxic effects.

The orange juice samples were prepared as for the mutagenicity assay (Ekasari et al., 1986a; chapter 4). The procedure for the conductance measurement was as follows: (1) Add 0.4 ml sample and 0.2 ml tester organism to a sterile Malthus tube of 2 ml size containing 0.2 ml sterile phosphate buffer pH 7.4 and 1.6 ml sterile pepton physiological salt solution. The tester organism was taken from a stock culture of *Salmonella typhimurium* TA 100 (Nutrient broth, Oxoid no. 2) with a titre of ca 1.6×10^6 cells/ml. The pepton physiological salt solution consisted of 0.1% pepton and 0.85% NaCl. (2) Mix gently on a Vortex mixer and fit into the instrument. The waterbath incubator was kept at 37°C. A separate preincubation period required for the plate assay was not necessary in this procedure in view of the long incubation time before detection.

Figure 5.5 shows the typical conductance curves obtained from orange juice samples heated at 93°C for various holding times. The unheated orange juice was detected first and the conductance curve obtained (a) had a sharp acceleration. The DT obtained with heated orange juice samples were in the range of 2.4-9 hr later than the DT obtained with freshly pressed unheated orange juice. DT depended on the amount of heat load, increasing with increasing heating time. At longer heating times the differences were more pronounced. The results indicate that for samples carrying a high amount of heat load, the conductance method is a promising supplement or even alternative to the mutagenicity assay.

SURVIVAL OF *S. typhimurium* TA100

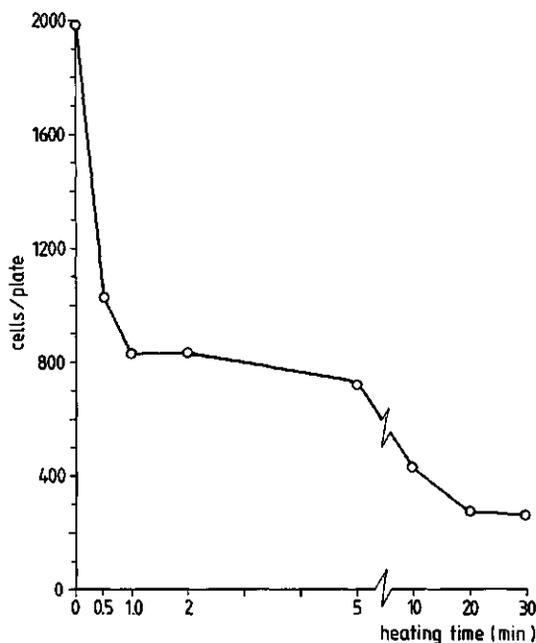


Figure 5.4 Survival of *Salmonella typhimurium* TA100 upon exposure to heated orange juices. Samples were laboratory prepared orange juice heated at 93°C (30 sec - 30 min) and assayed (0.2 ml serum/plate) for total plate count (see text). Inoculum used was ca. 50 cells/plate as appeared on control plates containing 0.9% NaCl solution.

Classification of commercial orange juices

In Table 5.2, 10 commercial samples are classified in four groups on the basis of their mutagenic responses interpreted as heat load. The presumed amount of heat load was judged by comparing the shapes of the resulting dose-response curves (not shown) with those in Figure 5.1.

Table 5.2 — Presumed Heat Load of Commercial Orange Juices based on the results of the modified Salmonella mutagenicity assay

Sample	Country of producer	Package	Product	Technological history	Presumed heat load ^a
A	United States	Pure Pak	Single strength	Pasteurized at 82°C for 4 sec; aseptic-fill	+
B	West Germany	Glass bottle	Single strength	Unknown	+
C	West Germany	Tetra Brik	Reconstituted	Aseptic fill	+
D	The Netherlands	Carton can	Frozen concentrate	Unknown	++
E	The Netherlands	Tetra Brik	Reconstituted	Aseptic fill	+++
F	The Netherlands	PKL-Combibloc	Reconstituted	Aseptic fill	+++
G	The Netherlands	Pure Pak	Reconstituted	Aseptic fill	+++
H1	The Netherlands	Glass bottle	Reconstituted	Tunnel-pasteurized at 74°C for 14 min	+++
H2	The Netherlands	Metal can	Reconstituted	Tunnel-pasteurized at 85°C for 10 min	++++
I	West Germany	Pure Pak	Reconstituted	Aseptic Fill	++++

^aRanged from lowest heat load (+) to highest heat load (+++++)

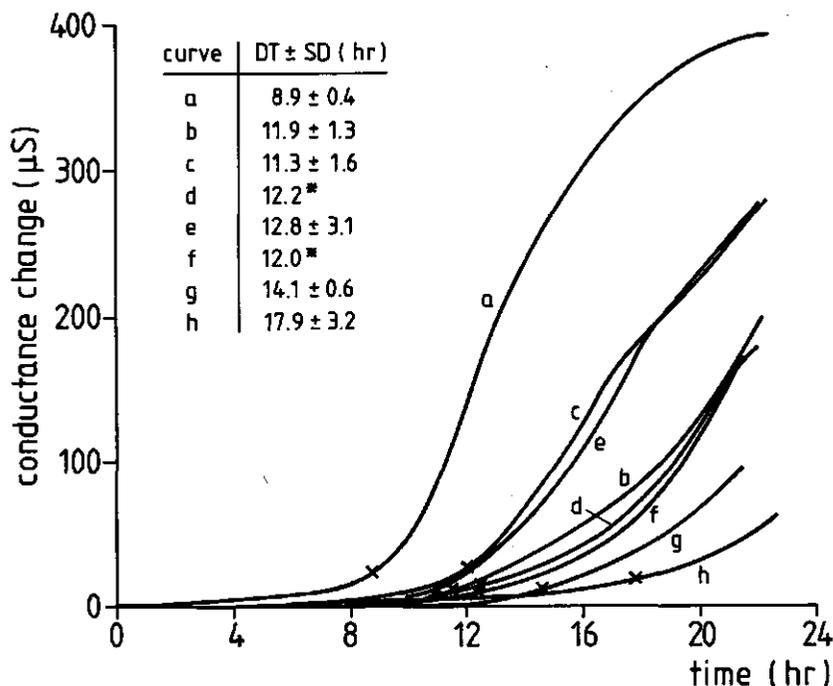


Figure 5.5 Conductance curves of *Salmonella typhimurium* TA100 with samples from laboratory prepared orange juice heated at 93°C for time zero (a); 30 sec (b); 1 min (c); 2 min (d); 5 min (e); 10 min (f); 20 min (g) and 30 min (h). Symbol x indicates detection time (DT; see text). Data listed ± SD are the mean of at least triplicates otherwise from duplicates (*).

The type of packaging and the label declaration ("from concentrate") allow speculations regarding the heat treatment of the samples. It can be seen that there is good agreement with the mutagenicity data. The frozen concentrate (D) ranks between the single strength juices (A and B) which have undergone pasteurization only and juices reconstituted from concentrate and heated once more for aseptic filling in carton (E, F, G). The classification of H1 and H2 is also justified by the information received from the manufacturer as specified in Table 5.2. Sample H2 in metal can has been

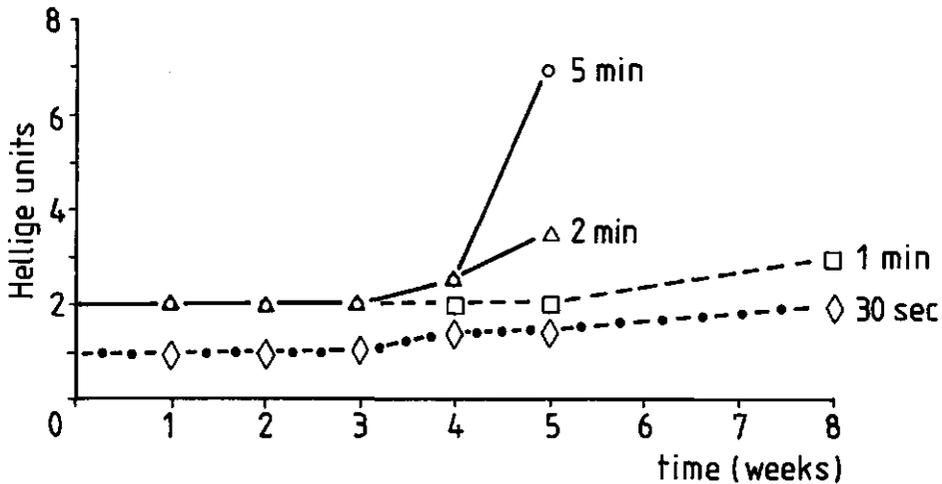


Figure 5.6 Color stability of heated orange juices during storage at 30°C. Samples were laboratory prepared orange juices heated at 93°C (30 sec - 5 min). They were measured for color on their alcohol filtrate (Meydav et al., 1977) using a Hellige Neo Comparator.

subjected to a higher temperature than sample H1 in glass bottle and has supposedly been kept at relatively high temperature to dry the can in order to avoid rusting. Sample I in Tabel 5.2 seems to be an exception, but may be explained by details of heat treatment not known to the authors.

Assessing inner quality

Figure 5.6 shows that juices with a higher heat load had a stronger tendency for browning. A similar effect was also reported by Mannheim and Havkin (1981) and Saguy et al. (1978). This means that the measurement of mutagenicity

and/or cytotoxicity is indeed an expression of the inner quality of a juice or concentrate.

It must be stressed that heat treated acidic orange juices neither show mutagenic nor cytotoxic effects. These properties are only obtained under specific conditions of the test.

Table 5.2 shows that juices do differ in inner quality which can be assessed by the mutagenicity assay. Whether these differences are relevant under prevailing systems of distribution is a question we cannot answer. However, if we would buy concentrated juices for reconstitution, we would certainly be guided by our test. Of course, the chemical nature of MIP, once known, may become the basis of a more accurate and simpler test.

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**CHAPTER 6: MUTAGENICITY AND POSSIBLE
OCCURRENCE OF FLAVONOL AGLYCONES
IN HEATED ORANGE JUICE**

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6 MUTAGENICITY AND POSSIBLE OCCURRENCE OF FLAVONOL AGLYCONES IN HEATED ORANGE JUICE

Indriati Ekasari¹, M.H. Bonestroo,¹ W.M.F. Jongen² & W. Pilnik¹

¹Department of Food Science, ²Department of Toxicology, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT

Orange juices were heated at 93°C for 1-30 min and hydrolysed with 1M HCl for 1 hr at 100°C. The resulting heat-treated orange juice and acid hydrolysate were analysed for flavonol aglycones (kaempferol and quercetin) and mutagenic activity towards *Salmonella typhimurium* TA 100 without S9-mix. Results show that mild heat treatments normally applied in the processing of orange juices are insufficient to liberate mutagenic flavonol aglycones from their glycosides. These findings support the hypothesis that heating produces Maillard intermediary products (MIP) which, after neutralisation to pH 7.4 and under specific preincubation conditions (4 hr at 37°C and pH 7.4), give rise to mutagenicity and cytotoxicity.

INTRODUCTION

Working on the development of a method to measure heat load in orange juice, we have demonstrated that heated orange juices have mutagenic and cytotoxic effects towards *Salmonella typhimurium* TA 100 under specific test conditions.

It is proposed that these effects are due to Maillard intermediary products (MIP; Ekasari et al. 1986a, b, 1988; chapters 4 and 5). Mazaki et al., (1982) reported that hydrolysates of citrus juices obtained with acid and enzymes were mutagenic by the Ames test. They suggested that the mutagenicity observed in the ether extract was due to flavonol aglycones such as kaempherol and quercetin detected in the hydrolysates.

In citrus fruit, flavonols are present as glycosides, which are not mutagenic but which may be converted to aglycones, for example, by acid hydrolysis (Brown, 1980; Rouseff, 1980; Uyeta et al., 1981). The pure aglycones quercetin from quercitrin (quercetin-3-rhamnoside) and rutin (quercetin-3rutinoside), and kaempherol from robinin (kaempherol-7-rhamnoside-3-galactorhamnoside) were reported to be mutagenic in the Ames test (Bjeldanes & Chang, 1977; Brown, 1980; Hardigree & Epler, 1978).

The present study was carried out to elucidate the possibility of involvement of mutagenic flavonols in our test system. Heat-treated orange juice (93°C, 1-30 min) and an acid hydrolysate were examined for the presence of kaempherol and quercetin and mutagenic activity towards *Salmonella typhimurium* TA 100 without S9-mix.

EXPERIMENTAL

Preparation of samples

Samples of laboratory-prepared orange juice (see Ekasari et al., 1986a; chapter 4), were divided into three portions and treated according to Fig. 6.1.

Treatment A resulted in a serum as usually prepared for our test system (Ekasari et al., 1986a; chapter 4). Treatment B resulted in an ether extract. Treatment C was an acid hydrolysis according to Mazaki et al. (1982) and resulted in an ether extract.

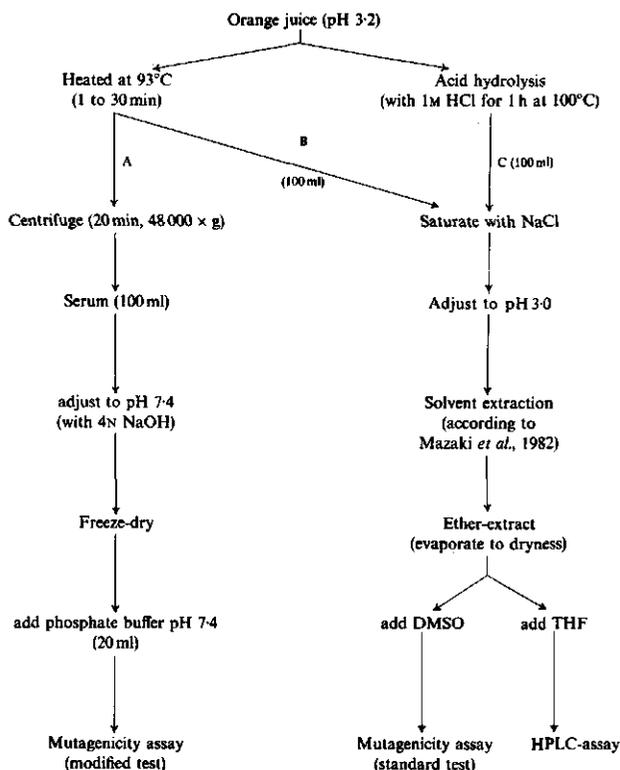


Figure 6.1. Sample preparation flow chart.

