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# Food science: basic research for technological progress

Proceedings of the symposium in honour of Professor W. Pilnik,  
Wageningen, Netherlands, 25 November 1988

J.P. Roozen, F.M. Rombouts & A.G.J. Voragen (Editors)



Pudoc Wageningen 1989

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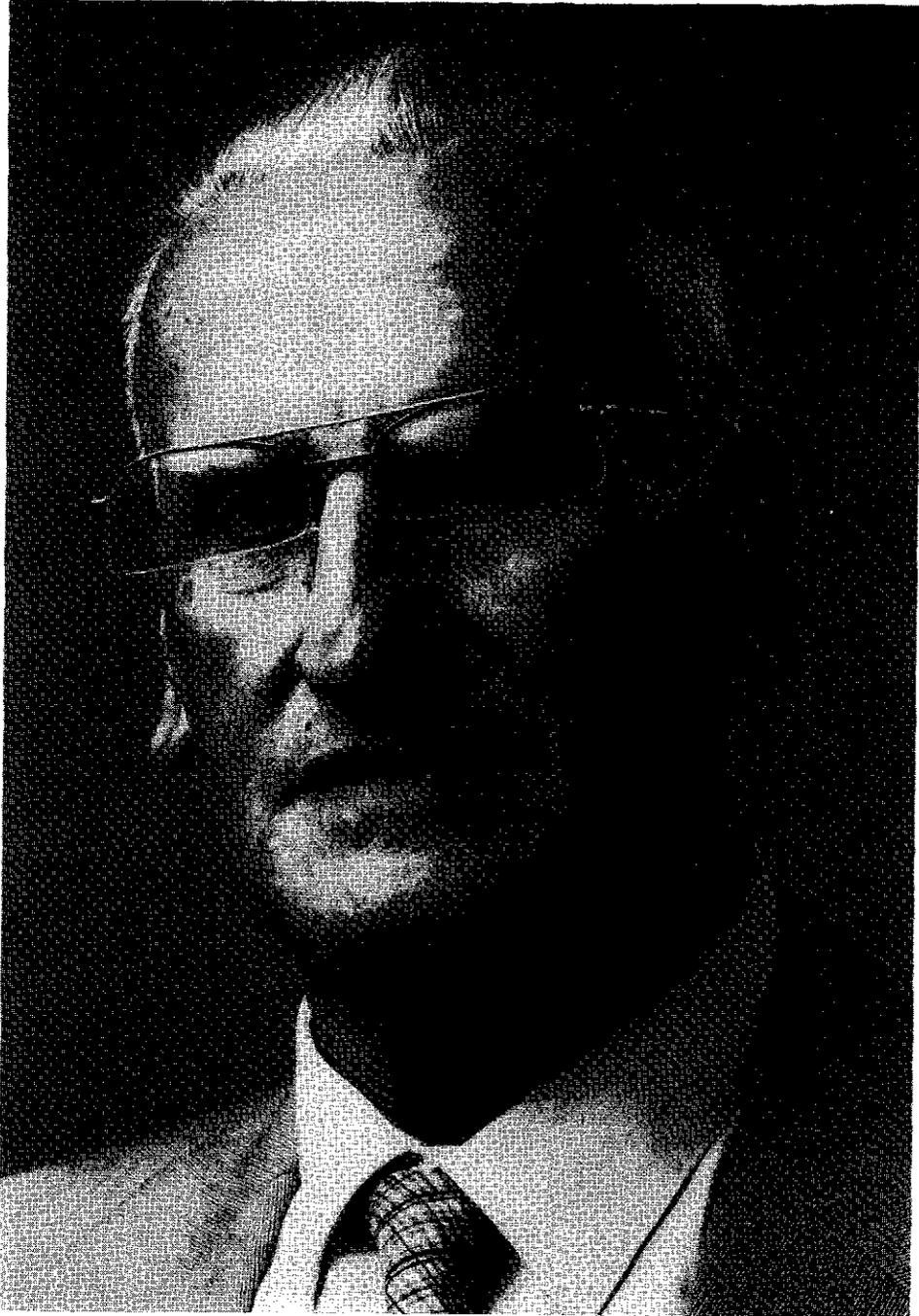
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Printed in the Netherlands.



*Professor Walter Pilnik*

Professor Walter Pilnik:  
a life dedicated to food science

This symposium was organized in honour of Professor Pilnik, on the occasion of the twenty-fifth anniversary of his appointment to the chair of Food Science at the Wageningen Agricultural University. This is remarkable in that Pilnik is the first and last to occupy this chair. This needs some explanation. In 1963 Professor Leniger developed from the existing curriculum of food technology two sub-curricula, namely that of food process engineering, and that of food science. In the same year, Professor Pilnik, through his appointment to the new chair of food science took responsibility for the food science sub-curriculum. In those early busy days, lecture courses and laboratory classes in food chemistry, food microbiology and quality control had to be developed, virtually from scratch.

In his education programmes, Professor Pilnik favoured the disciplinary approach. He has a special gift of extracting, from the enormous volume of knowledge on food products, the key processes of chemical, enzymological and microbiological nature. In this spirit, he familiarized his students with food products as complicated mixtures of compounds, which, depending on the conditions of storage and processing, may undergo various reactions affecting quality and nutritive value. In this spirit, he has trained many hundreds of young food scientists now active in all areas of food technology. In their approach to their profession, these graduates, many of whom participated in this symposium, truly form Pilnik's school of food scientists. His chair does no longer exist, but three chairs have emerged from it: one for food chemistry; one for food microbiology; one for quality assurance.

Professor Pilnik's ideas about teaching food science have drawn world-wide attention, as judged from the endless flow of food scientists from abroad visiting with him for discussions on development of research and education in food science.

In his research activities, Professor Pilnik is an equally remarkable and gifted personality. Up till now, twenty-three young scientists finished their doctoral studies under his guidance and supervision, and, although officially retired from university since 1 October 1988, several more doctoral theses have to be completed. In addition, numerous guest workers from many countries came to work with him for a shorter or longer period. He has been actively pursuing research projects in a very broad field, often in close collaboration with colleagues at the University, with food research institutes in the Netherlands and abroad, and with food industry.

He has been particularly successful in fruit and vegetable processing. His research efforts on structure--function relationship of pectic substances and other plant cell-wall polysaccharides have had a clear impact on fruit and vegetable technology. The unravelling of several mechanisms of cloud stability has greatly contributed to the quality of fruit juices. An even greater success was Pilnik's introduction of the enzymic extraction process for apple juices, one or two years later followed by enzymic liquefaction- and saccharification processes in manufacture of juices from fruits from tropical and moderate climates. These processes have been introduced world-wide. They are also a model for studies into new enzymic processes, such as hydrolysis of cattle feed for monogastrics, liquefaction and saccharification of biomass and improvement of baking quality of cereals and brewing properties of malt.

Undoubtedly the key to success in all these projects lies in the expertise that Pilnik's group built up in structure analysis of plant polysaccharides with enzymic and instrumental methods.

Studies into structure--function relationships of thickening and gelling agents have also contributed to the understanding of consistency in tomato paste and apple sauce as being primarily determined by pectin. Pectin appeared to be a valuable stabilizer in drink yoghurt, a new product that rapidly became very popular. Products such as frozen minced fish undoubtedly owe their success to the introduction of stabilizers to prevent them from losing drip and becoming tough, again an area in which Professor Pilnik and collaborators contributed significantly.

Studies with proteins were concerned primarily with hydrolysis to obtain products that could be used for fortification of foods and drinks. Enzymic hydrolysis was studied in relation to prevention of bitter taste, browning and removal of bean taste. As soya beans were used as protein source, the research group got involved with soya processing, particularly with the influence of heating processes on fat quality and on inactivation of trypsin inhibitor. Earlier studies with fats and oils were concerned with coffee lipids, but this rewarding research field had to be abandoned because of restrictions on staff.

Other major research concerns of Professor Pilnik and collaborators have included quality of fruit juices as influenced by heat charge and packing material through formation of Maillard intermediary products, and formation and identification of process flavours.

Pilnik's principle underlying all his research work is that it does not really matter what subject in food science one decides to work on, if only the job is done properly, which means that one has to work on the molecular level. However the inspiration has to come from an interest in product and process development. Indeed in most of Professor Pilnik's research work basic knowledge has been acquired, which allowed food industry to make rapid technological progress. It was just this apprehension which inspired us for the title of this symposium "Food Science: Basic Research for Technological Progress".

The organizing committee is pleased that the speakers to this symposium have so enthusiastically accepted to contribute to the proceedings. Covering different disciplines, they approach the theme of this symposium from different angles. Pilnik's staff and former students have contributed to these proceedings by submitting papers based on posters shown at the symposium. We are confident that the proceedings will provide a picture of the contribution of food science to modern society.

F.M. Rombouts  
J.P. Roozen  
A.G.J. Voragen

WALTER PILNIK - Curriculum Vitae

- 1921.07.17 Born in Zürich, Switzerland.  
1939-1945 Studies at Swiss Federal Institute of Technology, Zürich  
1943 Diploma chemical engineering.  
1945 Doctorate in technical sciences. Dissertation: Streaming birefringence of pectins in aqueous solutions.  
1945-1946 Chief chemist at Fischlin AG, Arth, Switzerland (fruit products).  
1946-1947 School of Hygiene and Tropical Medicine, London, postgraduate study in microbiology.  
1946-1949 Chief chemist at Koldinsky Ud, Pardubice, Czechoslovakia (pectin and fruit products).  
1950-1955 Director of research, Central Citrus Products Research Laboratory, Rehovot, Israel.  
1955-1964 Chief chemist, technical director and managing director, Obipektin Ltd., Bischofszell, Switzerland.  
1963-1988 Professor of Food Science, Head of Department of Food Chemistry and Food Microbiology, Wageningen Agricultural University, The Netherlands.

Pilnik was member of the Board of various Dutch Food Technology Institutes, member of the National Council of Agricultural Research and the Nutrition Council. Since 1968 he is Chairman of the Joint ECE/Codex Alimentarius Group of Experts on Standardization of Fruit Juices. He is a member of Chemical Societies and Food Technology Societies in The Netherlands, the United Kingdom, Switzerland and U.S.A. He has numerous connections with Universities, Food Science Institutes and Food Industries, in many countries.