Mutagenicity assays

The mutagenicity tests were performed according to our modified assay as previously described (Ekasari et al., 1986a; chapter 4) and the standard test which was an Ames test with 20 min preincubation at 37°C as described by Maron & Ames (1983). All mutagenicity tests were performed with *Salmonella typhimurium* TA 100 without S9-mix. Each test was carried out at least in triplicate. The numbers of spontaneous revertant colonies (114-152 colonies/plate) were subtracted from the numbers of induced revertant colonies.

Revertants induced by the positive control NQO (4-nitroquinoline-N-oxide) were $\geq 10,000$ revertant colonies/ μg . An extract was designated mutagenic if the number of induced revertants obtained was at least twice the number of spontaneous revertants (solvent control).

HPLC analysis of quercetin and kaempherol

A spectra Physics liquid chromatograph was used, equipped with an SP 8700 XR pump and a Kratos Spectroflow 773 variable wavelength detector (365 nm at 0.05 AUFS range).

A Merck reversed phase column (250 x 4.6 mm I.D.) self-packed with Lichrosorb 10 RP18 equipped with a Chrompack precolumn (100 x 2.1 mm I.D.) packed with Copell ODS 30-38 μm was used.

A gradient program (Table 6.1) was used. Quercetin and kaempherol were eluted in 29.15 min and 32.32 min, respectively; the flow-rate was 1.5 ml/min.

Table 6.1 Gradient Program: (A) 4% Acetic Acid in Methanol, and (B) 4% Acetic Acid in Water.		
Time (min)	A(%)	B(%)
0	10	90
9	10	90
24	50	50
30	60	40
35	60	40
36	100	0
42	100	0

RESULTS AND DISCUSSION

Table 6.2 presents kaempherol and quercetin content in the ether extract of orange juice sample (heated at 93°C for 1-30 min) and its acid hydrolysate obtained from acid

hydrolysis according to Mazaki et al. (1982). These analytical results were confirmed by complete recovery from spiked samples (data not shown). There was no detectable amount of either kaempferol or quercetin in orange juice samples heated at 93°C for various times and only a very small amount was observed after 30 min heating. The acid hydrolysate showed a higher amount of quercetin and kaempferol.

Furthermore, in agreement with the results of a previous study (Ekasari et al., 1986b), it was shown that heated orange juices under their natural acid condition were not mutagenic. Neither the serum (treatment A) nor the ether extract (treatment B) of heated orange juices (93°C, 1-30 min) showed any mutagenic activity in the Ames standard mutagenicity test (Table 6.3). These findings confirm that the heat treatments normally applied in the processing of orange juices are insufficient to liberate either kaempferol or quercetin from their sugar conjugates of the corresponding glycosides. The magnitude of the quercetin and kaempferol levels in the acid hydrolysate implicates that the concentration tested in the mutagenicity assays do not exceed

Table 6.2 Flavonol Aglycones in Ether Extract of Laboratory Prepared Orange juice.			
Treatment ^a	Kaempferol	Quercetin	Total
	(µg/ml single strength juice)		
B (unheated)	ND	ND	ND
B (93°C, 1 min)	ND	ND	ND
B (93°C, 2 min)	ND	ND	ND
B (93°C, 30 min)	0.16	0.11	0.27
C (acid hydrolysed)	1.8	5.5	7.3

a See Figure 6.1
 ND: not detected

1.1 µg/plate and 0.4 µg/plate, respectively. According to Bjeldanes & Chang (1977) and Hardigree & Epler (1978) quercetin is a direct-acting mutagen. However, the available amounts observed in treated orange juices (Table 6.2) would not be sufficient to cause mutagenic effects. Kaempherol requires metabolic activation by mammalian liver S-9 to become mutagenic. The cytotoxic effects observed in the acid hydrolysed sample (Table 6.3) probably are due to other compounds formed during the extreme acid treatment which resulted in a viscous dark-brown hydrolysate. We can conclude that the mutagenic flavonols i.e. quercetin and kaempherol are not involved in our test system.

Treatment ^a	Test system	Dose (ml/plate)	Revertants/plate
A (93°C, 1 min)	Modified	0.1	346 ^b
		0.2	268 ^b
A (93°C, 2 min)	Modified	0.1	225 ^b
		0.2	145 ^b
A (93°C, 2 min)	Standard	0.1	9
		0.2	27
B (93°C, 1 min)	Standard	0.05	8
		0.1	9
B (93°C, 2 min)	Standard	0.05	1
		0.1	10
B (93°C, 30 min)	Standard	0.05	8
		0.1	3
C (acid hydrolysed)	Standard	0.05	toxic
		0.1	severely toxic

a See Figure 6.1
b Indicates mutagenic

This supports our theory that heating produces Maillard intermediary products (MIP) which, after neutralisation to pH 7.4 and under the specific preincubation conditions described, give rise to mutagenicity and cytotoxicity (Ekasari et al., 1986a, b; chapter 4).

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**CHAPTER 7: PREDICTIVE VALUE OF MICROBIOLOGICAL
METHODS TO MEASURE HEAT LOAD;
COLOUR STABILITY OF COMMERCIAL
ORANGE JUICES DURING STORAGE**

Submitted for publication in J. of Food Science.

7 PREDICTIVE VALUE OF MICROBIOLOGICAL METHODS TO MEASURE HEAT LOAD: COLOUR STABILITY OF COMMERCIAL ORANGE JUICES DURING STORAGE

Indriati Ekasari¹, M.H. Bonestroo¹, W.M.F. Jongen² & W. Pilnik¹

¹Department of Food Science, ²Department of Toxicology, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT

Inner quality and colour stability of aseptically cold-filled orange juices packed in carton bricks were compared to that of hot-filled orange juices packed in glass bottles. Heat load measurements prior to storage were done using our microbiological methods, i.e. modified *Salmonella* mutagenicity assay and cytotoxicity test using Malthus conductance meter. Results showed that hot-filled orange juice carried a higher heat load than aseptically cold-filled orange juice. Colour changes during storage at 30°C were monitored by measurements of absorbances (420 nm) on the clear ethanol extract of the juice, and CIE:Y (lightness) values and citrus red (CR) values of the whole juice. There were lag periods for browning observed in both juices regardless of the filling method. Juice carrying a higher heat load had a shorter lag period.

INTRODUCTION

Most orange juices available on the market today are reconstituted from orange juice concentrates. Differences in

quality are sometimes observed after reconstitution and storage (Marshall et al., 1986; Sizer et al., 1988). Ekasari et al. (1988) suggested that an important factor in this respect is the inner quality of the juice, which depends on the heat load it has received during all processing steps up to the beginning of storage in the final consumer package. The expression inner quality is used to indicate that at that point no sensorial differences are noted, although differences in heat load are detected by microbiological methods.

So far our work has been addressed to the study of laboratory prepared single strength orange juices heat treated at a constant temperature (93°C) for various times (30 sec - 30 min). The present work was an accelerated shelf-life study of industrially reconstituted orange juices prepared for the market. Prior to storage the juices were assayed for their inner quality. Colour being considered an important quality aspect (Klim and Nagy, 1988; Mannheim and Passy, 1979; Robertson and Reeves, 1981), we followed colour changes during storage. The juices were stored at 30°C as recommended by Labuza and Schmidl (1985).

MATERIALS AND METHODS

Juice preparation

The juices were prepared from one batch of 64 °Brix commercial frozen orange juice concentrate. Reconstitution to 11.8 °Brix was performed in a fruit juice processing plant. The resulting single strength juice was divided to follow two different methods of pasteurization and filling: (1) aseptic cold-filled in 1 l laminated carton brick (95°C, 40 sec) and (2) hot-filled in 1 l glass bottle (85°C, 20 min) with slow cooling to 20°C. All juices were processed on the same date.

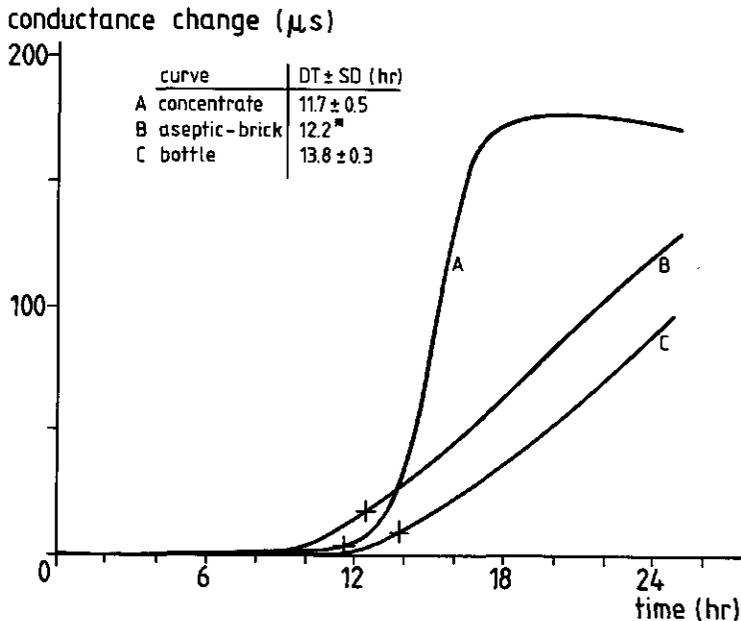


Figure 7.2 Conductance curves of *Salmonella typhimurium* TA 100 with samples of industrially reconstituted orange juices. Symbol (+) indicates detection time. Data listed \pm SD are the mean of at least triplicates otherwise from duplicates (*).

Colour stability during storage

Browning during storage is shown in Fig. 7.3. It is seen that aseptic brick packed juice had a lag period of 4 wk in which there is no difference in the absorbance value (420 nm) from the juice at time zero, whereas the bottled juice with the higher heat load started browning already after 2 wk. This may mean a longer shelf-life of the aseptic brick packed juice under commercial conditions.

The phenomenon of lag period was earlier observed by Karel and Nickerson (1964) and Mannheim and Passy (1979). They described the lag period as the period in which colourless compounds, which are preliminary to formation of brown compounds, are formed. These correspond to our theory of Maillard intermediary products (MIP) formation.

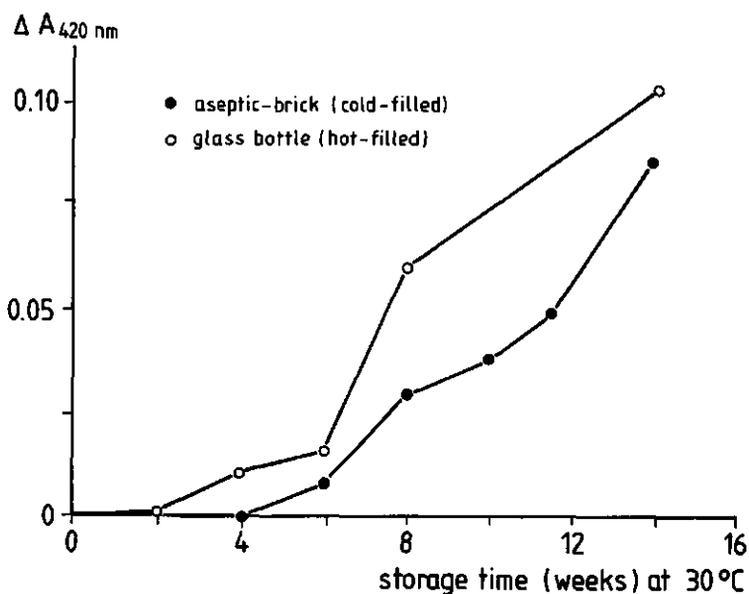


Figure 7.3 Browning development of industrially reconstituted orange juices upon storage at 30°C, as influenced by heat load.

The lag period was also reported in other fruit juice products, i.e. lemon juice (Clegg and Morton, 1965; Robertson and Samaniego, 1986), grape fruit juice (Lee and Nagy, 1988; Saguy et al., 1978), and apple juice (Toribio and Lozano, 1986).

Fig. 7.4 presents data of colour measurements. For the aseptic brick packed juice an increase in lightness (CIE:Y) and a decrease in citrus red (CR) values is observed during the first 3 wk of storage. This may be explained by reverse browning, probably due to trans-cis isomerisation of the carotenoids as has been described for tomato juice (Boskovic, 1979; Lovric et al., 1970). In the bottled juice this phenomenon is not detected in the colour measurement due to interference of the quickly starting browning reaction (Fig. 7.3). After the lag period the rate of browning (Fig. 7.3) and darkening (Fig. 7.4) appears to be similar in both juices. The fact that at a given time bottled juice is darker than aseptic brick packed juice must be ascribed to its shorter induction period.