Since 1952, Pilnik is married to Rena Shalem from Tel-Aviv. They have two sons: Ron-Daniel, born in 1960 and Ehud-Michael, born in 1962.

## Doctoral students and Dissertations

- A. Ruiter, 1969. Een chemische bijdrage tot de kennis van het roken van vis.
- J.P. Roozen, 1972. Inactivation of peroxidase, pectinesterase and alkaline phosphatase in polymers as a model for irradiation of dried foodstuffs.
- F.M. Rombouts, 1972. Occurrence and properties of bacterial pectate lyases.
- A.G.J. Voragen, 1972. Characterization of pectin lyases on pectins and methyl oligogalacturonates.
- D. Soedarmo Moeljohardjo, 1972. On the volatile flavour compounds of cooked trassi, a cured shrimp paste condiment of the Far East.
- E.J. Mulders, 1973. The odour of white bread.
- P.R. Beljaars, 1974. A contribution to the determination of aflatoxin B<sub>1</sub> quinine hydrochloride and L (+)-ascorbic acid in foodstuffs by quantitative in situ thin-layer chromatographic analysis.
- J. Castelein, 1974. Studie van de pectolytische afbraak van de mesokarp tijdens de fermentatie van koffie (coffea robusta).
- M.J.H. Keijbets, 1974. Pectic substances in the cell wall and the intercellular cohesion of potato tuber tissue during cooking.
- J.J.P. Krop, 1974. The mechanism of cloud loss phenomena in orange juice.
- P. Folstar, 1976. Studies on the composition of wax and oil in green coffee beans.
- T.H.M. Snoeren, 1976. Kappa-Carrageenan. A study on its physico-chemical properties, sol-gel transition and interaction with milk proteins.
- D.H. Liem, 1976. Analysis of cosmetics with regard to legislation.
- J.B.H. van Lierop, 1979. Determination of potentially carcinogenic compounds in food. Trace analysis of vinylchloride, vinylidenechloride, acrylonitrile, epichlorohydrin and diethylpyrocarbonate.
- C. Versteeg, 1979. Pectinesterases from the orange fruit - their purification, general characteristics and juice cloud destabilizing properties.
- W.W.A. Bergers, 1980. Glycoalkaloids and phenolic compounds in gamma irradiated potatoes. A food irradiation study on radiation induced stress in vegetable products.
- J.A. de Vries, 1983. Structural features of apple pectic substances.
- H.A.I. Siliha, 1985. Studies on cloud stability of apricot nectar.
- M. Kouzeh Kanani, 1985. Studies on the low temperature infrared heat processing of soybeans and maize.
- D.J.B. da Ponte, 1986. Effect of hydrocolloids and pretreatments on the keeping quality of minced fillets of whiting and cod during frozen storage.
- R. Heutink, 1986. Tomato juices and tomato juice concentrates: a study of factors contributing to their gross viscosity.
- G. Beldman, 1986. The cellulases of Trichoderma viride. Mode of action and application in biomass conversion.

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## Main papers

## FROM SPARTAN SIMPLICITY TO EPICUREAN DELIGHT: TECHNOLOGICAL CHALLENGES TO THE FOOD INDUSTRY

K. Venkat

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### Summary

The effects of economical trends, technological developments and lifestyle changes on the food industry are discussed. While United States national gross product is growing, the food expenditure is going down. Another interesting trend is that the United States are becoming a net importer of finished food products. Important factors in lifestyle changes of the consumers are: (1) they expect price-value, (2) they are looking for taste, variety and fresh foods, (3) they like convenience and natural foods, and (4) they attempt to reduce fat and cholesterol. Big organizations are basically incapable of doing innovation, therefore, the need for universities to address basic research problems is becoming more acute. Biotechnology will potentially impact every facet of the food industry: custom design of raw materials, introduction of foreign genes into plant and animal materials, development of sugar and fat substitutes. Product packaging is still an important part of the food costs. The consumer is convinced of the convenience of plastics, of which biodegradability and recycling are big problems. An emerging area is the interface between food and drug: more and more foods are becoming drugs and vice versa.

Descriptors: food expenditure, lifestyle changes, basic research problems, biotechnology, food industry trends, raw materials, gene transfer, sugar and fat substitutes, product packaging, food and drug.

### Introduction

It is a great pleasure and honour to be here and have the opportunity to share with you some thoughts on what the food industry is likely to face in the next several years. I have somewhat of a unique position with one foot in the industry and another one in the academic universe. Professor Pilnik too, in his own way, is a hybrid person in that he has worked on basic problems, but never forgot what the technological applications would be or, conversely, what technological problems really require basic scientific solutions. There were a number of possible areas I considered talking about. I finally decided to survey what is happening in the food industry and to set the stage for many different technological issues to be discussed in the symposium. So I have just picked out a few themes, questions, challenges and areas. I have not attempted to cover everything.

In doing so let me first show you certain trends and give you some background information on the state of the food industry. Unfortunately, I am going to be using a lot of data from the United States food industry. I tried hard to collect similar information for the European food industry but that was not easy. Even though it is United States information, I think the trends are equally applicable to Western Europe and perhaps other parts of the World too. Looking at the technological

trends and what the future is likely to be, I am reminded of a saying from the famous essayist E.B. White who said that the future is really not a unified dream but is like a mince pie, in that it is long in baking and never quite done! It is always difficult to talk about the future. First, let us look at a macrotrend and talk briefly about the role the food industry or food systems play in the overall economy.

Food Industry Trends

If you look at the United States national gross product over the past five years, you can see that it has been growing roughly 5 to 6% (Table 1). Now that the Presidential Election is over, I think people are going to face the trade deficit and the enormous debt of the United States. If we examine the food products expenditures over the last 5 years (Table 1), we see that while the GNP has been going at the rate of 6%, the food expenditure is going down. There is lots of speculation why - is it really because of reduction in food consumption (there is a little bit of that) or is it also an increase in actual income, so that as a percentage the values are smaller? This is rather significantly shown if one looks at an index of how much money a typical family spends over a week (Figure 1).

Table 1. Gross national product (GNP) and food products expenditures (FPE) 1983-1987.

Year	GNP		FPE	
	(\$ millions)	% increase	(\$ millions)	% increase
1983	3,405,700	base	421,888	base
1984	3,772,200	10.7	448,453	6.2
1985	4,010,300	6.3	472,799	5.4
1986	4,235,000	5.6	497,802	5.3
1987	4,487,700	6.0	515,776	3.6

Source: Bureau of economic analysis, United States Department of commerce

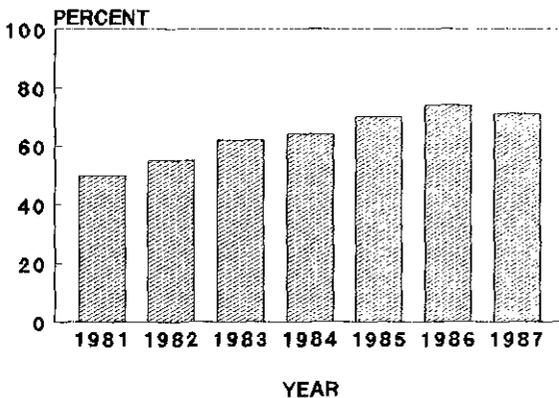


Figure 1. Weekly family grocery expenses January 1981/ January 1987. Source: Food marketing institute, "Trends update: 1987 consumer attitudes & the supermarket"

If you look at the last five or six years' data again, you can see that it is fairly constant. These figures represent the amount of money spent in buying things at the supermarket. However more and more people are eating outside the home and there is a projection that says that one out of every two meals will be taken outside the home by the year 1995 in the United States. Next, if you examine the food expenditures as a percentage of disposable income (Table 2), again you can see that it is fairly flat, somewhere around 16%. This indicates that the personal disposable income is increasing and that food costs have been fairly stable, i.e. inflation related food expenditures have not really gone up.

Table 2. Food expenditures compared against disposable personal income (billions of current dollars).

Year	Disposable personal income	Food expenditures	Percent
1966	525.9	109.0	20.7
1976	1252.6	236.2	18.8
1986	3022.1	497.8	16.5
1987	3181.5	514.7*	16.2

\* Estimated

Source: Bureau of economic analysis, United States Department of Commerce

Another interesting trend is that the United States is becoming a big importer of finished food products (Table 3). The balance of trade is about 6 billion dollars in the latest data. Again this is an indication that there is a lot of demand for products that are produced in Europe, many products, for instance, from Holland and Italy. This trend is likely to continue with the 1992 unification of Europe, and I think it is going to be quite significant in this part of the world.

Table 3. Food products balance of trade 1985--1987 (\$ millions).

Year	Exports	Imports	Balance
1985	10,254	15,976	-5,722
1986	11,485	16,500	-5,015
1987*	12,568	18,862	-6,294

\* Estimated

Source: United States Industrial Outlook 1988

What is happening to subsegments of the food industry? Here I want to show you two extreme examples. If you look at the breakfast cereal industry, again United States data over the last 6 years, you see that there is a compounded annual growth rate of about 6-7% (Table 4). Thanks in part to the great hoopla about dietary fibres, this industry is increasing quite significantly. Whereas if you look at the age-old conventional canning industry, you see that demand is flat, even going down. This is perhaps an indication of consumer perception. The consumer

thinks cans are still lined with lead. Canned goods have a negative image. Whereas the canning industry, for example, has historically developed nicely, we now have to find new methods of delivering the same canned goods in other forms, for instance frozen or refrigerated. This is not a simple problem in that we cannot take the same product and put it in different packaging. I think it is the old problem but perhaps in a new cover.

Table 4. Annual product shipments of breakfast cereals and canned foods.

Year	Value breakfast cereals (\$ millions)	Value canned foods (\$ billions)
1982	3,111	-----
1983	3,398	-----
1984	3,870	21.76
1985	4,379	22.71
1986	4,821	22.40
1987*	5,342	23.42

\* Estimated

Source: United States Department of Commerce; International Trade Assoc.