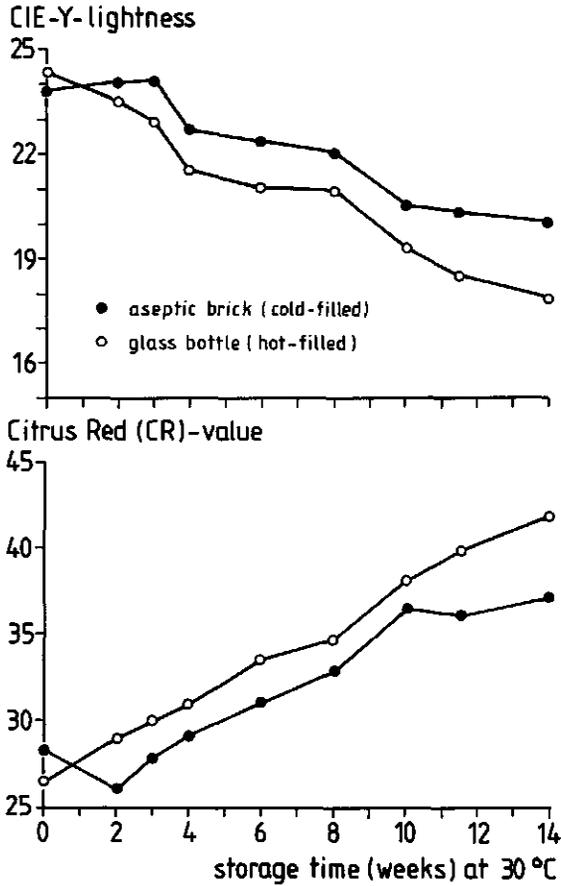


Figure 7.4 Changes of CIE:Y (lightness) values and citrus red (CR) values of industrially reconstituted orange juices upon storage at 30°C, as influenced by heat loads.

Conclusions

These accelerated shelf-life studies on industrially prepared orange juices show that the microbiological methods used to measure the heat load prior to storage, have a predictive value as has already been shown with laboratory prepared orange juices (chapter 5).

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CHAPTER 8: CHARACTERIZATION OF MUTAGENIC COMPOUND(S) IN HEATED ORANGE JUICE

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8 CHARACTERIZATION OF MUTAGENIC COMPOUND(S) IN HEATED ORANGE JUICE

Indriati Ekasari¹, H.E. Berg¹, W.M.F. Jongen² & W. Pilnik¹

¹Department of Food Science, ²Department of Toxicology, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT

Heated orange juice (93°C, 2 min) was fractionated using solvent extraction, vacuum distillation, ion exchange chromatography and gel filtration. The fractions were subjected to our modified *Salmonella* mutagenicity assay (pH adjustment to 7.4 and 4 hr preincubation at 37°C). Results showed that the mutagenic compound(s) are polar, non volatile, carry no charge and have molecular weights \leq 700 daltons. Three fractions separated by gel filtration showed a stronger mutagenicity than the serum; one fraction with molecular weight between 200-700 daltons possessed the highest mutagenic activity.

INTRODUCTION

In the preceeding papers (Ekasari et al., 1986a; 1986b; 1988; chapters 4 and 5) we reported that heat treated orange juices induced mutagenicity towards *Salmonella thymurium* TA 100 under the conditions of our modified *Salmonella* mutagenicity assay. The mutagenic response can be interpreted as heat load and its measurement gives an expression of inner quality of (concentrated) orange juice. On this basis we have been able to classify various commercial orange juices into four groups. Juices with a higher heat load show a stronger tendency for browning upon storage; similar

effect was also reported by other workers (Mannheim and Havkin, 1981; Marshall et al., 1986; Saguy et al., 1981). In the present study we attempt to define some properties of the compound(s) which cause mutagenicity. This will eventually lead to their identification. We, therefore, conducted fractionation of heated orange juice by vacuum distillation, solvent extraction, ion exchange chromatography and gel filtration to separate and concentrate the mutagenic compound(s). Since the strongest mutagenic response appeared in laboratory prepared orange juices heated at 93°C for 2 min, such juices were used for the purpose of this study. Juices heated for longer periods of time at this temperature (93°C) show a strong cytotoxic effect (Ekasari et al., 1988; chapters 4 and 5) and therefore were not included in the present study.

MATERIALS AND METHODS

Samples

Laboratory prepared orange juice heated at 93°C for 2 min as described by Ekasari et al. (1986a; chapter 4).

Preparation of the volatile and non-volatile fractions (vacuum distillation)

Heated orange juice (100 ml) was evaporated under vacuum at 60°C in a rotary evaporator until ca. 40 ml condensate was obtained. The resulting condensate (volatile fraction) and concentrate (non-volatile fraction) were adjusted to pH 7.4 with $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, filter sterilized (0.2 μm , Millipore) and tested for mutagenicity.

Ion exchange chromatography

Heated orange juice was centrifuged (48,000 x g for 20 min) and the serum, after pH adjustment to 7.4 (with 4 N NaOH) was chromatographed on a 9.5 cm x 2.0 cm column of Biogel CM (cation exchanger, Biorad) and a 12 cm x 1.6 cm column of Biogel DEAE (anion exchanger, Biorad). The columns were equilibrated with 6 M $(\text{NH}_4)_2\text{CO}_3$ solution; NH_4^+ and CO_3^{2-} were counter ions for CM and DEAE, respectively. Ten ml serum after two-fold dilution with distilled water was loaded onto each column and then eluted in 5 ml fractions with successively 5 column volumes of 0.0012 M $(\text{NH}_4)_2\text{CO}_3$ and 5 column volumes of 0.6 M $(\text{NH}_4)_2\text{CO}_3$. The eluates were collected and pooled into 3 portions called eluate I (fraction no. 1-10), eluate II (fraction no. 11-35) and eluate III (fraction no. 36-70); eluate I and II were obtained with the lower molar (0.0012 M) eluent. Each portion was freeze-dried, dissolved in 2 ml of 0.5 M phosphate buffer pH 7.4 (five-fold concentration), filter sterilized (0.2 μm , Millipore) and tested for mutagenicity.

Solvent extraction

Heated orange juice was centrifuged (48,000 x g for 20 min) and the serum, after pH adjustment to 7.4 (with 4 N NaOH), was freeze-dried in a 100 ml portion. The freeze-dried serum (ca. 12 g) was extracted in a Soxhlet apparatus, first with 250 ml of petroleum ether for 3 hr and then successively with chloroform and methanol in the same manner. Each solvent fraction was evaporated to dryness in a rotary evaporator under vacuum at 35°C. The freeze-dried serum (unextracted), the solvent extracts and the ultimate residue were dissolved in 20 ml 0.5 M phosphate buffer pH 7.4, i.e. five-fold concentration with regard to the 100 ml serum used. The aliquots were filter sterilized (0.2 μm , Millipore) and tested for mutagenicity.

Gel filtration

Heated orange juice was centrifuged (48,000 x g for 20 min) and the serum, after pH adjustment to 7.4 (with 4 N NaOH), was chromatographed at 2°C on a Biogel P2 from Biorad (100-200 mesh; M.W. cut-off 100-1800 daltons). The gel material was packed into the column, 100 cm x 2.6 cm; for each run 2 ml of sample was loaded onto the column. Elution was carried out with sterilized distilled water at a flow rate of 25 ml/hr. Fractions (3 ml) were collected and measured for U.V. absorbance at 210 nm (Carl Zeiss M4QIII spectrophotometer). The pool were divided into four portions (see Fig. 8.2), i.e. fractions no. 40-69, no. 70-110, no. 111-130 and no. 131-170. Each portion was freeze-dried, dissolved in 0.5 M phosphate buffer pH 7.4 to original volume of sample (2 ml), filter sterilized (0.2 µm, Millipore) and tested for mutagenicity. In order to estimate the molecular weight of fractions the elution curve (Fig. 8.2) was compared with the one established with a water solution containing galactan (M.W. > 2000), stachyose (M.W. = 666), raffinose (M.W. = 504), sucrose (M.W. = 342) and glucose (M.W. = 180) where in each fraction the phenol sulfuric acid test (Dubois et al., 1956) was performed.

Mutagenicity assay

Modified *Salmonella* mutagenicity assay (4 hr preincubation at 37°C and pH 7.4) as described by Ekasari et al. (1986a; chapter 4). *Salmonella typhimurium* TA100 without S9-mix was used. Mutation ratio (number of induced revertant colonies per plate divided by number of spontaneous revertant colonies per plate) ≥ 2.0 indicates positive mutagenic response. The numbers of spontaneous revertants were in the range of 96-178 colonies/plate. Revertants induced by the positive control (4-nitroquinoline-N-oxide) were $\geq 1000/0.1$ µg per plate.

Each experiment was done at least twice and each concentration of the sample was tested at least in triplicate.

RESULTS AND DISCUSSION

Vacuum distillation

Table 8.1 shows the results of the mutagenicity test on condensate and concentrate. Mutagenic activity was only observed in the concentrate indicating that the mutagenic compound(s) are non volatile at pH 7.4. In this connection the mutagenicity of commercial concentrated orange juices (Ekasari et al., 1986a; chapter 4) should be noted.

Table 8.1 Mutagenicity of volatile and non volatile fraction from heated orange juice				
	Mutation ratio ^b			
	Dose (ml/plate)			
Fraction ^a	0.1	0.2	0.3	0.4
CONCENTRATE (NON-VOLATILE)	2.6*	3.2*	2.8*	2.5*
CONDENSATE (VOLATILE)	1.0	0.9	0.9	0.9

a See Material and Methods (vacuum distillation).
b Asterisk indicates positive mutagenic response.

Ion exchange chromatography

As shown in Table 8.2, mutagenicity was observed only in the first few fractions of the lower molar eluent in both anion and cation exchanger. This indicates that the mutagenic compound(s) were not bound to the columns and,

therefore, directly washed out from the exchanger beds. Thus, the mutagenic compound(s) appeared to carry no charge.

Table 8.2 Mutagenic activity of fractions from ion exchange chromatography of heated orange juice

Fraction ^a	dose ml/plate	Mutation ratio ^b	
		anion exchanger	cation exchanger
Eluate I	0.10	2.6*	2.9*
	0.20	2.3*	1.7
	0.45	1.2	0.6 (toxic)
Eluate II	0.10	1.1	1.2
	0.20	1.2	1.1
	0.45	1.3	1.2
Eluate III	0.10	1.1	1.1
	0.20	0.8	0.9

a See Materials and Methods (ion exchange chromatography).

b Asterisk indicates positive mutagenic response.

Solvent extraction

Fig. 8.1 presents the dose-response curves of the mutagenic activity of the unextracted serum, the ultimate residue, and the petroleum ether extract. Results from chloroform and methanol extract (not shown) were similar to petroleum ether extract; in all cases no mutagenic activity was observed. Furthermore, mutagenic effect was observed in the unextracted serum and the ultimate residue (water extract). Together with the results from ion exchange chromatography (carry no charge) this suggests that the mutagenic compound(s) are polar.

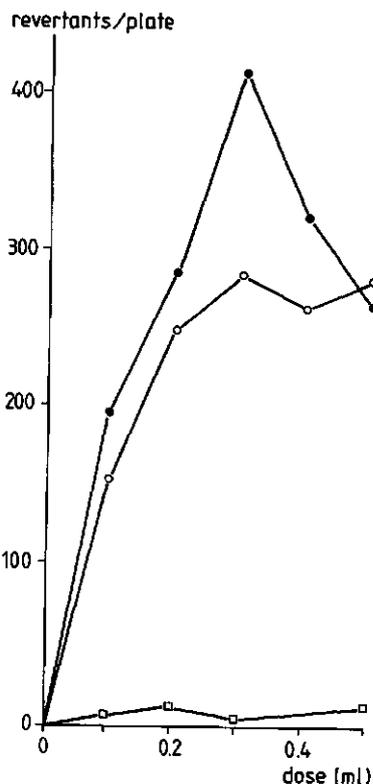


Figure 8.1 Dose response curves of mutagenic activity of (●) unextracted serum, 5x concentrated; (○) ultimate residue (water extract); (□) petroleum ether extract. For details, see Materials and Methods (solvent extraction). The numbers of induced revertants/plate are corrected for the number of spontaneous revertants.

Gel filtration

Fig. 8.2 shows the elution profile of serum of heated orange juice obtained with distilled water and measured for absorbance at 210 nm. Fig. 8.3 presents dose-response curves of the mutagenic activity of the pools. Mutagenic activity was observed starting from fraction no. 70 up to 170 (Fig. 8.2). It should be noted that the pools had a much stronger mutagenic activity than the unfractionated serum indicating a

concentration of the mutagenic compound(s). The pool of fractions no. 70 until 110 shows the strongest mutagenic activity. The molecular weight of these mutagenic fractions estimated from the elution profile from the same column with carbohydrates (not shown; see Materials and Methods) was found to be $200 < M.W. < 700$.

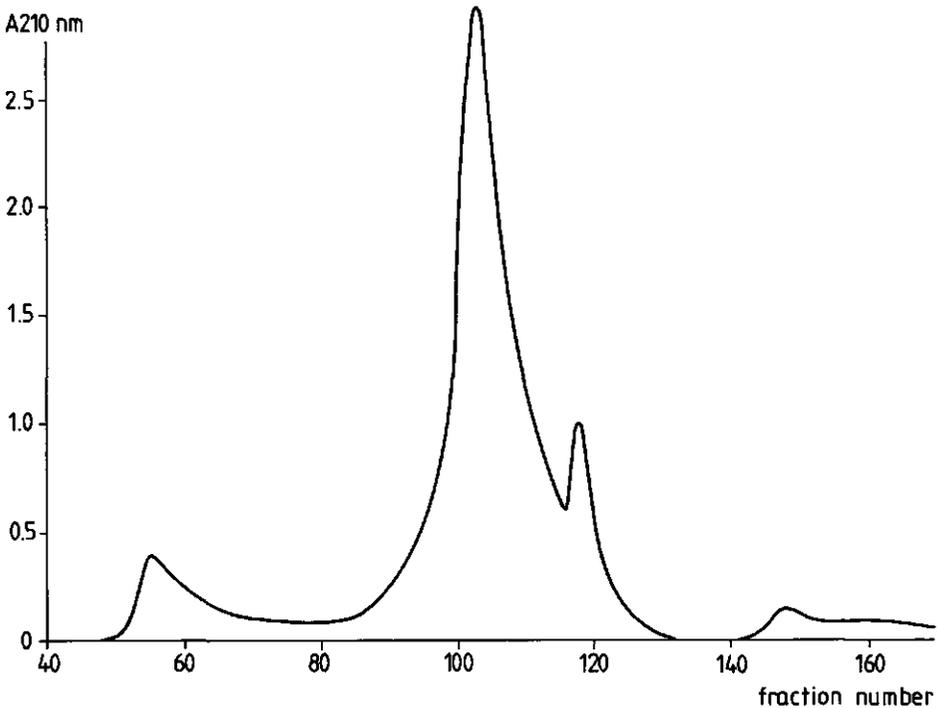


Figure 8.2 Gel filtration profile of serum of heated orange juice (93°C, 2 min) on Biogel P2. Column: 100 cm x 2.6 cm; eluent: sterilized distilled water; flow rate: 25 ml/hr; fraction size: 3.0 ml.

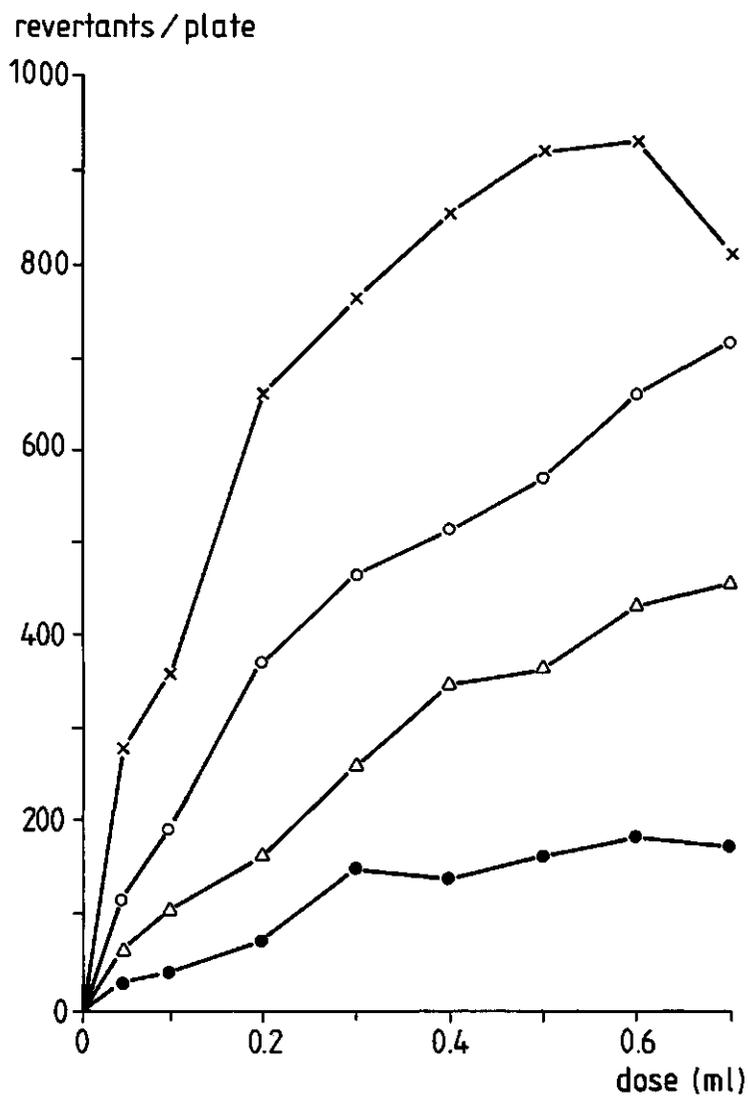


Figure 8.3 Dose-response curves of mutagenic activity of (●) unfractionated serum; (Δ) fractions no. 131-170 (M.W. \leq 200); (○) fractions no. 111-130 (M.W. \leq 200); (x) fractions no. 70-110 ($200 <$ M.W. $<$ 700). For details, see Fig. 8.2 and Materials and Methods (gel filtration). The numbers of induced revertants/plate are corrected for the number of spontaneous revertants.

In conclusion, partial characterization of the mutagenic compound(s) has been achieved and shows them to be polar, non-volatile, carrying no charge and having molecular weights between 200-700 daltons. Gel filtration appeared to be a good method to obtain fractions from heated juice which are strongly mutagenic. This is an important prerequisite for further investigations to isolate mutagenic compound(s) in order to determine their molecular structure.

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**CHAPTER 9: THE USE OF AN AUTOMATED SYSTEM
FOR MUTAGENICITY TESTING TO ASSESS
THE INNER QUALITY OF HEAT TREATED
ORANGE JUICES**

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9 THE USE OF AN AUTOMATED SYSTEM FOR MUTAGENICITY TESTING TO ASSESS THE INNER QUALITY OF HEAT TREATED ORANGE JUICES

Indriati Ekasari¹, C.J.M. Hoenderboom¹, W.M.F. Jongen² and W. Pilnik¹

¹Department of Food Science and ²Department of Toxicology, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT

The Cobas Bact automated microbiological growth analyser was used to explore the feasibility for automation of a *Salmonella* mutagenicity assay for heat load measurement of orange juice. The results indicated that endogenous growth factors, e.g. histidine present in whole orange juices and cytotoxic effects of the heated juices disturb the turbidometric assay system. Good results were obtained with gel filtration and HPLC fractions of the juice. It is concluded that for the screening of fractions in view of collecting material for the chemical identification of mutagenic Maillard intermediary products (MIP) the automated system may be helpful.

INTRODUCTION

We have described a modified version of the *Salmonella* mutagenicity plate incorporation assay to assess the inner quality of heat treated (pasteurized) orange juices (Ekasari et al., 1986a, 1988a; chapters 4 and 5). Data obtained with both laboratory prepared and commercially available orange juices show a correlation between heat load and mutagenicity. It is proposed that the mutagenicity of the heat

treated juice is due to Maillard intermediary products (MIP), which are not themselves perceived sensorially but which are the precursors reacting further to form brown pigments and off-flavours. The test can be used by manufacturers of orange juice for screening purposes when purchasing concentrates and for testing processing conditions in their factories, both with a view to shelf-life prediction, i.e the inner quality of the juices. In order to be able to test samples at large industrial scale, we envisaged to automate our method.

The objective of the present study was to explore an alternative system to our plate method (Ekasari et al., 1986a; chapter 4) which would provide the same approach of methodology but increase the capacity. In recent years several automated microbiological growth analysers that are designed to the handling of large numbers of samples have been developed. To detect growth different end-points are being used such as conductivity (Malthus instrument; Ekasari et al., 1988a; chapter 5) and turbidity. In the present investigation the Cobas Bact, an automated microbiological growth analyzer that monitors turbidity was used. This instrument was generously made available to us by Hoffman-La Roche (The Netherlands). In this article the results of this study are presented and the possibilities and limitations of the method are discussed.

MATERIALS AND METHODS

The Cobas Bact principle

A detailed description of the instrument is given by Arni et al. (1985), Dollenmeir (1985), Göcke and Schupbach (1986). The maximum capacity per trial is 800 individual samples. The system offers the option of monitoring growth as an increase in absorbance at various wave lengths. For our pur-

pose absorbance had to be measured at 564 nm to avoid interference of developing browning with turbidity measurements. The test is taking place in a disposable plastic rotor (\pm 9 cm diameter) consisting of a central chamber of 5.4 ml capacity and 16 peripheral measuring compartments, each with a capacity of 400 μ l. The central chamber is filled with 5 ml growth medium, 0.1 ml of 0.5 M phosphate buffer pH 7.4 and 0.1 ml tester bacterial suspension. The measuring compartments are filled with test substances and/or positive control at various doses up to a maximum of 100 μ l. For the mutagenicity test, one compartment of every rotor is filled with 100 μ l sterile distilled water which serves as negative control. After sealing, the rotor is immediately loaded to the Cobas Bact apparatus. By an initial centrifugation 300 μ l of the content of the central chamber is automatically distributed (centrifugal pipetting) into every compartment. Between the absorbance measurements the rotor is deposited in an air incubator (37°C) on a continuously rotating paternoster elevator. The results are recorded by computer (IBM PC/2 model 60 using Xenic system) and printed as growth curves.

The bacterial tester strain

Salmonella typhimurium strain TA 100 without S9-mix was used. The test culture was reisolated from master plates (Maron and Ames, 1983; chapter 3) and grown in nutrient broth (Oxoid no. 2) for 6 hr. It was further diluted with saline solution (0.9% NaCl) to the desired concentrations of cells.

Growth medium

Unless stated, otherwise the growth medium used was Vogel-Bonner medium E (Vogel and Bonner, 1956) supplemented with glucose (2%) and biotin (0.5 μ g/ml). This composition was

similar to that used for the plate assay. The amount of histidine (L-His) added is indicated in the figure legends.

Samples

Mutagen NQO (4-nitroquinoline-N-oxide): this was prepared by dissolving 1 mg NQO directly in 1 ml DMSO and then diluting with saline solution (0.9% NaCl) to the desired concentration as detailed in the figure legends (dilution factor $\geq 10^6$).

Orange juices: these were prepared according to Ekasari et al. (1986a; chapter 4).

Mutagenic fractions: Gel filtration fractions were a pool of mutagenic fractions of heated orange juice (93°C, 2 min) prepared by the same method as described in Chapter 8 (Ekasari et al., 1988b). On these fractions the ninhydrine test for the presence of amino acids was performed. Mutagenic fractions collected from three similar runs (6 ml of samples elution) were pooled, freeze dried, and dissolved in 1.2 ml 0.5 M phosphate buffer pH 7.4 (five-fold concentration). The aliquots were filter-sterilized (0.2 μ m, Millipore), tested for mutagenicity in Cobas Bact and injected (100 μ l) into HPLC for further fractionation.

HPLC fractions were prepared by eluting aliquots of mutagenic fractions obtained by gel filtration (see above) on a Spectra Physics HPLC system (SP 8770) equipped with a Kratos 773 variable wave length detector. The columns and conditions used were the same as those described by Voragen et al. (1988) to fractionate MIP from model systems of apple juice. These were an experimental column of Aminex HPX-42H (300x7.8 mm i.d.; Bio-Rad labs, Richmond, CA, USA) equipped with precolumn AG 50W-X4 (50x4.6 mm i.d.). Elution was performed with 0.01N H₂SO₄ (0.6 ml/min) at 30°C. The fractions were collected on the basis of retention time (one-minute fraction; 0.6 ml). Sulfate ions in fractions were precipi-

tated by addition of saturated Ba (OH)₂ solution. After centrifugation and washing of the pellets, the collected supernatants were brought to pH 7.4 with diluted NaOH solution and subsequently freeze dried.

To obtain aliquots with soluble solids concentration equal to single strength orange juice, the freeze-dried fraction was dissolved in 0.5 ml of 0.5 M phosphate buffer pH 7.4. The fraction were then individually tested for mutagenicity in Cobas Bact (70 µl/compartment).

RESULTS AND DISCUSSION

Effect of varying histidine (His) concentration and initial test cells number

The Cobas Bact assay is a kinetic system in which the bacterial growth is monitored by a turbidometry system. Auxotrophic growth and prototrophic growth of the induced revertants are recorded as increasing absorbance. For measuring mutagenicity, it is crucial to establish a system that can clearly distinguish auxotrophic growth from prototrophic growth. In the plate test, prototrophic growth appears as prominent thick round colonies on the agar plate at the end of the incubation period. Auxotrophic growth does not show as colonies but as the so-called background lawn which is not counted.

Auxotrophic growth of the tester bacteria *Salmonella typhimurium* TA 100 depends on the presence of the amino acid histidine (His). A trace amount of His is required to enable the bacterial cells to divide a few times so that they can carry the initial mutation over to the next generation of cells (Maron and Ames, 1983; chapter 3).

In turbidity measurements the amount of His in relation with the initial bacterial cells becomes a critical factor. Initial experiments were carried out by adding varying propor-

tions of inoculum and His together with growth medium to the rotor. As Fig. 9.1(A and B) indicate the higher the His con-

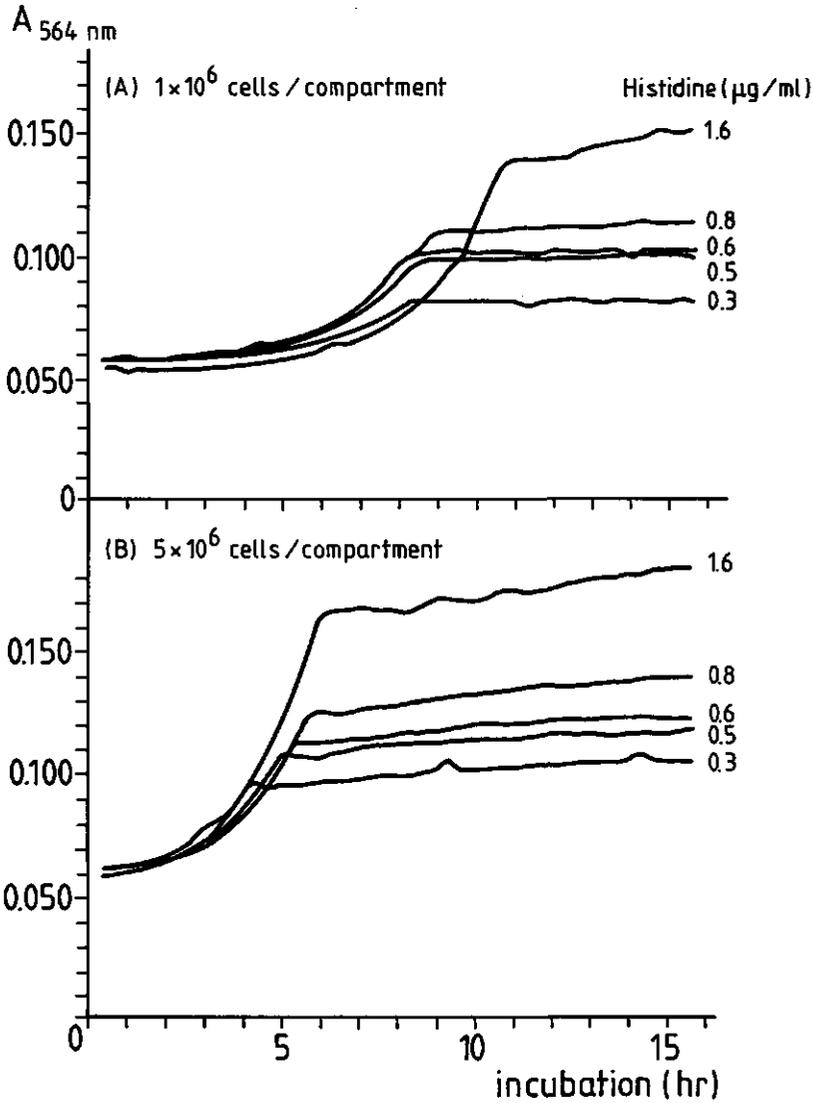


Figure 9.1 Effects of histidine (His) on auxotrophic growth of *Salmonella typhimurium* TA 100 on the Cobas Bact assay.

centration, the higher the absorbance. The growth curves show that at the same level of His concentration, the use of tester cells of 5×10^6 /compartment resulted in an earlier cessation of auxotrophic growth than that of 1×10^6 /compartment.