### Lifestyle changes

Let us now examine certain consumption patterns and demographics. We expect one out of two meals to be taken outside the home. What is even more interesting is that there will be more and more single-family homes. Food is becoming the central focus of many social activities; and as an ageing population requires a unique mechanism of feeding them, there will be more and more institutional feeding. If we project this over the next ten years, a key theme is emerging: you have to bring the food to the people! People are not always going to come and buy this in a supermarket. That is going to introduce a lot of challenges not only in the production or packaging of the food but in the whole delivery system, the whole distribution system. In Germany, for example, you can actually call a free telephone number and they can deliver a refrigerated meal to your home within a few hours.

The consumers are also crying out for taste and variety. No matter what you really do, what excellent technology you have, people are not going to buy things unless you can really deliver taste and variety. Another major trend that is emerging is a shift toward prepared ethnic foods. This is the fastest growing segment in the food business in America, for instance Chinese foods, Indian food and Thai food. The consumer is also demanding new standards in quality.

When we talk of quality, the consumer is more and more looking for fresh foods and often thinks that refrigerated foods are probably the closest to being fresh. As a result the whole area of refrigerated or chilled foods is certainly changing dramatically. Looking again at 'lifestyle changes', people are becoming health-conscious. This can be illustrated by a couple of interesting statistics about the consumption of meat and poultry in the United States over the last few years. I need not tell you that beef consumption is going down all the time and that beef is being replaced by poultry and fish. (Table 5). All forms of

seafood consumption are really growing quite rapidly.

Table 5. Meat and poultry per capita consumption (lbs.).

Year	Beef	Pork	Chicken	Turkey	Veal	Lamb	Total
1985	78.8	62.0	57.6	12.1	1.8	1.4	213.7
1986	78.4	58.6	58.8	13.3	1.9	1.4	212.4
1987	75.5	58.9	62.5	15.2	1.5	1.3	214.9
1988*	71.8	62.6	65.3	17.4	1.5	1.3	219.9

\* Estimated

Source: United States Department of Agriculture (February 1988)

Another major factor in life-style changes is convenience as is shown by the information on frozen foods (Table 6), especially increasing in popularity with the advent of the microwave oven. Table 6 also illustrates the point I made earlier, namely the volume of ethnic food (e.g. Oriental, Mexican). Continuing on these 'life-style changes', people are also concerned about consuming natural foods. Freshness is important and additives, artificial colours and flavours are major concerns to them. Another factor is, of course, weight-consciousness. It is estimated that today there are something like 60 million women in America on some kind of a diet control, a dieting programme.

Table 6. Sales of selected frozen foods (in millions of dollars).

Item	1987	Change from 1986 (%)
Meat	154	-3.9
Fish	1000	+13.5
Mexican dishes	263	+25.2
Mexican dinners	97	+8.4
Oriental dishes	223	-17.1
Oriental dinners	47	-32.0
Vegetables with butter sauces	76	+3.7
Vegetables with cheese sauce	94	+20.5
Vegetables without cheese sauce	95	0
International vegetables	126	+7.5

Source: SAMI, New York, NY

Fat consumption appears to be another major area of concern. People are consciously attempting to reduce fat. The United States Government recommends that no more than 30% of the energy intake be in the form of fats. In addition, there is the concern about cholesterol and people who have used vegetable materials are really concerned about cholesterol. The consumer, alas, is confused between different claims about cholesterol, vegetable or animal fats and saturated or unsaturated oils. It is interesting to look at what the consumer is saying and what the food industry is trying to do. On the one hand, the consumer does not want fat

and on the other hand, they go and buy the extra-heavy-fat chocolate ice cream. This is very intriguing, and reminds me of the German saying: "Was wir sind ist nichts. Was wir suchen ist alles".

#### Food industry consolidation

Let us leave the consumer and focus for a moment on what is happening in the food industry itself. There have been incredibly numerous mergers, acquisitions, and consolidations in the last few years. Look at what has happened to General Foods, Kraft, Pillsbury, RJR/Nabisco and so on. These 'megamergers' have resulted in 'hyper-corporations'. There used to be a time that \$1 billion in sales was a big deal but today \$1 billion does not even qualify for being in the league. What is the significance of this to the theme of this conference? Bigger organizations are basically incapable of innovation. New innovations come from smaller organizations. There is an interesting statistic from the U.S. Department of Commerce that over 75% of all innovations come from companies employing 15 people or less. In my opinion, this is going to be even more important and more critical, particularly in the food industry. Therefore, the need for universities to address basic research problems is becoming more acute. Large companies are not likely to support basic R&D programs anymore.

#### Technology trends/issues

There are several technological areas which present great opportunities and challenges. The first is biotechnology and there is a lot of effort in this University to build a major centre in food-related biotechnology. Biotechnology would potentially impact every facet of the food industry all the way from the production of agricultural raw materials to the distribution of the product to the consumer - in other words, ingredients, processing methodology preservation and distribution. The era of 'designer raw materials' is about to dawn on us. Conventional approaches worked on field-related problems such as disease resistance and pesticide tolerance. The primary benefits of this went to the farmer and the benefit to the food processor was secondary. Today we really can begin to custom-design raw materials and develop, for instance, maize for corn oil, animal feed, corn starch or corn sweeteners. Biotechnology offers opportunities to change the composition of agricultural produce:

- agronomic traits
- designer raw materials
- animal genetics
- processor-related targets
- consumer-oriented targets.

For example, the more solids there are in tomatoes today, the more advantageous it is to processing. An increase of its solids content by 1% is probably worth 80 to 90 million dollars per year for the Californian crop alone. So this is the kind of economic advantage that biotechnology can really provide. This cuts across any number of crops and specific traits and, from the consumers standpoint, can give the opportunity to provide naturally derived materials and improve the composition or protein balance or introduce the right kinds of amino acids into various crop materials. In maize this has already been done: the increase in tryptophan content of maize proteins. From the consumer's standpoint, product differentiation is also becoming a major issue. The consumer today is concerned about the use of chemical pesticides and herbicides. It is possible through biotechnology to introduce pesticide and herbicide

resistance into various crops and this is really going to be a great attraction. What are the challenges to this agricultural biotechnology area? On the one hand, we have the technologies that can do these interesting things. On the other hand, we also have the problems of excess production in the Western World: the milk lakes, the butter mountains and the excess commodities that have been produced and that are in storage everywhere. The key question is how to reward the farmer adequately. So the name of the game today for the introduction of biotechnology is value-added agriculture. That is not just producing a commodity but really, making products with specific characteristics for a segment of the market. And this is where everything is going. Leading that whole area is the ability to change the composition of natural, raw materials, for example changing the monounsaturated to polyunsaturated fatty acid ratio in an oil-seed crop.

Next to the whole area of safer agricultural inputs, another large area where basic research is really needed is to understand the function and the role of major enzymes in natural agricultural materials. Historically the food industry has taken care of this by a blanching treatment. That is a rather primitive way of doing things because we do not know what the role of these enzymes are. It is also not just a matter of turning these enzymes on or off by e.g. antisense DNA. The enzymes are there in the first place for some function and we need to understand that it is not a matter of really taking a sledge hammer approach. Research challenges of agricultural raw materials are

- value added raw materials
- composition changes
- safer agricultural inputs
- low-cost hybrid seeds
- basic understanding of the function of key enzymes
- regulatory mechanisms of multi-gene traits
- vectors for gene introduction
- plant gene expression
- influences of physiological and environmental factors
- mechanisms of nitrogen fixation.

The fundamental mechanisms of multi-gene traits are still not properly understood. Pesticide tolerance is normally a single-gene trait. In other words, the particular character or trait is controlled by a single gene. But many things of interest are controlled by many different genes. It is unknown what these genes are, where they are located and what their regulatory mechanisms are. How are these things expressed? What are the central points of gene regulation? We also need better vectors for the introduction of foreign genes into plant material and a better understanding of plant gene expression at molecular level. Molecular biologists want to work on all these exciting things. They should work together with people in the field. They cannot work in isolation. We have to understand the impact of physiological and environmental factors. Bearing in mind the complex regulatory mechanisms, nitrogen fixation is a dream: some day we would like to teach every plant to fix its own nitrogen. But we are far from unravelling the fundamental biochemistry. In animal genetics, the whole endocrinological or hormonal function is something that we need to understand clearly. We need to understand susceptibility to disease and how we can control it. Knowledge is lacking particularly for fish. A lot of the things we know today about feed utilization efficiency is empirical. We need to understand this more mechanistically. With regard to food-borne micro-organisms, we need better DNA probes, better mechanisms to detect salmonella and other

microbial food contaminants. Research challenges in animal genetics are

- understanding hormonal function
- gene-transfer technologies
- disease proneness and management
- enhanced efficiency of feed utilization
- DNA probes for rapid detection of food-borne micro-organisms

For ingredients, many exciting things are going on. In addition to aspartame, there are, in the area of alternative sweeteners, new materials with superior heat stability, better taste profiles and better functionality. Fat substitutes or fat mimetics simulate fat in smoothness, texture, creaminess and yet are low in energy. Olestra is a sucrose polyester which appears to meet all of the criteria for a good fat mimetic. If this product is approved, it will revolutionize the food industry by giving fantastic tools to tailor-make some exciting new products. Other fat-mimetic products would certainly be introduced, perhaps by genetic engineering of proteins or polysaccharides. This is going to be the next generation genetic engineering. In the first generation of genetic engineering, we produced proteins, usually therapeutic proteins. In one of our own laboratories, we have successfully produced a genetically engineered bacterium to overproduce certain amino acids, namely an aromatic amino acid (PHE), which has a solubility of about 34 g/l under physiological conditions. The organism overproduces this compound in a way that the amino acid actually crystallizes in the medium.

#### Packaging

Once you have produced the product, packaging is still a large part of the food costs. These costs can be as much as 40-45% of the total manufacturing cost. Consumers in America are convinced of the convenience of plastics and are looking for superior plastics. Here Europe leads the United States significantly. Some of the challenges that are left follow: plastic bottles are difficult to run in a factory. You can run glass bottles at 800 bottles a minute, but the same line will only produce 200 plastic bottles a minute. We need to improve the productivity of these systems. We are also looking for superior clarity, clarity approaching that of glass. Improvements in multi-layer coextruded bottles, retortable containers are all beginning to emerge. But in my opinion the biggest factor is still cost, which we need to bring down. Issues include:

- multi-layer co-extrusion
- retortable plastic containers
- aseptic packaging
- tamper-evident packages
- biodegradability
- recycling requirements.