Once the auxotrophic growth ceases, the growth curve remains a plateau. It can be expected that prototrophic growth of revertants induced by a mutagenic substance will rise beyond this plateau. This was seen by Arni et al. (1985) when measuring the mutagenicity of a large number of compounds in this way. Our results shown on Fig. 9.1 suggest to apply the combination of 5×10^6 tester cells/compartment with $0.8 \mu\text{g}$ His/ml medium. Gocke and Schüpbach (1986) also concluded that this His concentration was the maximum which can be used before disturbing prototrophic growth. On this basis we first tested 4-NQO (4-nitroquinoline-N-oxide), a direct acting mutagen used as positive control in our test.

Experiments with NQO (4-nitroquinoline-N-oxide)

Various concentrations of NQO (0.1pg-5ng) were loaded to the rotor compartments. The results are presented in Fig. 9.2. They show distinct prototrophic growths induced by different amount of NQO. The effects are seen to be dose related: the higher the NQO concentration, the earlier the appearance of prototrophic growth (detection time). At the lowest concentration (0.1 pg), there is no prototrophic growth observed.

Experiments with orange juices of various heat load

Neither histidine nor biotin nor glucose was added to the medium considering the sugars and amino acids content of orange juice. Fig. 9.3 shows results of these experiments. A prolongation of auxotrophic growth was observed in all cases when compared with Fig. 9.2. We suspect that this is

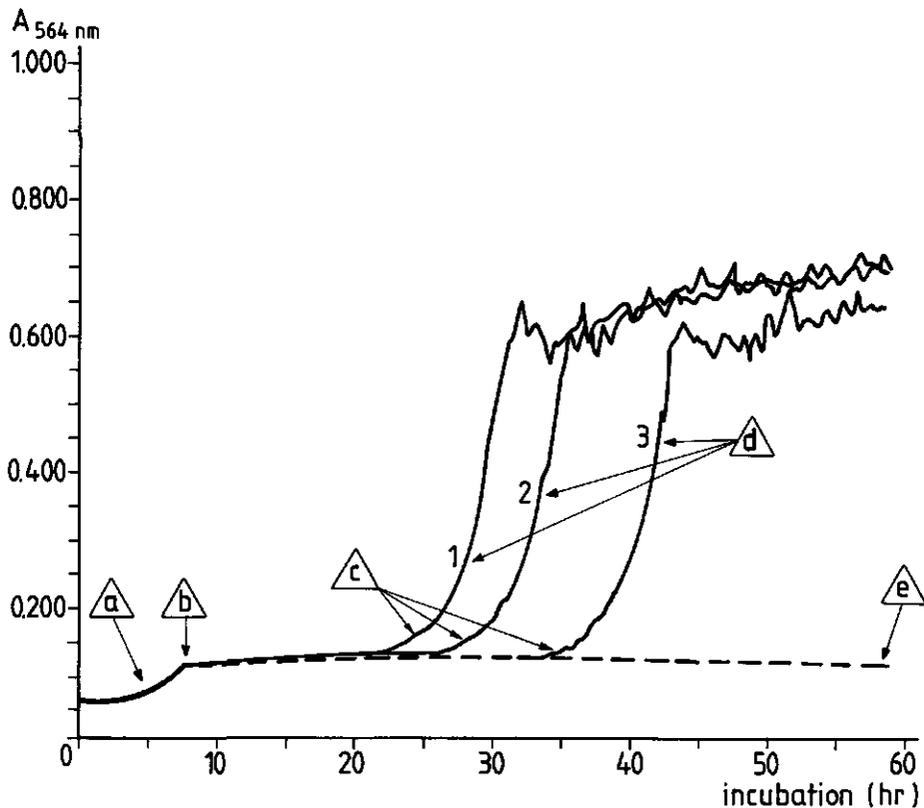


Figure 9.2 Growth curves of *Salmonella typhimurium* strain TA 100 upon exposure to the direct acting mutagen NQO (4-nitroquinoline-N-oxide) in the Cobas Bact assay.

- a) Auxotrophic growth.
- b) The cessation of auxotrophic growth.
- c) The start of prototrophic growth (detection time).
- d) Prototrophic growth of revertants induced by NQO. Concentrations per compartment: 5 ng(1); 1 ng(2); 0.1 ng(3).
- e) Negative control.

Inoculum used was ca. 5×10^6 cells/compartment. His (0.8 $\mu\text{g}/\text{ml}$) was added to the growth medium (see Materials and Methods).

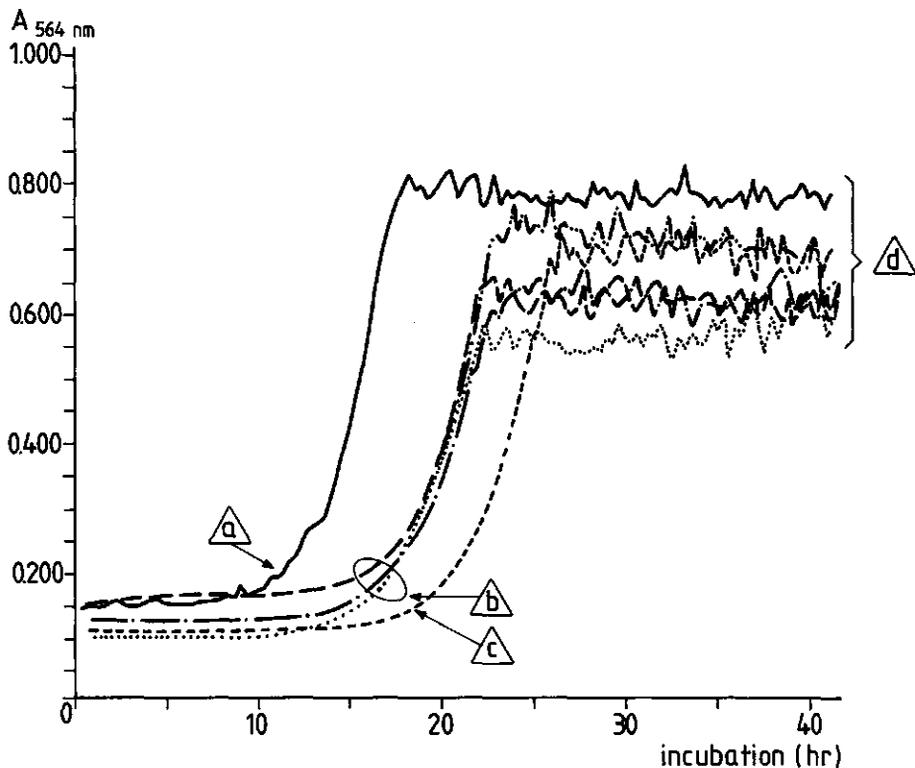


Figure 9.3 Growth curves of *Salmonella typhimurium* TA 100 upon incubation in serum of heat treated orange juices in the Cobas Bact assay.

a-c: The delay of auxotrophic growth in samples a (unheated orange juice), b (orange juices heated at 93°C for 1, 2, 5 and 20 min) and c (orange juice heated at 93°C for 30 min);

d: The cessation of growth in samples a, b and c.

The medium added was only Vogel-Bonner E solution and the inoculum used was ca. 5×10^6 cells/compartment. See Materials and Methods for sample preparation. Samples used were 70 μ l/compartment and brought to 100 μ l by adding 30 μ l saline solution of 0.9% NaCl.

due to the presence of excessive amounts of endogenous growth factors, e.g. His in the orange juice samples, which results further in an excessive auxotrophic growth, creating a suspension of high cell density in the compartment. Consequently, limitations of other nutrients, oxygen or simply lack of space will prevent following secondary growth (prototrophic) from revertants. This possibility is also described by Falck et al. (1985). It is seen that plateau curves remained flat during the incubation period irrespective of the amount of heat load (Fig. 9.3: d). This means that these recorded growth curves cannot be analyzed for the occurrence of revertant growth. The Cobas Bact liquid assay system with turbidity measurement, therefore is not suitable for measuring heat load of orange juice due to the presence of endogenous growth factors in the juice. In the plate assay, we have demonstrated that these factors do not disturb our test system (Ekasari et al., 1988a; chapters 4 and 5).

An explanation for the heat load related delay in auxotrophic growth (points b and c in Fig. 9.3) with heat treated orange juices is found in their previously shown cytotoxicity (Ekasari et al., 1988a; chapters 4 and 5) which is in good agreement with the present finding. Delay times in auxotrophic growth of toxic samples is also shown by other investigators (Falck et al., 1985).

Experiments with mutagenic fractions of heated orange juice

In previous work (Ekasari et al., 1988b; chapter 8), we found that gel filtration chromatography on Biogel P2 is a good method to separate a mutagenic fraction from other juice components. Amino acids were not eluted in this fraction as indicated by the ninhydrine test (data not shown). The mutagenicity of this fraction is indeed shown on the Cobas Bact system. The growth curve in Fig. 9.4 demonstrates auxotrophic and prototrophic growths as expected. Further

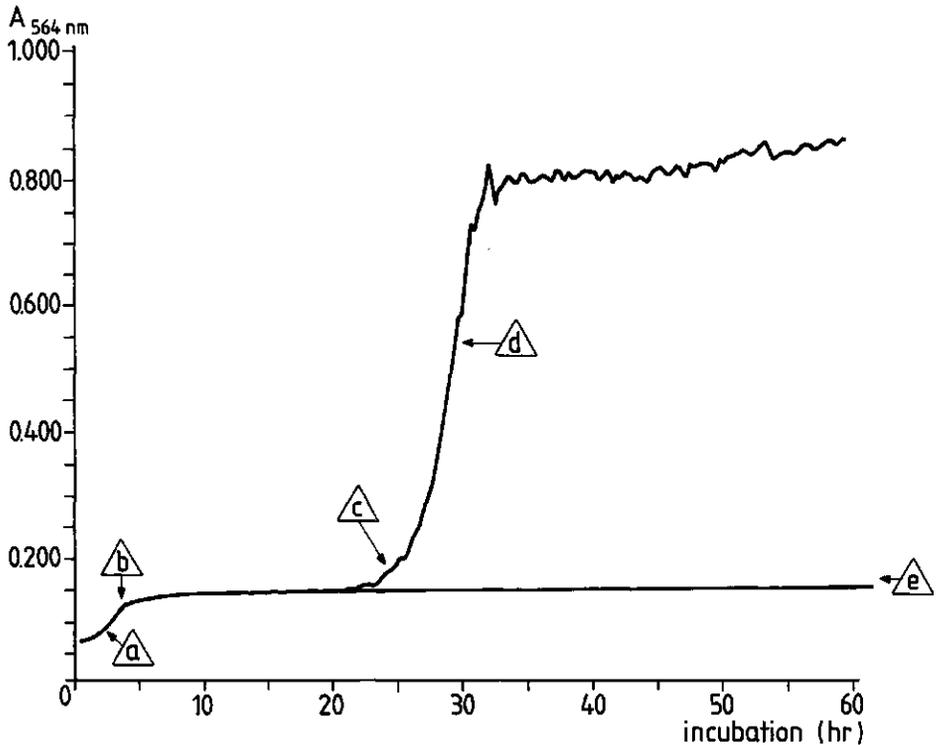


Figure 9.4 Representative growth curve of *Salmonella typhimurium* strain TA 100 upon exposure to a mutagenic fraction of heated orange juice (93°C, 2 min) in the Cobas Bact assay.

- a) Auxotrophic growth.
- b) The cessation of auxotrophic growth
- c) The start of prototrophic growth (detection time).
- d) Prototrophic growth of revertants.
- e) Negative control.

The growth medium was supplemented with 0.8 µg His/ml and the inoculum used was ca. 5×10^6 cells/compartiment. The mutagenic fraction was obtained by gel filtration (see Materials and Methods). Sample used was 70 µl per compartiment. Saline solution (0.9% NaCl) was used to bring total sample volume to 100 µl.

experiments were done on HPLC fractions (see Materials and Methods). Mutagenic fractions (confirmed by plate test, data not shown) were again obtained and showed growth curves similar to Fig. 9.4.

Table 9.1 shows dose related detection times for NQO as previously noted (Fig. 9.2). This tendency is also observed for the mutagenic fractions (gel filtration) of heated orange juice, although the results are statistically not significant. It should be noted that the concentration range for the orange juice fractions was 1:3 but for NQO 1:50. It is further seen on Table 9.1 that all the compartments of a rotor used for testing NQO showed prototrophic growth. This was not the case for the orange juice fractions.