Although aseptic packaging has long been around, I think the idea of using aseptic packaging for particulate materials is still a dream and not yet available. We need to focus on that. I also want to emphasize briefly a couple of other aspects of plastic packaging material. These are the problems of biodegradability and the recycling of plastics. They are not trivial. They are major problems in the United States and probably also in parts of Europe. So besides developing superior-performance plastic, we also have to understand how we are going to deal with it once it is used.

## Computer technology

All kinds of exciting things are happening in information technology. The food industry is somewhat slow to pick up the value of this but is now beginning to realize the benefits that computer technology can bring:

- factory-floor automation
- machined vision
- robotics
- translating information into knowledge
- profitability.

What really matters is computer power which is increasing at an incredible rate. Until about three years ago, the compounded monthly-not annual - rate of increase in computer capability was 3%. But as of two years ago, this has shifted to an astonishing 5 per cent compounded growth per month. I believe the challenge to the food industry is the adaptation of this kind of technology in a flexible way. Going and buying the computer is the easy part. What to do with it is the more difficult part. There have been a lot of bad examples. That is because we have neglected the human interface and these interfaces are of key importance. This requires a new breed of people with different kinds of training.

Clearly, there are other areas of opportunity and challenge we cannot cover all of them here:

- food-drug interface markets
- strategic response to fragmented distribution channels for convenience foods
- electronic data interchange in the supermarket
- the ultimate plastic container
- the new horizons in separation technology
- domesticating migratory fish species
- the next-generation microwave
- food safety and bugbusters.

But I want to leave you with a few interesting thoughts. An emerging area is the interface between food and drug. More and more foods are becoming drugs and vice versa and such products have a great market opportunity. We also need a better understanding of distribution systems and shelf-life. The availability of electronic data at the supermarket terminal today is revolutionizing that end of the business and home shopping offers new channels of distribution. We have to develop new energy-efficient areas of separation technology. These are new opportunities.

One of the examples of new opportunities that I use within my own company, which is a large processor of tuna fish, is domesticating tuna. The opportunity applies also to other fish species. Another challenge is the next generation of microwave products. We need to understand the interaction of the packaging materials to the microwave energy and to the food product. The problem of food safety remains. Not everything that comes out of the refrigerator is as safe as a lot of consumers believe!

These kinds of new products translate into other research needs and research challenges:

- structure and function of receptor sites
- neurotransmitters, appetite and behaviour
- food intake and cancer
- food consumption and other diseases
- basic methodology in nutritional sciences
- combination preservation technologies
- economical production of flavours/fragrances through plant-cell culture
- energy savings with non-thermal processing
- better understanding of 'texture'
- in-line sensors
- water interaction with food components
- microwave effects on product quality
- 'why' questions versus 'how' questions.

In my opinion, these areas are going to explode because a large amount of basic science is beginning to emerge. We also need to know more about the relationship between food consumption and diseases, particularly between food intake and cancer. All kinds of claims and hypotheses are being made but we really need to understand these relationships in a more fundamental way. That leads me to a plea for better basic methodology in the nutritional sciences. A lot of research going on in nutrition is still empirical. Sensor technology presents an exciting challenge because in the food industry we often measure things not because they are important but because we can.

Finally, we also need more fundamental knowledge about the interaction of water with food components and of the interactions between food components themselves and how these relate, for instance, to product quality and microwave effects.

#### Closing comment

Let me quote Yeats: "The intellect of man is forced to choose perfection of the life or of the work. And if you take the second, must refuse a heavenly mansion waiting in the dark".

Professor Pilnik chose of a life of imperfection not with respect to his work but in how he devoted his time! And in this imperfection, he has provided great inspiration to generations of food scientists and food engineers.

#### Acknowledgment

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## UNIVERSITY AND INDUSTRY - A MATRIMONY OR A CONCUBINAGE ?

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### Summary

This paper expresses the views of a fruit technologist after 27 years of experience in the food industry. It explains what industry expects from the university on both fields: education and research. But also the contributions of industry to the university are mentioned.

Descriptors: food industry; food science; university education.

### Introduction

It is a great pleasure and honour for me to address you on the occasion of the 25th Anniversary of the appointment of Professor Pilnik to the Chair of Food Science at the Agricultural University Wageningen. I would like to talk to you on a subject I am particularly acquainted with and in which I have cooperated very closely with the person we are honouring here today for his work over a quarter of a century.

The profession of food technologist is most interesting for me as nourishment for man represents more than just providing the body with the daily requirement of energy. In a wider sense, the food technologist is responsible for the physical wellbeing of man, which is in turn responsible for mental wellbeing.

Correct nourishment means the most attractive provision possible of all the necessary substances in the correct amounts needed for proper functioning of the human body. These amounts can vary considerably from one to another. Generally the right combination of food substances is considered as a condition for health and wellbeing, but we all know that our body can, fortunately, deal with considerable abuse, differing widely from the ideal combination.

But food in the right composition must also be free from any harmful substances, so that the raw materials must be produced correctly and food must not be subject to deterioration on its way to the consumer. The food technologist is also responsible for providing the population with an even and steady flow of foodstuffs over the whole year. This means that he has to ensure that the raw materials harvested are not spoiled, but are maintained on good quality over the year, or at least for longer periods, and remain available for human consumption.

The food technologist is therefore simultaneously mathematician, physician, chemist, biologist, microbiologist, chemical engineer, and so on. But can he really be all that?

The Swiss Society of Food Science and Technology, the professional organization of Food Scientists and Technologists, formed a commission 20 years ago to consider all questions of education especially for the then pending revision of study plans. In order to talk to the food industry, we enquired how a food technologist should be trained best to fulfil expectations of the industry. The answers were most interesting, but they hardly formed one point of view.

There were too many special wishes. But there was a general basis, which could be described as follows:

A food technologist should be able to approach a problem and solve it on a scientific basis. I believe that this statement is still valid. At the time, the opinion was held that the food technologist could solve problems himself with the assistance of existing literature and his own work. Today I would say that he should be able to approach a problem with his competence and draw on the expertise of specialists.

#### Education of food technologists

This takes me right into the middle of the subject relationship between university and industry, which is really what I want to talk about. One of the duties of a university is to assure a proper education of experts and provide them with the basic knowledge, which will permit the young graduate to find occupation in one of the segments of the food industry. Part of such education normally requires also a certain period in industry, which should give the student the possibility to acquaint himself with actual processes, of which he has learnt academically. Such practical periods are most certainly an important but sometimes problematic service of the food industry to the training of young food technologists, problematic because it may sometimes be necessary to divulge some particular know-how, which one does not want to find used in a competitive organization two or three years later.

But education is not complete at graduation. In addition to special product knowledge, which now has to be obtained, further developments on all scientific fields should be followed too. And this brings me to a point which, in my opinion, is getting more and more problematic.

The young food technologist, who assumes a function in the food industry will soon find, that the daily tasks require all his time and that there remains little or no time for study of literature. And when he sees the immense number of publications, he could resign himself to apathy. A solution to restrict oneself to one speciality field. I know that it is often difficult, even for academic scientists to review the flood of scientific publications in all the different languages. I believe that it would be an important and valuable task of the university, in the interest of the further education of the graduate, to reduce the tons of publications to a synopsis of the few important new criteria and do away with the rest of the ballast. Even then there would still be enough to read. Who can solve this problem?

#### Food research in the universities

But training of food technologists is only one of the relationships between university and industry. Research, with which I would like to deal now, is as important as training, although it does not always get the attention it deserves. Industry often profits from the results of research in certain fields, for instance space travel, not knowing in the slightest of the background of results.

But what about the actual research in the food industry for their own purposes? I think that only a few of the large food groups carry out research on a broad scientific basis, whilst the large part of food producing companies restrict themselves purely to product development and quality control. Most certainly the main reason for this is financial. The food industry has the reputation not to be subject to crises, but to work on margins

of only small profit. A further factor is the fast but expensive development of laboratory equipment, which causes smaller firms to give up research as such. So this already provides the answer on whose shoulders research rests:

- the large food groups
- the universities.

As the results of industry's own research are mostly kept secret and only those results are published that cannot be industrially used, the larger part of the food industry depends on the results of research at universities. But does the university carry out the research that concerns the individual food producer? Do we not all pay taxes and therefore have the right that research necessary for us is carried out for the share we pay?

The question of who is to decide on the research programme of a university department or academic institution has been discussed extensively during the last 20 years and the answer has not always been the same. Personally I have a clear opinion.

A food research institute should, at least in the widest sense, carry out research in relation to practical problems and concentrate in the interest of efficiency to some main sectors, which are laid down by the head of the institute, having regard for general industrial requirements. But where the problems are (i. e. in which direction research is wanted or necessary), the head of the institute can only find out through close contact with those responsible in industry. It is therefore absolutely wrong, to isolate the professor in an ivory tower and to keep him away from profit-greedy industry. In the food sector, the consumers profit in the end from improvements in food processing and thereby obtain better nutritional values, reduced costs, better and healthier preserving methods and the prolonged shelf-life, improvements in methods of analysis, methods of ascertaining adulteration of foodstuffs and so on. All this is made possible through research.

#### Commissioned research

But there are surely some individual problems that are not dealt with in an institute. There is then the possibility of commissioning an institute to carry out research on a particular problem. Normally institutes are only too willing to accept commissioned research, having regard for the chronic lack of funds, provided of course such research fits into the general programme of the institute.

This very often leads to a fruitful cooperation for both sides, although we cannot lose sight of one negative aspect of outside research, that secrecy of results is difficult, if not impossible. This can lead to dissatisfaction, as the one, who pays for research, would also like to be the only one entitled to its results. Nevertheless the commissioning of research projects is still relevant. Very often, medium and larger companies are making use of this possibility, even if they may have their own research laboratories.

In order to slow down the concentration of foodprocessing firms into fewer and larger food groups, it would surely be the laudable task of universities to come to the aid especially of small and medium foodprocessing plants and give them the necessary scientific and research support.

## Informing the media about food science

A further major task, which in my opinion is not sufficiently taken on by universities, is informing the public about the evaluation of risks in food for human consumption. Hardly a week passes without an actual or apparent food scandal, which is published by our beloved media in banner headlines. The defence put up by industry is received with pitiful smiles, as the public expects that this industry has acted unethically with greed for profits and with utter negligence towards the consumer. If, however, science takes a position, then the effects are much more favourable, as the public does not then assume that such statements are made from financial motives.

Surely, there is nobody, who would not permit the press to draw public attention to nuisances. Unfortunately, however, we find that especially in the popular dailies and weeklies a journalism is presented without any elementary knowledge of the subject, but with the imputation that the food industry is the branch of the economy that spoils wonderful natural products whose original condition would guarantee permanent health, that destroys all major nutrients by the use of chemistry and all sorts of processes and adds poison intentionally in immense amounts to these products as food additives.

I know that this is rather bluntly formulated, but it represents a tendency. Thanks to ever improving analytical techniques, we are reaching further and further into areas of unknown dimensions concerning the composition of food-stuffs and we find substances whose existence in food was unknown. It is of course not good, if we find poisonous substances in our food, which are of environmental origin and we must do everything within our power to reduce the content of such substances to a minimum. The presence of such substances, however, does not yet give any evidence about actual danger to health, very often they are less harmful than "natural" poisons, not to mention the microbial toxins. Unscientific publications about this problem cause considerable worries in the population and one can understand that people then ask what one could still eat, since everything is poisoned. This is then immediately followed by the demand for the prohibition of everything, pesticides as well as additives. This is an obvious consequence of the feeling of insecurity among the public. But we all know that the production of food for the nourishment of the world's population would not be possible without pesticides and preservatives, even not in the industrialized world, where we live today in abundance.

## Resistance to change

I now turn to a further subject, that has occupied my mind lately. There are certain circles wanting to turn back the wheel of time, being against every new development, for instance, in the sector of plant and animal production. Not only should there be laws against the use of new techniques, but also research connected with these subjects should be forbidden. Such ideas coming from people with full tummies represent a mockery for those who starve and, when we consider the expected growth of the world's population, such an attitude is irresponsible.

Under such circumstances an active and continuous public campaign by universities is indispensable. This task cannot be left to politicians only, because they lack the specialized knowledge and usually they accept as true anything that furthers their own ideology.

On the other hand, only financial interests are attributed to the food industry in everything they do. I refer here to the repeated attacks against Nestlé.

For that reason, it is only science, which has the possibility to inform objectively and with the necessary credentials, taking into account worldwide conditions.

### Conclusion

Dear Walter, I am sure that many of the points I mentioned now in my speech are not unfamiliar to you, because they are the result of my personal experience after 25 years of cooperation between university and industry, and after 27 years of friendship with you (figure 1). Quite a number of connections exist between university and industry, and one can exist only with difficulty without the other. The connections between the partners are fruitful for both sides; they are interesting and must be based on mutual trust. Sometimes one party profits a bit more from the other and sometimes there are tensions, e. g. when one wants more and more money, or when the other makes unethical request. There are also things that are only whispered into ears behind closed doors, things which concern both partners only.

One could ask, whether we have a lifelong connection between an university and a food company? In other words, a marriage? I don't think so. It is rather a concubinage, in which the partners remain together as long as there is satisfaction and happiness and they can separate without any formalities when the relationship has lost its purpose. A new partner can also have attractions and can lead to a new and fruitful relationship for both parties. Is that not so?

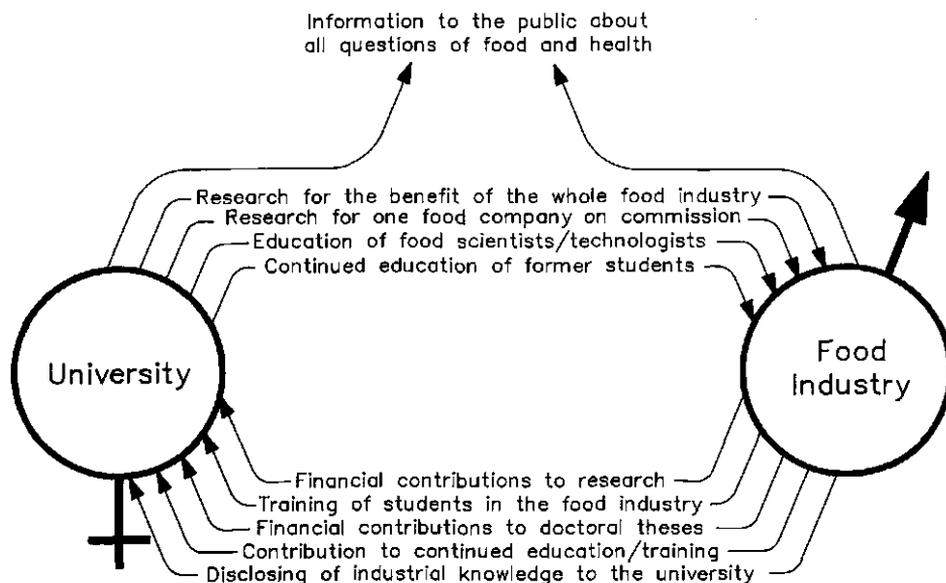


Figure 1. Relations between university and industry.

## SAFETY OF FOOD: THE VISION OF THE CHEMICAL FOOD HYGIENIST

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### Summary

The lack of safety of food includes both microbiological and chemical components. Control of risks of non-microbiological nature is the task of the chemical food hygienist. In this review, an inventory of some chemical hazards is presented and briefly discussed.

The concepts hazard and risk are defined. Risk management should rely on a holistic approach and be based upon the ALARA principle. It is recommended that the interest of the chemical food hygienist will include management of hazards from toxic compounds of microbiological origin formed in foods as a result of mishandling. For this purpose, chemical food hygienists should closely cooperate with food microbiologists and food technologists.

Descriptors: chemical food hygiene; hazardous compounds in food; risk assessment; risk management

### Introduction

The intention of this paper is to deal with safety of food - not with health-promoting attributes of food. The health impact of food consumption is actually greatly affected by the consumer's choice. It is well established that many consumers in the Western World do not make that choice in such a way that their food intake ('diet') can be considered healthy. They ingest too much fat, too little fibre, too much salt but, above all, too much food. Irrespective of this abuse of food, the consumer is entitled to safe food. The reason for this is that, though the public can determine its food intake, it cannot assess itself whether the food eaten is safe (Mosselet al., 1984).

When discussing 'safe food', thoughts go, in the first place, to microbial safety, and not without reason. Microbiological contamination and colonization are major food risks and even advanced countries seem unable to bring this problem under control. The true incidence of food-transmitted disease of microbial etiology, for The Netherlands, may be some hundreds of thousands (Beckers, 1988). It is of course true that, though serious complications sometimes follow, most food infections and intoxications are rather mild. Many result from unhygienic treatment of food in restaurants and the consumer's home (Bryan, 1982). Particularly, hygienic production of raw staple foods of animal origin, including above all pig and poultry meat (Mosselet, 1988), ought to be a matter of continuous care for the food microbiologist, the food technologist and the caterer alike.

## Essentials of risk management

Hazards are defined as conditions that cause harm to the consumer under certain circumstances. The medical and social impact of a hazard depends of course on the probability that harm is caused by the hazard; this is called the risk.

In a report of the Royal Society in Britain, risk is defined as "the probability that a particular event occurs during a stated period of time, or results from a particular challenge" (Royal Society, 1983). Applied to toxic substances in food, risk is also the probability that the continuous presence of potentially hazardous substances lead to any harm, during a stated period of time.

This can be depicted by the scheme (Figure 1).

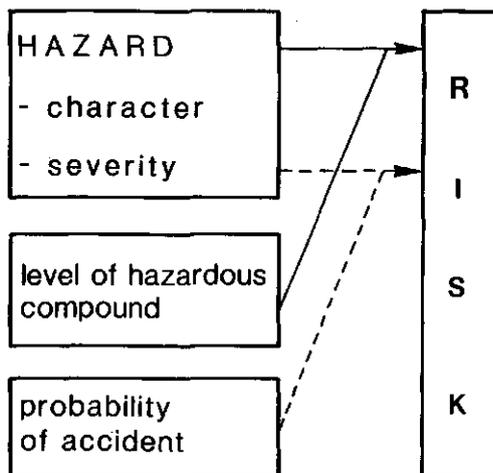


Figure 1. Factors leading to health risks related to hazardous components of foods.

The term hazard applies only to an event or condition that has been empirically demonstrated to endanger human health.

Such real hazards are disparate from perceived risks (Slovic, 1987). Untoward effects entailed by food consumption are no exception to this rule. Actual hazards prevailing in food consumption have been ranked by, for instance, Wodicka (1977). His ranking is as follows:

- Microbiological
- Nutritional
- Environmental contaminants (pollutants)
- Natural toxicants
- Pesticide residues
- Food additives

Microbial contamination was situated at the top of the list as constituting the greatest hazard, and nutritional problems arising from inappropriate choice of foods and suboptimal intakes ranked second.

It must be emphasized that ranking as such does not give a fair representation of the different risks, as the gap between nutritional hazards and those from environmental contaminants is a large one. It was suggested by Hall (1971) that the hazard from environmental contamination was of the order of a thousandth of that associated with nutritional and microbiological hazards and that the hazard from pesticides and food additives is a hundredth less again.

Far more important, however, is that the perceived hazard ranking by the public (Hall, 1971) is completely different from the real situation. Consequently, it is an important task for public health experts to convince the public that its ranking is not in agreement with reality. The perceived ranking, according to Hall, is as follows:

- Food additives
- Pesticide residues
- Environmental contaminants
- Nutritional hazards
- Microbiological hazards
- Natural toxicants

Control of risks is one of the main assignments of the food scientist. This, in turn, requires first and foremost quantification of health risks. This, however, is often somewhat inaccurate. However it represents the only effective approach to control hazards in food intake (Mossel, 1988; 1989).