Table 9.1 Summary of results obtained by testing NQO (4-nitroquinoline-N-oxide) and pool of mutagenic fractions (gel filtration) of heated orange juice with Cobas Bact test.

Dose tested per compartment ^a	Detection time (hr) ^b	Number of positive compartments ^c
<u>NQO</u>		
0.1 ng	35 ± 2.3	15
1 ng	26 ± 1.7	15
5 ng	23 ± 1.5	15
<u>Mutagenic fractions of heated orange juice</u>		
35 µl ^d	24.6 ± 1.7	11
70 µl ^d	25.7 ± 2.6	9
100 µl	23.5 ± 1.2	3

a) Number of compartments tested was 15 per rotor.

b) The start of prototrophic growth

c) Showing prototrophic growth.

d) Saline solution (0.9% NaCl) was used to bring the total sample volume to 100 µl.

CONCLUSIONS

The results of this study indicate that the automated system with turbidometric technique is not suitable for a mutagenicity assay to measure heat load of whole orange juice as such. Fractionation which gives promising results unfortunately is tedious and therefore not suitable for large scale usage as a routine test. Furthermore the correlation of mutagenicity of fraction with heat load of the juice has not been tested. However, possibilities may exist for other fruit juices with a low level of endogenous growth factors. Of course for the screening of fractions in view of collecting material for the chemical identification of mutagenic MIP the automated system may be helpful.

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CHAPTER 10: BALANCE BETWEEN MUTAGENICITY AND ANTIMUTAGENICITY IN HEATED APLLE JUICES

Based on the paper entitled, "Antimutagenic effects of apple juices: interference with heat load measurement by microbiological methods".

Presented as an award paper (George F. Stewart International Research Paper Competition), at the 50th Anniversary Meeting of the US Institute of Food Technologists (IFT), June 24-29, 1989, Chicago, Illinois, USA.

10 BALANCE BETWEEN MUTAGENICITY AND ANTIMUTAGENICITY IN HEATED APPLE JUICES

Indriati Ekasari¹, W.M.F. Jongen² & W. Pilnik¹

¹Department of Food Science, ²Department of Toxicology, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

ABSTRACT

Whole heated clear apple juice subjected to our modified *Salmonella* mutagenicity assay did not show any mutagenic response. However, when fractionated one fraction obtained from gel filtration did show a dose-related mutagenic response. This suggests the presence of antimutagenic factors. The Ames standard mutagenicity test was used to investigate the antimutagenic activity of apple juice samples against the direct acting mutagens NQO and MNNG. There was a dose and heat load dependent reduction in the mutagenicity of both mutagens, indicating the presence of antimutagenic factors. These findings add a new aspect to the health value of apple juice.

INTRODUCTION

Browning of apple juices during heat treatment and storage has been reported to be a consequence of Maillard type reactions. Such reactions cause a reduction of shelflife (Babsky et al., 1986; O'Beirne, 1986; Toribio and Lozano, 1984, 1986). The apple juice industry is in need of a test to detect these reactions at an early subsensory stage.

HMF (5-hydroxymethylfurfural), a Maillard intermediate has been proposed as an indicator of heat abuse in apple juice because it can be determined easily and rapidly (Toribio and

Lozano, 1987). However, no significant correlation between HMF content and browning of apple juices has been found (Voragen et al., 1988; Wucherpfennig and Burkardt, 1983). The complexity of Maillard reactions (Hodge, 1953; Namiki and Hayashi, 1983; Nursten and O'Reilly, 1986) makes it difficult to find a simple chemical method for the detection of intermediates responsible for browning.

Being successful in measuring heat load of orange juices using our modified *Salmonella* mutagenicity assay (Ekasari et al. 1986, 1988a; chapters 4 and 5), we attempted to apply it to apple juice. In preliminary studies using the same method (Geesink, 1987; Postmus, 1989), whole clear apple juices heated at 93°C for 30 sec - 30 min were investigated. Unlike heated orange juices, neither mutagenic nor cytotoxic response could be observed. A fraction obtained from gel filtration, however, did show mutagenic activity indicating that antimutagenic factors might be present in whole apple juices. These would presumably interfere with the mutagenicity assay. While our objective is still the measurement of heat load, this study has acquired a further dimension, investigation of antimutagenic factors in apple juices.

MATERIALS AND METHODS

Preparation of apple juice samples

The apples used were of the Golden Delicious cultivar. Juices were laboratory prepared following Fig. 10.1. For the microbiological assays, the heated or unheated juices were treated as follows: adjust to pH 7.4 with 4 N NaOH, filter (0.2 μ m, Millipore), freeze dry, and dissolve in 0.5 M phosphate buffer pH 7.4 to obtain 5x concentration.

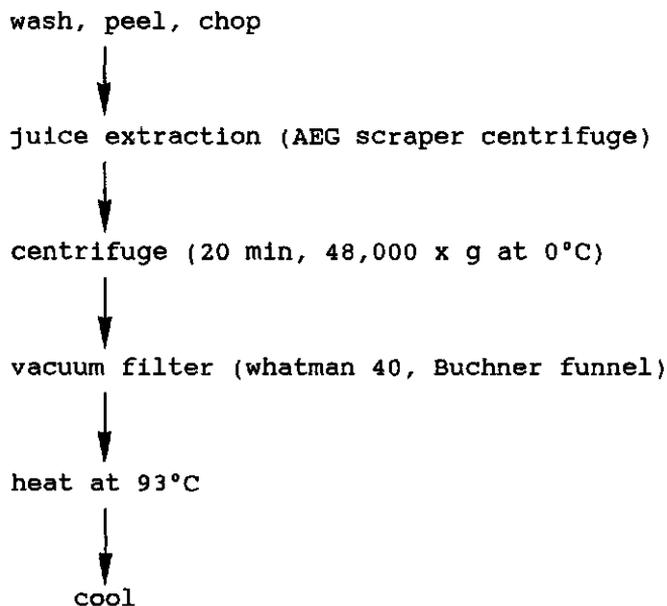


Figure 10.1 Laboratory preparation of clear apple juice

Gel filtration chromatography

Heated (93°C, 2 min) clear apple juice was chromatographed after pH adjustment to 7.4 (with 4 N NaOH) on a 2.5 cm x 32.5 cm column of Biogel P2 (Biorad, 200-400 mesh, M.W. cut-off 100-1800 daltons) at 2°C. For each run, 2 ml of sample was loaded into the column. Elution was carried out with sterilized distilled water at a flow rate of 18 ml/hr. Fractions (1.5 ml) were collected in a fraction collector and pooled into four portions according to U.V. absorbance (U.V. detector, Uvicord S, LKB 2138; 206 nm) as shown in Fig. 10.2. Each pool was freeze dried, dissolved in 0.5 M phosphate buffer pH 7.4 to the original volume of sample (2 ml), filter sterilized (0.2 μ m, Millipore), and tested for mutagenicity. In order to estimate the molecular weight (M.W.)

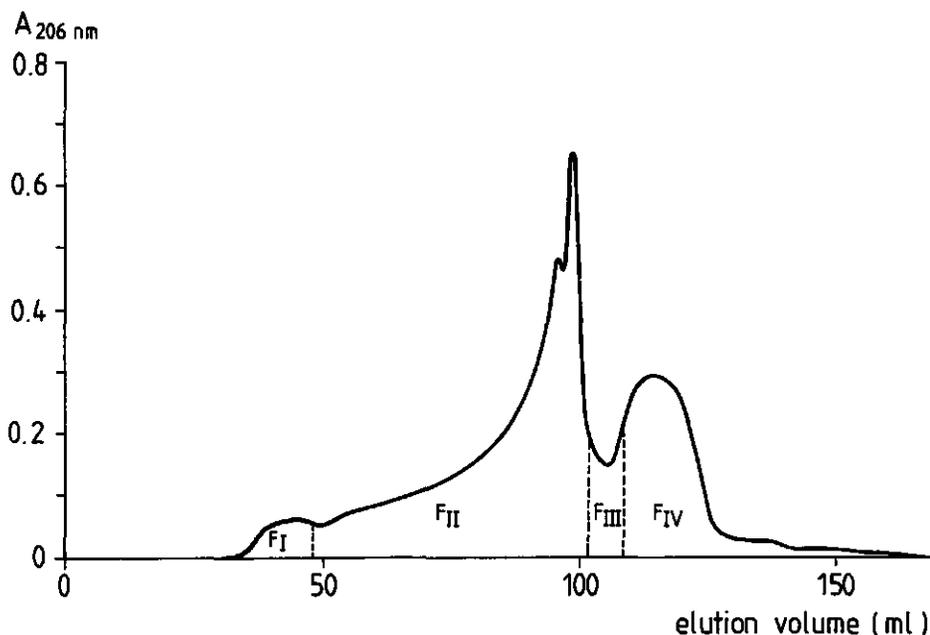


Figure 10.2 Gel filtration profile of heated clear apple juice (93°C, 2 min) on Biogel P2. Column: 2.5 cm x 32.5 cm; eluent: sterilized distilled water; flow rate: 18 ml/hr. See Materials and Methods for details of samples.

of fractions the elution curve (Fig. 10.2) was compared with the one established with a solution containing galactan (M.W. > 2000), stachyose (M.W. = 666), raffinose (M.W. = 504), sucrose (M.W. = 342) and glucose (M.W. = 180), where in each fraction the automated orcinol test (Tollier and Robin, 1979) was performed.

Preparation of mutagens

Solutions of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) and 4-NQO (4-nitroquinoline-N-oxide) were prepared by dissolving 0.01 g mutagens directly into 1 ml DMSO (dimethylsulphoxide) and then diluting with 0.9% NaCl to a concentration of 10 µg and 1 µg per ml, respectively.

Mutagenicity assays

These were performed with *Salmonella typhimurium* TA 100 without S-9 mix. The modified *Salmonella* mutagenicity assay (Ekasari et al., 1986; chapter 4) with 6 hr preincubation at 37°C and pH 7.4 was used to test apple juices and fractions of apple juice obtained from gel filtration chromatography. The Ames standard mutagenicity test employing the direct plating procedure (Maron and Ames, 1983) was used to determine mutagenic activity of MNNG and 4-NQO. Prior to plating 0.1 ml of each mutagen solution was mixed with 0.1 ml to 0.5 ml apple juice samples (5x concentrated) in a sterile 13 mm x 100 mm capped culture tube. After 10 min standing in the dark the following additions were made to each tube: 0.1 ml of 0.5 M phosphate buffer pH 7.4, 0.1 ml bacterial culture (1×10^8 cells), an appropriate amount of saline solution (0.9% NaCl) to give a final volume of 1.3 ml, 3 ml of molten top agar (45°C). The contents of the tubes were mixed on a Vortex mixer and poured onto selective agar plates. The plates were then incubated at 37°C for 48 hr. As a negative control, 1.1 ml of saline solution, and as a positive control, 0.1 ml of each mutagen solution plus 1 ml of saline solution were used. Revertants induced by 1 µg MNNG and 0.1 µg 4-NQO were 1505 and 1368 colonies, respectively.

Determination of cell survival upon exposure to apple juice samples

This was done following the method described above (direct plating) with the exception that (1) the bacterial culture used was diluted with pepton physiological salt solution (0.85% NaCl and 0.1% pepton) to a titre of ca. 3×10^3 to 5×10^3 cells/ml and (2) each selective agar plate was enriched with 1.5 mg histidine (His). Each experiment was done at least twice and each concentration of the sample was tested at least in triplicate.

RESULTS AND DISCUSSION

The results of mutagenicity tests are presented in Table 10.1 and Fig. 10.3. As Fig. 10.3 indicates, whole clear apple

Table 10.1 Mutagenicity of fractions of heated clear apple juice. Fractions obtained by eluting whole clear apple juice heated at 93°C for 2 min on Biogel P2 (see Materials and Methods for details).

Fraction no. (see Fig.10.2)	Mutation ratio ^a		
	amount of sample (ml)		
	0.1	0.2	0.4
F I	1.1	1.1	1.1
F II	1.1	1.1	1.7
F III	1.1	1.5 ^b	1.7 ^b
F IV	2.1 ^b	4.6 ^b	5.8 ^b

^a Mutation ratio: number of induced revertant colonies per plate divided by number of spontaneous revertant colonies per plate.
^b Indicates positive mutagenic response (Mutation ratio > 2.0). The number of spontaneous revertants was 181 colonies/plate.

juice showed no mutagenic response but a fraction of the same juice obtained from gel filtration did show a strong mutagenic activity. The shape of the dose response curve and the molecular weight range of this mutagenic fraction ($180 < \text{M.W.} < 700$: estimated from a calibration curve made for oligomeric carbohydrates from the same column) is quite similar to that obtained from heated orange juice previously reported (Ekasari et al., 1988b; chapter 8). These results suggest that antimutagenic factors are present in whole apple juices which inhibited the expression of mutagenicity. Such factors have been described in literature as natural constituents of various fruits, vegetables and legumes (Fukuhara et al., 1981; Morita et al., 1978) or as compounds formed during heat treatment of browning reaction systems (Kim et al., 1986; Powrie et al., 1986).

The antimutagenic capacity of these factors, present in apple juice were tested on two direct acting mutagens. Figs. 4B and 5B show the antimutagenic properties of unheated and heated apple juices towards MNNG and 4NQO, respectively. The antimutagenic effects are seen to be dose and heat load related.