Control of microbiological risks is often within reach, provided hazardous commodities are processed for safety (Mossel, 1984). It is quite striking that the public is often opposed to such processes because of concern that these may lead to a substantial loss of nutritive value - an other example of a perceived hazard. Bender (1987) has recently refuted such concerns in a well-documented review.

A careful critical evaluation of chemical hazards in food revealed that the risks concerning these hazards are small (Pilnik and Folstar, 1979; Gray, 1986). Nonetheless these hazards should not be neglected but should be subject to continuous care by the chemical food hygienist. It includes intervention by introduction and maintaining of procedures that reduce risk to a level "as low as reasonably achievable" (the 'ALARA' principle). Reasonably means that undue financial burdens to society should be avoided. Achievable means that such intervention should not only be attainable but also maintainable.

Furthermore the approach is holistic. This means taking into account (i) hazards occurring all along the complete production line; (ii) every hazardous event that determines an adverse health effect. A recent example of a holistic worst-case quantitative approach was suggested by Mossel (1989).

In following this pursuit, one must never overlook and must actively aid that not a single human activity, including eating and drinking, is free or can ever be free from all hazards. So a 'zero risk' never exists and the best achievable target is reducing risks to the ALARA level.

#### Enumeration of chemical hygiene problems

As stressed in the Introduction, 'chemical hygiene', just like microbiological hygiene, must be practised in order to keep food safe. The following is a listing of compounds present in foods that need some closer inspection:

- Naturally occurring food components
- Biocontaminants (as justified below)
- Environmental contaminants
- Contaminants resulting from animal treatment
- Pesticide, herbicide and fungicide residues
- Additives
- Contaminants resulting from processing and preparation
- Components migrating from packaging materials
- Products of reactions occurring during processing and storage

#### Naturally occurring toxic food components

In earlier times, man had to learn by experience which plants or parts of plants could be ingested without subsequent ill effects and which not. This was based merely on intuition, just as it is in other primates. Some harmful and even seriously poisonous components occur in plants, which make them unsuitable for human consumption. If so, one can remove them by techniques such as heating, extraction or simply by moistening. For instance, the cassava root and some types of beans contain cyanogenic glycosides, which can be removed by moistening the ground raw material, in order to hydrolyse the nitrile group from the carbohydrate moiety, and by subsequent heating to expel the hydrogen cyanide produced by hydrolysis. Far more stable are the solanidine glycosides in potatoes and tomatoes. Glucosinolates, which are present in a variety of plants such as cabbage, brussels sprouts and cauliflower, are split in the gastrointestinal tract, giving rise to goitrogenous compounds (Hambraeus, 1982).

Natural compounds acting as potent, acute poisons have been identified by experience so that at present it is generally known that products containing such compounds should not be eaten. This is what man taught his children. However problems still arise with compounds that do not cause acute harm, but will produce adverse effects by chronic exposure. It requires specific expert knowledge to find out whether components of this type are present in foods and which parameters influence their content, i.e. under which conditions they may give rise to morbid effects. So, in addition to the consumer's general ignorance of the risk of food contamination by pathogenic organisms (Mossel, 1984), the risk of natural toxicants is little realized either. The scientific preoccupation with natural toxicants in food should therefore be increased.

#### Biocontaminants

The consumer is similarly unfamiliar with the potential presence of biocontaminants in food. Biocontaminants include toxic substances produced by living organisms in food ('biotoxins'), but particularly those compounds that may occur in meat, milk or eggs through ingestion by livestock of contaminated feed - mostly by colonizing moulds - from which the compounds under consideration are carried over. Such food is not colonized itself and rendered toxic by toxin-producing micro-organisms. It is contaminated with toxic substances produced elsewhere.

A classical example is aflatoxins which had originally been produced in components of mixed animal feed such as groundnut meal, and which is carried over to milk. It has been said that one of the components, i.e., aflatoxin B<sub>1</sub>, belongs to the most potent carcinogens known (Wogan, 1973) and that the carcinogenicity of the metabolite aflatoxin M<sub>1</sub>, although lesser, is large as well. However, such assessments require a high level of competence, as is demonstrated by the more recently upflared discus-

sion on the carcinogenicity aspect of the health impact of aflatoxin ingestion (Stoloff, 1987).

#### Environmental contaminants

For this type of contaminants which, in most cases, are notably persistent (Lindsay & Sherlock, 1982), chronic effects are the main concern, except if acute effects are serious. Fortunately, in most cases the levels of environmental contaminants in food are low. The risks caused by toxic elements in food, such as lead, mercury and cadmium, are considered to be small, and so are the risks caused by xenobiotics, i.e. chlorinated hydrocarbons such as organochlorine pesticides.

But the situation may suddenly change. A dramatic example is the disaster caused by a brominated biphenyl preparation, marketed as a flame retardant (Senn, 1982). In 1973, by mere coincidence, a mixture of polybrominated biphenyls (PBBs) was inadvertently mixed with livestock feed, which then was sold to unsuspecting farmers. Many farm animals, primarily dairy cows, ingested the contaminated feed and then died, because PBBs are persistent, cumulative and relatively toxic compounds. But most survived and ended up in the food channel, just like their milk. This mix-up remained undetected for at least seven months, and by then almost everybody in the area had been exposed to PBB to a certain extent. This event has become known as the "Michigan story", as it happened in the State of Michigan, in the United States. The novel "PBB - an American tragedy" written by Edwin Chen (1979) provides ample information about this disaster for those who are interested in more details.

It is impossible to ensure that such events never happen again. Obviously such risks can be reduced by various measures. The general policy of prevention should be that persistent substances which show a tendency to accumulate in adipose tissues of man and animals should not be produced at all; with this preventive approach such substances cannot enter the food chain in course of time. Unfortunately, PCBs are still in production now, though in lesser amounts.

Hence there is a need for every country to have a 'disaster centre' to deal with severe food contamination, should it occur. In such a centre, experts should be employed who are authorized to launch an action programme as soon as the first signs of such a serious pollution become detectable. Such a 'crisis team' should be maintained in stand-by position by regular exercises, like military forces. The team should consist of highly qualified analysts with modern equipment, and of authorities with a mandate to take immediate measures, on the basis of reliable and relevant measurements. Maintaining and training such a team is, of course, expensive. But it should be remembered that a lot of money is spent in training fire brigades, the police and military forces too.

#### Residues from animal and crop treatment

In principle, all preparations administered to animals will lead to residues in edible tissues, milk and eggs, if the time between administration and slaughter is too short. Moreover, preparations administered parenterally may reside at the site of injection for some time. The problems with veterinary medicines in food have recently been mentioned, amongst others, by Truswell (1988) and, as for meat, reviewed by Haagsma (1988).

The situation is similar when the time between treating crops with pesticides and the moment of harvesting is too short. Attention has to be paid to the considerable toxicity of some of these pesticides, fungi-

cides and herbicides.

The significance of residues resulting from animal treatment is, in some cases, over-emphasized. A striking example is that of growth-promoting factors, e.g., estradiol-17 $\beta$ , or preparations that increase the milk production, e.g., bovine somatotropin (BST). Provided that consumption of an infection site will be excluded, the risks of hormones are negligibly small or virtually absent (Reid, 1980; Taylor, 1983). The same holds, in all probability, for the application of BST (Hart, 1985; McBride, Burton & Burton, 1988).

#### Food additives

Food additives have been subject to extremely careful (and expensive!) laboratory screening before they are used (Pilnik & Folstar, 1979). In their review on avoidable risks of cancer, Doll and Peto (1981) consider the risks caused by these substances as very small. Nonetheless, there is much concern within the consumer's mind about food additives. Such concerns are often stimulated by press coverage in which the safety of food additives is questioned. These additives are suggested to be of 'chemical' nature and consequently dangerous. It is not told, however, that all food is made up of 'chemicals'. Food specialists have therefore the obligation to explain to the consumer that the distinction as 'chemical' is incorrect. A substance can be described according to concepts of chemistry, physics or otherwise, and this holds for both compounds of natural and synthetic origin. Moreover, the public need to be made aware that it is not the origin of a substance that determines its biological effects, which depend solely on chemical structure.

Nevertheless, the origin of food additives such as colorants and preservatives remains a perennial point of discussion: should both synthetic and natural additives be used or shall we confine ourselves to natural products? This question needs to be asked, not only because there is a continuing demand from the consumer's side for 'natural' additives, but especially because no additive is completely free from impurities. Products of chemical synthesis should be purified, eliminating starting materials and compounds resulting from side-reactions. 'Natural' compounds should be purified as well in order to remove accompanying substances that have no place in the final product. Generally spoken, purification is more difficult and more complicated for 'natural' additives, as it is also much more problematic to characterize the raw material, which may contain a great many of ill-defined components whose toxicity is largely unknown. In products of chemical synthesis the presence of ill-defined and toxic components cannot be completely excluded either; but, as a rule, the situation is not characterized by such an extreme complexity. Either way, purification can be considered as an artificial treatment, that may also be regarded as objectionable in the eyes of consumers who will in principle only accept so-called natural products. This would lead to the refusal of all additives, which would of course result in a severe limitation of marketed food products. Such extreme opinions do not alter the fact that many food additives of natural origin may be valuable ingredients as well in the manufacturing of modern food products too.

#### Contaminants resulting from food processing and preparation

During processing and culinary preparation, food may come into contact with potentially hazardous substances. A well known example is the contamination with polycyclic aromatic hydrocarbons, as a result of the de-

position of smoke particles, which may occur during grilling or barbecuing of meat. In charcoal-grilled meat, benzo(a)pyrene is sometimes found at contents of more than 100 micrograms per kilogram. These high contents may be partly explained by the dripping of fat onto hot coal where it is subjected to pyrolysis; benzo(a)pyrene and other carcinogens thus formed are partly deposited on meat surfaces. If only a little amount of fat is available to drip from the meat, this deposition is obviously much smaller (Doremire, Harmon & Pratt, 1979). However it is extremely difficult to establish more accurately the risks caused by ingestion of food treated in this way, as information about the release of benzo(a)pyrene from ingested soot particles in the gastrointestinal tract is lacking. This surely merits future research.

Other examples can be given, such as the uptake of traces of disinfectants used for cleaning food equipment. This kind of contamination can be considered of minor health impact and can be avoided by maintaining GMPs. This may hold for more compounds in this category.