Further more, the survival curves (Figs. 4A and 5A) and examination of the background lawn of the plates under the stereo phase-contrast microscope confirmed that the inhibitory effects observed were not due to a cytotoxic effects on the tester bacteria. This is in contrast to orange juice in which cytotoxic effects are noted at high dose and heat load (Ekasari et al., 1986a, 1988a; chapters 4 and 5). A possible explanation is that the factors present in whole (heated) apple juice do not only inhibit mutagenic activity but also are capable of reducing cytotoxic effects in general. As shown by Morita et al. (1978) apple juice inhibits the activity of indirect acting mutagens such as tryptophane pyrolysates, probably by binding to the parent compounds. This

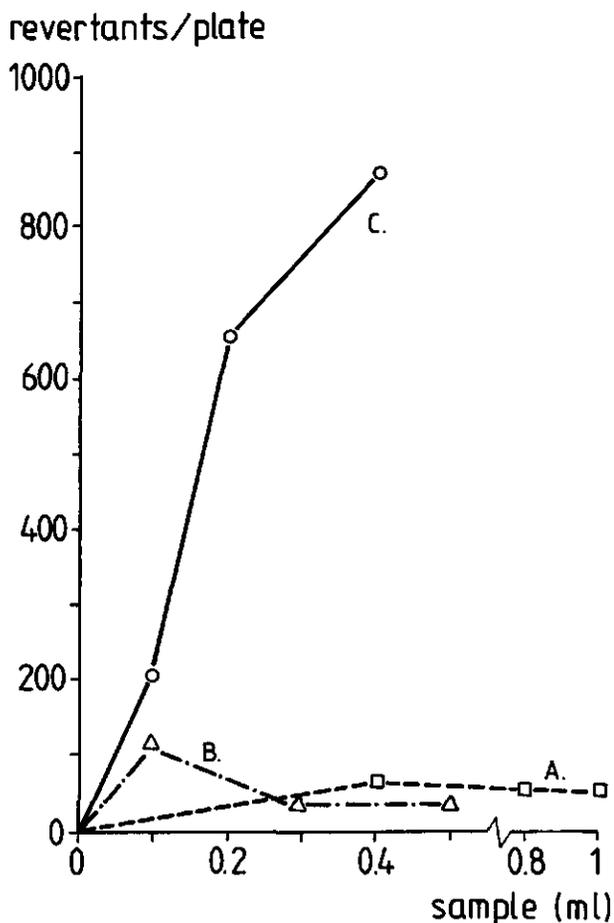


Figure 10.3 Dose-response curves of the mutagenicity of apple juice samples heated at 93°C for 2 min. (A) unfractionated whole clear apple juice (single strength) (B) whole clear apple juice (5x concentrated) (C) fraction F IV (see Fig. 10.2) obtained from gel filtration of A. The mutagenic activity was determined using modified *Salmonella* mutagenicity assay (Ekasari et al., 1986) with 6 hr preincubation time. The number of spontaneous revertants was 181 colonies/plate.

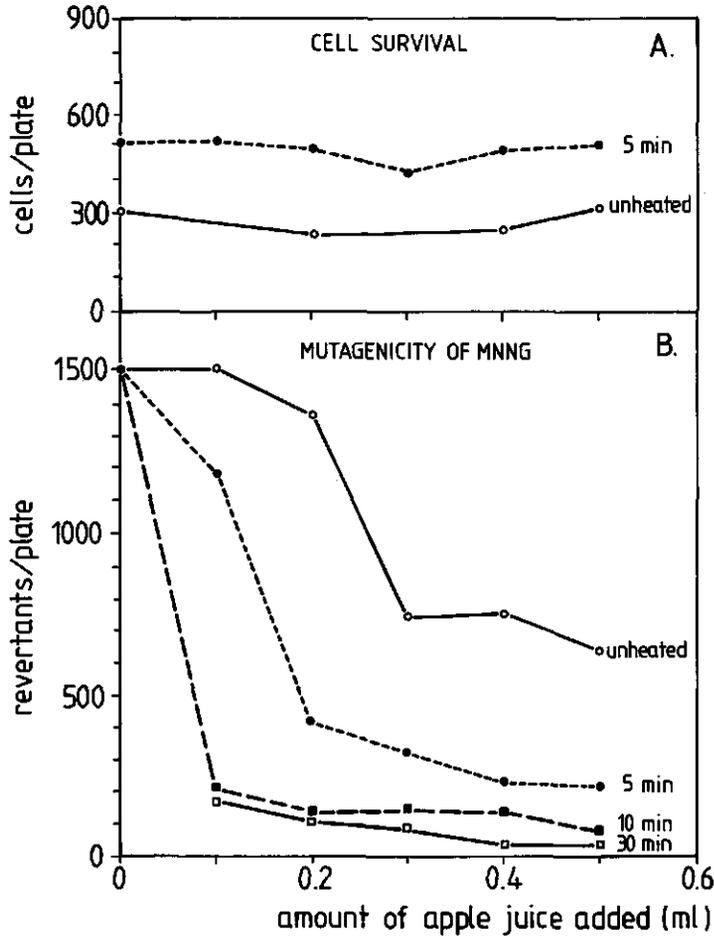


Figure 10.4 (A) Survival of *Salmonella typhimurium* TA 100 upon exposure to clear apple juice samples.
 (B) Inhibitory effect of clear apple juice samples on the mutagenicity of MNNG towards *Salmonella typhimurium* TA 100 in the Ames test. Samples used were 5x concentrated unheated and heat treated (93°C) juices, as indicated in the graphs (see Materials and Methods for details). The number of spontaneous revertants was 98 colonies/plate.

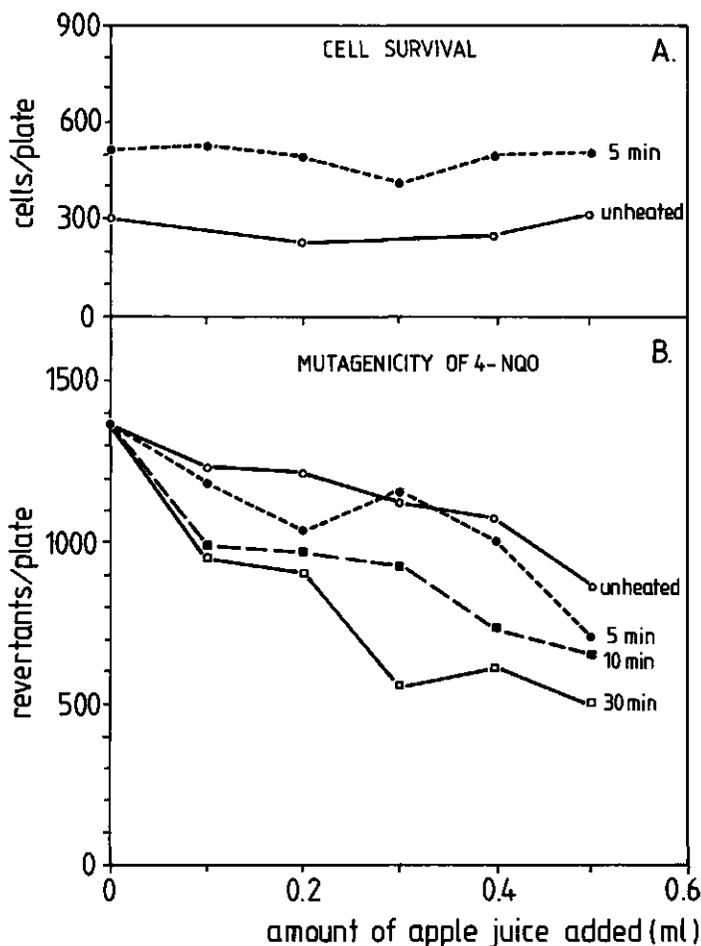


Figure 10.5 (A) Survival of *Salmonella typhimurium* TA 100 upon exposure to clear apple juice samples.
 (B) Inhibitory effect of clear apple juice samples on the mutagenicity of 4-NQO towards *Salmonella typhimurium* TA 100 in the Ames test. Samples used were 5x concentrated unheated and heat treated (93°C) juices, as indicated in the graphs (see Materials and Methods for details). The number of spontaneous revertants was 135 colonies/ plate.

indicates that in (heated) apple juices factors which can bind to compounds with different chemical reactivities are present. Furthermore, it should be noted that the chemical composition of apple juices is different from that of orange juices (Lee and Wrolstad, 1988; Wallrauch and Faethe, 1988b) particularly in the ascorbic acid content and free amino acids spectrum (Wallrauch and Faethe, 1988a) which are precursors for Maillard reaction products (Babsky et al., 1986; Poll, 1983). Since heat treatment implicates a complex series of chemical reactions, the formation of cytotoxic compounds even under similar conditions may differ quantitatively and qualitatively in the two types of juices.

CONCLUSION

These studies allow the following conclusions:

- (1) Separation of the mutagenic factors from other apple juice constituents might give a possibility to determine heat load in apple juices as a measure of the inner quality.
- (2) Antimutagenic factors are present in apple juices. There is a dose and heat load dependent reduction in the mutagenicity of MNNG and 4-NQO in the presence of apple juices. These findings add a new aspect to the value of apple juices as a healthy food.

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11 SUMMARY AND CONCLUDING REMARKS

The aim of this thesis was to develop methods to measure Maillard intermediary products (MIP) in (concentrated) fruit juices which are related to their inner quality.

Orange juice and apple juice were chosen because of their popularity.

Although intensive research efforts have been done for many years, there is still a lack of knowledge over specific chemical structures of MIP. Therefore microbiological methods were introduced in this study.

Chapter 1 presents literature data on the enormous increase in consumption of orange juice and apple juice in the USA and Europe. Also it discusses the relation between MIP formation and heat treatment.

From the flow diagram for processing of orange juice as shown in Fig. 1.1, it can be seen that the formation of MIP resulting from processing cannot be avoided as heat treatments are necessary for enzyme inactivation and prevention of microbiological spoilage. A higher heat load will create more MIP. The term inner quality was chosen to define the shelf-life expectation of a good exterior quality fruit juice which, under equal external circumstances, depends on the type and concentration of MIP. The MIP's themselves are not perceived sensorially, but are the precursor compounds which react further to form brown pigments and off-flavours. Therefore, to a large extent, the shelf-life of (reconstituted) juices depends on the concentration of MIP at the moment of packing. Thus the concentration of MIP is considered as an indicator for the inner quality of fruit juices. A simple quantitative method for MIP detection would be useful to the fruit juice industry for evaluating the inner quality of concentrated juices used as raw material and for examining conditions of heat treatment in their own plants, taking into consideration the heat load which

the juices carry from previous heat treatments.

Chapter 2 describes Maillard-type reactions in fruit juices resulting in non-enzymatic browning. The involvement of compounds such as acid-catalyzed break-down products of reducing sugar and ascorbic acid, are also discussed.

In chapter 3 the principles and methodological aspects of the test methods used in the investigations, i.e. *Salmonella* mutagenicity and cytotoxicity assays are described.

The discussions from chapter 3 are extended to chapter 4 and 5. These deal with experiments to determine whether it is possible to use the mutagenicity assay to determine the heat load of orange juice.

Chapter 4 presents data on *Salmonella* mutagenicity testing of orange juice with strain TA100. This bacterial strain is widely used in mutagenicity testing of aqueous Maillard-reaction products. Using the original protocol of the assay, no mutagenic response could be obtained. However, a mutagenic response was observed in heated orange juice when the assay was modified, including pH adjustment of samples to 7.4 and 4 hr preincubation at 37°C and pH 7.4. It must be emphasized that heat-treated orange juice under natural acidic conditions is not mutagenic. The mutagenic effects obtained are dose related and related to the heat load and can be ascribed to Maillard-intermediary products (MIP).

In chapter 6 the possibility that the mutagenicity comes from mutagenic flavonol aglycones was investigated. During heat treatment there is a possibility that kaempherol and quercetin are released from their corresponding glycosides, i.e. robinin and quercitrin and/or rutin, respectively. The results from chapter 6 show that at conditions normally applied to process orange juice, the level of the concentration of kaempherol and quercetin is not sufficient to interfere with the mutagenicity assay. This confirms that the mutagenic effect must be ascribed to MIP. The data presented in chapter 4, Appendix (table 4.A1) allow the following conclusions:

1. that mutagenic MIP are formed during the preincubation period from precursors present in the heated orange juice and
2. that due to their weak mutagenicity these must be preincubated together with tester bacteria.

The mutagenicity is observed to depend on heat treatment and dose (Figs. 5.1 and 5.2). For both factors, an increase in mutagenicity is followed by a decrease due to cytotoxic effects.

The extension of the mutagenicity to a cytotoxicity assay is described in chapter 5. Similar to the mutagenic effect, a cytotoxic effect was observed only under the specific test conditions, i.e. pH adjustment of samples to 7.4 and 4 hr preincubation at 37°C and pH 7.4. Kinetic measurement on the cytotoxic effect was done using Malthus conductance meter.

Increase in conductance is associated with bacterial metabolism and growth. The time required to reach a sharp acceleration of conductance changes is defined as detection time (DT). It is assumed that DT is influenced by cytotoxic effects. Chapter 5 shows that DT correlates with heat load; the unheated juice had the shortest DT and the longest DT was the juice with the highest heat load.

The conductance method seems to be a promising supplement and even an alternative to the mutagenicity assay, particularly for testing samples carrying a relatively high heat load.

Using the dose-response curves presented in Fig. 5.1 as reference, commercial and industrial orange juice samples were classified according to their presumed heat load (Chapter 5, table 5.2). Frozen concentrate ranks between the directly filled single strength juice (pasteurized once) and the reconstituted juice (concentrated and pasteurized).

Chapter 7 presents data from an accelerated shelf-life testing of industrially reconstituted orange juice prepared for the market. Two methods of filling were applied, i.e.

hot and aseptic-cold. Lag periods for browning were observed irrespective of the filling methods. It is shown that juice carrying a higher heat load had a shorter lag period. The microbiological methods used to measure heat load prior to storage, i.e. modified *Salmonella* mutagenicity assay and cytotoxicity assay (using a Malthus instrument) do indeed have a predictive value for shelf-life. This was also observed earlier in a study using laboratory prepared orange juice (Fig. 5.6).