#### Compounds migrating from packaging materials

Plastics usually contain a variety of compounds of low molecular weight such as plasticizers, stabilizers, lubricants, unreacted or partially reacted monomer and impurities (Crompton, 1979). Most of these have lipophilic properties and tend to migrate from the packaging material into the lipid constituents of the packed food. Not all such compounds are harmful to the consumer, but transfer of considerable amounts of these chemicals from the packaging material to the food should, of course, be avoided once again by adherence to GMPs.

Some monomers require special attention. This applies particularly to vinyl chloride, which has strong carcinogenic properties (Feron et al., 1981).

Canned food may be contaminated with lead originating from can solder. The lead content of canned beef, for example, is substantially higher than that of fresh beef (Maggi et al., 1979).

#### Reaction products formed during processing and storage of foods

Every food product contains some compounds whose chemical stability is limited and which may enter, under certain circumstances, in chemical reactions. Heating particularly causes or accelerates such reactions, which may sometimes produce harmful compounds. Oxidation and polymerization reactions in fats or oils leading to such compounds are known for many years (Kaunitz & Johnson, 1973; Wolfram, 1979).

Since the late 1970s, some highly mutagenic compounds have been detected that result from pyrolytic reactions in foods, with proteins as the major sources. Although these components are only formed under extreme conditions, in particular when proteinaceous food is charred, it has been found that formation of mutagens may also occur with milder cooking (Alink et al., 1988). However, recent studies indicate that the consumption of normally cooked food, including meat, does not cause any measurable risk to human health (Alink & Kuiper, 1988).

The well known Maillard reaction between carbohydrates and proteins or amino acids may produce components that may sometimes prevent harmful effects but which may also be hazardous in some respect. Kato recently stated (1988) that much is yet unknown about hazardous effects of the Maillard reaction and that much more information is needed.

Many more examples can be given. In the late 1960s it was observed that nitrite, which is customarily added to meat products as an anti-

microbial preservative and colour stabilizer, might participate in reactions leading to the formation of highly carcinogenic compounds, the N-nitroso compounds or nitrosamines (Hotchkiss, 1987). This occurs in particular when these meat products are more or less severely heat-treated. Fortunately, this reaction can be prevented by the addition of ascorbic or isoascorbic acid (Walters, 1980).

Physical methods of preservation, e.g., gamma-irradiation of foods, may also initiate chemical reactions that alter the product to some extent. It must be stated, however, that these reactions do not necessarily lead to hazardous compounds. Irradiated food can generally be recognized as safe, as is emphasized by numerous statements (e.g. World Health Organisation, 1983). If questions about safety concerning gamma-irradiation are advanced, it should certainly also be taken into account that the same applies to a number of heat-processed products, which then require at least as much attention.

The food chemist has to emphasize that a foodstuff is a dynamic system in which, under many circumstances, chemical reactions may take place that could lead to possibly hazardous changes. Thorough chemical knowledge of 'systems' is required to assess the health impact of suchlike changes of the chemical constituents of foods.

#### Other challenges for the chemical food hygienist

The activities of chemical hygienists are not restricted to control of non-microbiological hazards.

Certain outbreaks of illness due to microbial activity in food are rightly termed 'food poisoning'. The toxins that cause these adverse effects have often been identified (Mossel, 1988) and can be assessed by chemical analysis. To phrase it academically: food hygienists should not only be interested in food intoxications but also in food intoxications.

For example, it is not of significance for health whether mycotoxins have been formed by mould growth on the food itself or that they originate from animal feed and are subsequently carried over to edible tissues. It is the measured presence of toxic substances in food that determines their health risk. The food chemist has the equipment to determine toxins in food that are produced by bacteria or by moulds, irrespective of whether they are relatively simple compounds (such as histamine and other pressor amines) or complex compounds such as proteins. Immunochemical methods, in particular, can be helpful in the analysis of these toxins, either in direct analysis or by isolation by means of immuno-affinity chromatography.

Close and cordial cooperation between food microbiologists, chemists and technologists is imperative to improve the overall safety of food.

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#### References

- Alink, G.M. et al.; 1988. Mutagenicity of food pellets from human diets in The Netherlands. *Mutat. Res.* 206:387-393.
- Alink, G.M. & Kuiper, H.A.; 1988. Personal communication.
- Beckers, H.J.; 1988. Incidence of foodborne diseases in The Netherlands:

- Annual summary 1982 and an overview from 1979 to 1982. *J. Food Prot.* 51:327-334.
- Bender, A.E.; 1987. Nutritional changes in food processing. In "Developments in Food Preservation", Vol. 4. S. Thorne Ed. Elsevier Applied Science, London/New York, p. 1-34.
- Bryan, F.L.; 1982. Foodborne disease risk assessment of foodservice establishments in a community. *J. Food Prot.* 45:93-100.
- Chen, E.; 1979. PBB: an American tragedy. Prentice Hall, Inc. Englewood Cliffs, N.J., 329 p.
- Crompton, T.R.; 1979. Additive migration from plastics into food. Pergamon Press, Oxford, 234 p.
- Doll, R. & Peto, R.; 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Nat. Cancer Inst.* 66:1191-1308.
- Doremire, M.E., Harmon, G.E. & Pratt, D.E.; 1979. 3,4-benzopyrene in charcoal grilled meats. *J. Food Sci.* 44:622-623.
- Feron, V.J. et al.; 1981. Lifespan oral toxicity study of vinyl chloride in rats. *Food Cosmet. Toxicol.* 19:317-333.
- Gray, J.; 1985. How safe is your diet? *Chem. & Ind.*: 146-148.
- Haagsma, N.; 1988. Control of veterinary drug residues in meat - A contribution to the development of analytical procedures. Thesis, Utrecht, 157 p.
- Hall, R.L.; 1971. Information, confidence, and sanity in the food sciences. *The Flavour Industry* 2:455-459.
- Hambraeus, L.; 1982. Naturally occurring toxicants in food. In: "Adverse effects of foods", E.F.P. Jelliffe & D.B. Jelliffe Eds. Plenum Press, New York/London, p. 13-36.
- Hart, I.C. et al.; 1985. The effect of injecting or infusing low doses of bovine growth hormone on milk yield, milk composition and the quantity of hormone in the milk serum of cows. *Anim. Prod.* 40:243-250.
- Hotchkiss, J.H.; 1987. A review of current literature on N-nitroso compounds in foods. *Adv. Food Res.* 31:53-115.
- Kato, H.; 1989. Nutritional and physiological effects of Maillard reaction products. In: "Trends in Food Science", Proc. 7th World Congress of Food Science and Technology, H.G. Ang Ed. Singapore Institute of Food Science and Technology, in press.
- Kaunitz, H. & Johnson, R.E.; 1973. Exacerbation of heart and liver lesions in rats by feeding of various mildly oxidized fats. *Lipids* 8:329-336.
- Lindsay, D.G. & Sherlock, J.C.; 1982. Environmental contaminants. In: "Adverse effects of foods", E.F.P. Jelliffe & D.B. Jelliffe Eds. Plenum Press, New York/ London, p. 85-110.
- Maggi, E. et al.; 1979. Mercury, chromium, lead and organochlorine pesticide residues in some food products of animal origin: A review. *Meat Sci.* 3:309-319.
- McBride, B.W., Burton, J.L. & Burton, J.H.; 1988. The influence of bovine growth hormone (somatotropin) on animals and their products. *Res. Dev. Agric.* 5:1-21.
- Mossel, D.A.A.; 1988. Impact of food-borne pathogens on today's world, and prospects for management. *Anim. Hum. Health* 1:13-23.
- Mossel, D.A.A.; 1989. Listeria in foods - its determination, enumeration and control. *J. Food Microbiol.* 8, in press.
- Mossel, D.A.A. et al.; 1984. Microbiological quality control. In "Quality control in food industry", Vol. 1. 2nd Ed. S.M. Herschdoerfer Ed. Academic Press, London etc., p. 79-168.
- Pilnik, W. and Folstar, P.; 1979. Entwicklungstendenzen in der Lebensmitteltechnologie. *Dtsch. Lebensm. Rundsch.* 75:235-248.

- Reid, J.F.S.; 1980. Significance of natural oestrogen-implanted beef to human health. Proc. Conf. The use, residues and toxicology of growth promoters. Dublin, p. 24-31.
- Royal Society; 1983. Risk Assessment: Report of a Royal Society Group. The Royal Society, London.
- Senn, C.L.; 1982. The Firemaster incident. In: "Adverse effects of foods", E.F.P. Jelliffe & D.B. Jelliffe Eds. Plenum Press, New York/London, p. 129-134.
- Slovic, P.; 1987. Perception of risk. *Science* 236:280-285.
- Stoloff, L.; 1987. Carcinogenicity of aflatoxins. *Science* 237:1283. Response by B.N. Ames and R. Magaw, *ibid.*, p. 1283-1284.
- Taylor, W.; 1983. Risks associated with the exposure of human subjects to endogenous and exogenous anabolic steroids. In Proc. Symp. "Anabolics in Animal Production", Office International des Epizooties, Paris. E. Meissonnier Ed., p. 273-287.
- Truswell, A.S.; 1988. Veterinary medicines in animal foods - Reassuring findings but surveillance should go on. *Br. Med. J.* 297:1216.
- Walters, C.L.; 1980. Nitrosamines in meat products. In: "Developments in meat science", Vol. I. Ed. R. Lawrie. Applied Science Publishers, London, p. 195-217.
- Wodicka, V.O.; 1977. Food safety - Rationalizing the ground rules for safety evaluation. *Food Technol.* 31 (9):75-77, 79.
- Wogan, G.N.; 1973. Aflatoxin carcinogenesis. In: *Methods in cancer research*, Ed. H. Bush. Academic Press Inc., New York, p. 309-344.
- Wolfram, G.; 1979. Neue Ergebnisse zur Ernährungsphysiologie erhitzter Fette - Allgemeiner Überblick. *Fette, Seifen, Anstrichm.* 81:559-562.
- World Health Organisation; 1981. Wholesomeness of irradiated food, Technical Rep. Ser. No. 659. WHO, Geneva.