In chapter 8, the compound(s) responsible for the mutagenicity in heated orange juice are characterized as follows: polar, non-volatile, carrying no charge and having molecular weights between 200-700 daltons. Gel filtration appeared to be a good method to obtain fractions from heated juice which are strongly mutagenic. This is an important prerequisite for further investigations to isolate mutagenic compound(s) in order to determine their molecular structure.

Chapter 9 discusses the feasibility for automation of our modified *Salmonella* mutagenicity assay (chapter 4) by using the same approach of methodology but increasing the capacity. The Cobas Bact, an automated microbiological growth analyzer that monitors turbidity was used. The results indicated that endogenous growth factors, e.g. His present in whole orange juice and cytotoxic effects of the heated juice, disturb the assay system. It is concluded that the automated system with turbidometric technique is not suitable for the purpose of measuring heat load of whole orange juice as such. For the screening of fractions with the aim of collecting material for the chemical identification of mutagenic MIP, this system may be helpful, because of the fact that good results were obtained with gel filtration and HPLC fractions of the juice.

Chapter 10 discusses the balance between mutagenicity and antimutagenicity in heated apple juice. Unlike heated orange juice, apple juice was found to have a negative mutagenic balance indicating the presence of antimutagenic factors.

These results allow the following conclusions:

1. Separation of the mutagenic factor from other apple juice constituents might give a possibility of determining heat load in apple juices as a measure of the inner quality.
2. Antimutagenic factors are present in apple juice. There is a dose and heat load dependent reduction in the mutagenicity of MNNG and 4-NQO in the presence of apple juice. These findings add a new aspect to the value of apple juice as a healthy food.

In conclusion it can be stated that fruit juices do differ in inner quality (Table 5.2; Figs. 7.1, 7.2, 10.4 and 10.5). Juices with a higher heat load have a stonger tendency for browning (Figs. 5.6 and 7.3). This means that the measurement of mutagenicity and/or cytotoxicity is indeed an expression of the inner quality of a (concentrate) juice. Hence the tests can be used by manufacturers of orange juice for screening purposes, for instance when purchasing concentrates as raw material and for testing processing conditions in their factories, both in view of shelf-life prediction. Of course, the chemical structure of MIP, once known, may become the basis of a more accurate and simpler test for the assessment of inner quality of fruit juices.

SAMENVATTING EN CONCLUSIES

Het doel van het onderzoek, beschreven in dit proefschrift, was het ontwikkelen van methoden om intermediaire Maillard reactieproducten (MIP) te meten in geconcentreerde vruchtesappen aangezien hun aanwezigheid bepalend is voor de innerlijke kwaliteit van het sap.

Vanwege hun populariteit is voor sinaasappelsap en appelsap gekozen.

Ofschoon er gedurende vele jaren intensief onderzoek is verricht naar de chemische structuren van MIP, is men er tot op heden niet in geslaagd de verantwoordelijke structuren op te helderen. Om deze reden is in dit onderzoek gekozen voor het gebruik van microbiologische methoden.

In Hoofdstuk I zijn literatuurgegevens opgenomen die een enorme toename laten zien in de consumptie van sinaasappelsap en appelsap in zowel de Verenigde Staten als Europa. Tevens wordt de relatie tussen de vorming van MIP en hittebehandeling besproken.

Het flow-diagram over de bereiding van sinaasappelsap (Fig. 1.1) laat zien dat de vorming van MIP als gevolg van de behandeling van sinaasappelsap onvermijdelijk is aangezien hittebehandelingen nodig zijn voor enzym inactivatie en het voorkomen van microbiel bederf. Een grotere hittebelasting zal een grotere vorming van MIP tot gevolg hebben. De innerlijke kwaliteit is afhankelijk van het soort en de hoeveelheid MIP. MIP zijn zelf niet sensorisch waar te nemen, maar reageren verder tot de vorming van bruine pigmenten en stoffen die smaakbederf veroorzaken.

In dit verband wordt het begrip innerlijke kwaliteit geïntroduceerd en gedefinieerd als de houdbaarheidsduur van een vruchtesap met goede uiterlijke kwaliteitskenmerken.

Om deze reden zal de houdbaarheid van sappen grotendeels afhangen van de hoeveelheid en soort MIP op het moment van verpakken. Op deze manier bezien vormt de hoeveelheid MIP een indicator voor de innerlijke kwaliteit van vruchtesap-

pen. Een eenvoudige kwantitatieve methode om MIP te bepalen zou daarom een nuttig instrument zijn voor de vruchtesap-industrie om de houdbaarheid van (geconcentreerde) sappen te bepalen en om hitte behandelings condities tijdens de fabricage processen te controleren.

Hoofdstuk 2 geeft een overzicht van Maillard reacties in vruchtesappen die leiden tot niet-enzymatische bruining.

De betrokkenheid van verbindingen zoals de zuurcatalyseerde afbraakproducten van reducerende suikers en ascorbinezuur wordt eveneens besproken.

In hoofdstuk 3 worden de principes en enkele methodologische aspecten besproken van de testmethoden die tijdens het onderzoek gebruikt zijn zoals de *Salmonella* mutageniteits-test en cytotoxiciteitstest. De discussie van hoofdstuk 3 wordt voortgezet in de hoofdstukken 4 en 5. In deze hoofdstukken worden experimenten besproken die nagaan of het mogelijk is met behulp van een mutageniteitstest de hittebelasting van sinaasappelsap vast te stellen.

In hoofdstuk 4 worden de gegevens gepresenteerd van mutageniteitsonderzoek van sinaasappelsap met *Salmonella typhimurium* stam TA 100. Deze stam wordt veel gebruikt bij onderzoek naar de mutageniteit van wateroplosbare Maillard-reactie produkten. Wanneer het standaard testprotocol wordt gebruikt wordt geen mutageniteit waargenomen. Echter wanneer het protocol wordt gemodificeerd geeft verhit sinaasappelsap, in tegenstelling tot onverhit sap, wel een mutagene respons. De veranderingen bestaan uit een pH aanpassing tot 7,4 en een preincubatie van tenminste 4 uur bij 37°C en pH 7.4. De mutagene respons die dan wordt gevonden is dosis en hitte belasting gerelateerd en is dus gecorreleerd met de aanwezigheid van MIP. Het dient benadrukt te worden dat verhit sinaasappelsap bij zijn natuurlijke lage pH niet mutageen is bevonden.

In hoofdstuk 6 wordt de mogelijkheid onderzocht dat de waargenomen mutageniteit afkomstig is van mutagene flavonol aglyconen. In theorie is het mogelijk dat tijdens de hitte-

behandeling kaempherol en quercetine worden vrijgemaakt van hun corresponderende glycosides robinine en quercitrine en/of rutine. De resultaten tonen aan dat onder de condities die normaal worden gebruikt bij de bereiding van sinaasappelsap de hoeveelheid kaempherol en quercetine niet voldoende is om op enige wijze te interfereren met de mutageniteitstest. Dit bevestigt de mogelijke rol van MIP bij de waargenomen mutageniteit. Deze gegevens hebben tot de volgende hypothese geleid:

1. Mutagene MIP worden gevormd tijdens de preincubatie vanuit precursors die in verhit sinaasappelsap aanwezig zijn.
2. Vanwege hun zwakke mutageniteit is preincubatie in aanwezigheid van de bacteriën noodzakelijk.

De waargenomen mutageniteit is afhankelijk van zowel dosis als hittebelasting en voor beide factoren wordt een toename in mutageniteit gevolgd door cytotoxische effecten.

In hoofdstuk 5 wordt hieraan aandacht besteed.

Evenals mutageniteit worden cytotoxische effecten alleen waargenomen onder de specifieke testcondities en het bleek noodzakelijk om cytotoxische kinetisch te bepalen met gebruik van de Malthus geleidsbaarheidmeter.

Een toename in geleidbaarheid is geassocieerd met bacteriële groei en metabolisme. De tijd die nodig is om een exponentiële toename van de geleidbaarheid te bereiken wordt gedefinieerd als detectietijd (DT). Het ligt voor de hand te veronderstellen dat de DT waarde wordt beïnvloed door cytotoxische effecten. In hoofdstuk 5 wordt beschreven dat dit inderdaad het geval is: onverhit sap heeft de laagste DT waarde en de hoogste DT wordt gemeten bij het sap met de grootste hittebelasting.

De geleidsbaarheidmeting lijkt een veelbelovend supplement en misschien zelfs een alternatief voor de mutageniteitstest te zijn, speciaal voor monsters met een relatief hoge hittebelasting.

Bevroren concentraat zit dan precies tussen het oorspronkelijke sap (eenmaal gepasteuriseerd) en het aangengd sap (geconcentreerd en gepasteuriseerd).

In hoofdstuk 7 worden gegevens gepresenteerd betreffende een versneld houdbaarheidsonderzoek van industrieel bereid sinaasappelsap afkomstig uit concentraat. Er zijn twee soorten afvulmethodes gebruikt, te weten heet en aseptisch-koud. Voor beide soorten werd onafhankelijk van de gebruikte methode een "lag"-periode gevonden voordat bruining meetbaar was. Voor het sap met een hogere hittebelasting was de "lag"-periode korter hetgeen tot de conclusie leidt dat de gebruikte microbiologische methoden nl. de gemodificeerde *Salmonella* mutageniteitstest en de cytotoxiciteitstest inderdaad een voorspellende waarde bezitten t.a.v. houdbaarheid.

Dit was eerder ook gevonden voor sinaasappelsap monsters bereid op het laboratorium.

In hoofdstuk 8 word(t)en de verbinding(en) verantwoordelijk voor de mutageniteit in verhitte sappen chemisch gekarakteriseerd: polair, niet-vluchtig, ongeladen, met een molekuul gewicht tussen 200-700 daltons. Gelfiltratie bleek een geschikte methode om sterk mutagene fractie's uit sinaasappelsap te verkrijgen. Dit is een belangrijk aspect voor verder onderzoek gericht op isolatie van de mutagene verbinding(en) en structuuropheldering.

Hoofdstuk 9 gaat over de mogelijkheid om de gemodificeerde *Salmonella* mutageniteitstest te automatiseren en te integreren met de cytotoxiciteitstest. Hierbij wordt hetzelfde principe gevolgd en er werd gebruik gemaakt van de COBAS-BACT en geautomatiseerde bacteriële-groeimeter die de turbiditeit meet. Endogene groeifactoren met name His zoals ze aanwezig zijn in het complete sap in combinatie met cytotoxische effecten maken het onmogelijk om met het complete sinaasappelsap te werken.

De conclusie is dat het geautomatiseerde systeem niet bruikbaar is voor onderzoek met het sap zelf maar dat het systeem

bruikbaar is voor het screenen van fracties voor onderzoek naar de identificatie van de precursors.

Hoofdstuk 10 handelt over de balans tussen mutageniteit en antimutageniteit in verhit appelsap. In tegenstelling tot sinaasappelsap bleek appelsap een negatieve mutageniteitsbalans te bezitten hetgeen wijst op de aanwezigheid van antimutagene factoren. Voor appelsap geldt dat:

1. Scheiding van de mutagene factor van andere componenten aanwezig in appelsap biedt de mogelijkheid om ook hier de hittebelasting als een maat voor de innerlijke kwaliteit te gebruiken.

2. Er zijn antimutagene factoren aanwezig in appelsap.

Appelsap veroorzaakt een dosis gerelateerde afname van de mutageniteit van stoffen zoals MNNG en 4-NQO. Deze aspecten voegen een nieuw aspect toe aan de waarde van appelsap als gezond voedingsmiddel.

Concluderend kan worden gesteld dat vruchtensappen in het algemeen verschillen in hun innerlijke kwaliteit waarbij sappen met een hogere hittebelasting sneller bruinkleuring te zien geven. Dit betekent dat het bepalen van de mutageniteit en/of cytotoxiciteit inderdaad een reflectie is van de innerlijke kwaliteit zoals die hier is gedefinieerd. De test is dus een bruikbare methode voor producenten van sinaasappelsap voor screenings doeleinden zoals bij inkoop van geconcentreerde sappen en om productieprocessen te controleren. Vanzelfspreken zal wanneer de chemische structuur van de betreffende MIP is opgehelderd dit de basis worden voor een nauwkeuriger en eenvoudiger methode om de innerlijke kwaliteit van vruchtensappen te bepalen.

CURRICULUM VITAE

Indriati Ekasari received her Master (Ir.) degree in Food Science and Technology from the Agricultural University, Bogor, Indonesia, in Feb. 1977, with distinction. In May 1979, she acquired the International Award for Graduate Study competition from the East-West Center, Honolulu, USA, which enabled her to obtain a Master of Science (MSc.) degree in Microbiology from the University of Hawaii at Manoa, Honolulu, USA, in Aug. 1982. In summer of 1980, she was a visiting research fellow at the Department of Food Science and Technology, Cornell University, Geneva, New York, USA, working on rapid ethanol fermentation. In 1981, she was a research intern at the East-West Resource Systems Institute, Honolulu, working in the Energy Systems Project. In 1983, she worked in the Research & Product Development Department, H.J. Heinz Company, Elst, The Netherlands. Since April 1984, she has been working in the Laboratory of Food Chemistry and Microbiology, Department of Food Science, Wageningen Agricultural University, The Netherlands, conducting research on non-enzymatic browning in fruit juices. In 1985, she began the research for the present doctorate thesis. In recognition of her research achievements, she received the following awards:

- 1) the 1984-1985 IFUW (International Federation of University Women) award,
- 2) the 1987 J.A. Glerum Prize for Food Technology (sponsored by the Thomasen & Drijver Verblifa Company), and
- 3) the 1989 George F. Stewart International Research Paper Competition Award, presented by the US Institute of Food Technologists (IFT).