MICROBIOLOGICAL SAFETY ASSURANCE OF DRIED NEUTRAL pH SPECIALITY FOODS OF VEGETABLE ORIGIN

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Summary

Fragile fruit and vegetable products sparingly dried at temperatures below 40 °C in vacuo have become a component of compounded dried foods that is much in demand. Commodities with a pH below about 4 do not present any health risk of microbiological nature, but those with a much higher pH do. As is the rule, microbiological safety assurance of the latter has to rely on the design of longitudinally integrated good manufacturing practices (GMP), validated by a "holistic" risk analysis, i.e. covering any critical point or stage between raw material and ingestion, hence including reconstitution.

Methods for monitoring end-products -- both with respect to internal auditing and compliance with Government or customers' requirements -- are recommended. These include techniques for the counting and identification of the target organisms: mesophilic and thermotrophic Enterobacteriaceae, the Clostridium group, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes and mould spores. Special analytical precautions required when examining dried products containing the rapidly spreading Bacillus alvei group are discussed. Results obtained on products manufactured by GMP are presented.

To resolve -- or even better to avoid -- the all too frequent disparate, if not conflicting, opinions about reference values ("standards"), which often mar good relations between producers and customers, the precepts applicable to the interpretation of microbiological-analytical data and the ecologically valid approach to the assessment of target values are presented. In essence, these should be empirically derived from levels that are

- safe, as established by holistic risk analysis;
- attainable and maintainable by GMP.

Reference values should moreover always include tolerances called for by

- unavoidable minor fluctuations in contamination levels under industrial conditions;

- limited intrinsic precision and accuracy of the particular microbiological-analytical technique in every instance.

Descriptors: dried foods; infant foods; holistic quantitative risk analysis; reference values; Listeria monocytogenes; Staphylococcus aureus; thermotrophic Enterobacteriaceae; Bacillus cereus; Clostridium spp; mould propagules.

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## Introduction

Dried foods of vegetable origin have been popular commodities since primitive times. However, until about 1920, their sensorial quality left much to be desired. At that time, significant improvements in drying technology were introduced. Steps taken included blanching to inactivate tissue enzymes, careful attention to avoid local scorching during dehydration and, if necessary, packaging in nitrogen or CO<sub>2</sub> to avoid oxidation of lipids. They markedly improved the hedonistic rating of the final product (van Arsdal, 1963; 1964; Leniger & Bruin, 1977; Cunningham, 1982; Mujumdar, 1987).

The ideal process of food dehydration would, of course, be freeze-drying, because it greatly spares native structures and naturally occurring constituents of fragile foods. Freeze-drying is only feasible in a few instances, because of its high cost (Goldblith et al., 1975; Mellor, 1978). A reasonable substitute is drying on a vacuum belt (Mujumdar, 1987). In this process, the temperature of the food product generally does not exceed 40 °C.

Vacuum belt drying and its many modifications are applied to a variety of specialty foods and particularly food components. As long as the pH of commodities does not exceed 3.8 (Chung & Goepfert, 1970; Smelt et al., 1982; Tanaka, 1982; Ferreira & Lund, 1987; Young-Perkins & Merson, 1987), no substantial microbiological problems are to be anticipated. However when vacuum belt drying is used for commodities of markedly higher pH, potential microbiological hazards have to be taken into account.

Pathogenic bacteria have occasionally been isolated from dried foods, which were mainly of animal origin (Collins et al., 1968; Craven, 1978; Rowe et al., 1987). Nonetheless vegetable raw materials may also be contaminated with

- soil organisms such as C. botulinum (Kautter et al., 1978; Arnon et al., 1979; Seals et al., 1981; Kautter et al., 1982; MacDonald et al., 1985) or B. cereus (Kim et al., 1971; Blakey et al., 1980);
- pathogens of enteric origin if poor practices are followed in their manufacture (Geldreich & Bordner, 1971; Keoseyan, 1971; Wright et al., 1976; Tamminga et al., 1978; Bockemühl et al., 1984; Rude et al., 1984; Doyle & Schoeni, 1986; Garcia-Villanova et al., 1987).

Pathogens generally survive well under conditions of low water activity ( $a_w$ ) (Li Cari & Potter, 1970; Ray et al., 1971; Christian & Stewart, 1973; Uzelac & Stille, 1977; Badanry et al., 1985; Rockland & Beuchat, 1987), which justifies microbiological alertness.

This paper records the results of about twenty years of investigations in microbiological safety assurance and monitoring of dried speciality foods of vegetable origin manufactured by sophisticated techniques, pioneered and elaborated by Professor Walter Pilnik.

## Strategy of microbiological safety assurance of delicately dried products

The assurance of the safety, quality and acceptability of food commodities in general terms has to rely entirely on management of production.

In management of microbiological safety, the critical points in production lines must first be identified (Bauman, 1974). These mostly concern contamination with organisms, subsequent growth and, for enterotoxin formation, also metabolic activities of pertinent organisms (Bryan et al., 1971; Mossel, 1988). Such potential hazards must subsequently be brought under control by measures of longitudinally integrated intervention (Mossel, 1983b; Mossel, 1988; Silliker et al., 1988). These include - meticulous selection and where required decontamination of raw materi-

als

- prevention of contamination throughout
- storage of intermediates allowing microbial growth, at temperatures that arrest proliferation of organisms of concern.

Practices considered satisfactory must be validated by quantitative risk assessment (Mossel & Drion, 1979; Mossel & Dijkman, 1984; Shapton, 1988). Such an analysis should, like measures of intervention, extend throughout the production and distribution line and most definitely, in dried foods, also include reconstitution (Mossel et al., 1973). Consequently this validation procedure is aptly termed holistic quantitative risk assessment (Mossel, 1989). It is summarized in Table 1. The entire process of design, validation and any rectification and rechecking of procedures

Table 1. Terminology used in, and the strategy followed when applying holistic quantitative risk assessment (HQA) and subsequent risk management.

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### I. Semantics

**Hazard:** event or condition which has been empirically demonstrated to endanger human health.

**Severity:** character and magnitude of the impact of a given hazard

**Risk:** probability of occurrence of a hazard.

**Worst case scenario:** presupposition of extreme, though by no means imaginary hazard/risk situations.

**Holistic:** taking into account every hazardous event that can occur throughout the cycle that determines an adverse health effect.

**ALARA:** magnitude of risk as low as reasonably achievable, i.e. attainable and maintainable.

**Management:** reducing the impact of a hazard to an acceptable level.

### II. Fundamentals

1. No human activity, including eating and drinking is, or can ever be free from all hazards. Consequently in essence "zero risk" cannot exist.
2. Estimation of health risks is often inaccurate. Nonetheless it represents the only effective approach to allaying concerns of the public and public health authorities alike; irrespective of these being realistic or perceived only.
3. HQR-assessments (a) start from "worst case scenarios", i.e. analyse the impact of situations and events that are exceptional though not figments of the imagination; (b) have to include all critical situations, potentially occurring within the cycle of events that results in adverse health effects.
4. If a risk analysis demonstrates unacceptable hazards, this should prompt emphatic recommendations for intervention, i.e. risk management.
5. Effective intervention is defined as the introduction of procedures allowing to attain an "acceptable (ALARA) risk".
6. The expenditure of risk management may not be trifling in some instances. However it must be weighed against associated benefits such as (i) diseases averted; (ii) avoiding undue financial burdens to society at large; (iii) preventing unjustified anxiety amongst the public which is hard to allay after the event.

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After Mossel, 1988 & 1989

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results in what is generally termed good manufacturing and distribution practices (GMP).

Attempts at safety assurance relying on examination of final products alone are sometimes still practised. These will of course not affect, let alone allow, management of, such microbial hazards. Inspection can do no more than close the stable after the horse has bolted. However, monitoring end-products as a last step in integrated safety assurance is extremely useful. In this instance, examination of samples allows (i) checking on adherence to previously elaborated good manufacturing practices; (ii) timely detection of incidentally going-out-of-control of essentially well managed processes (Habraken et al., 1986). If unfavourable results are obtained, the laboratory's responsibility does not stop upon forwarding these to management. Rather immediate feed-back to production lines is essential, including (i) suggestions for intervention without delay; (ii) offering assistance in validating the efficacy of any measures taken to rectify observed deficiencies (Mossel, 1988).

When applying these general principles to vacuum-dried specialty food products, the crucial point is that their manufacture does not include exposure to heat, which would substantially reduce initial counts of bacteria, viruses, parasites, yeasts and moulds. The microbial profile of a run of sparingly drying a challenged vegetable product of pH about 6 by the Pilnik procedure is presented in Table 2. Consequently safety assurance has to rely entirely on the remaining elements of GMP (Mossel & Shennan, 1976). Raw materials will have to be carefully selected and when required to be processed for safety before being dried. Careful cleaning and disinfection of the plant has to be ensured to reduce infectious pressure on lines to an extremely low level; and this has to be monitored by frequent microbiological spot checks. As illustrated by Fig. 1, microbial profiles of products during the drying process have to be followed meticulously in order to assess subsequently, by holistic quantitative

Table 2. Fate of various types of relevant micro-organisms during GMP processing followed by various reconstitution procedures of sparingly dried vegetables, for dry matter of vegetable paste of pH 6.0 and for vegetable powder.

The commodity was challenged with a soil inoculum to allow reliable assessment of changes in the microbial association during the chain of events (Mossel, 1983a).

	log <sub>10</sub> of count of colony-forming units (g <sup>-1</sup> )				
	paste before drying	powder	reconstituted paste before 10% dm at room temperature		
			2 h	6 h	20 h
Mesophilic aerobic colony count	4.7	4.8	5.5	6.5	9.2
Caseolytic micro-organisms	4.3	3.2	4.4	4.8	8.4
Enterobacteriaceae 30 °C	3.4	<1.0	2.5	3.3	5.7
Enterobacteriaceae 42 °C	2.6	<1.0	2.0	3.0	5.2
<u>Enterococcus</u> spp. (Lancefield-d)	4.2	3.6	4.1	4.3	5.7
<u>Bacillus</u> spp.	4.2	3.8	3.9	4.2	5.2
Yeasts and moulds	3.2	3.0	3.2	3.3	4.4
<u>Clostridium</u> spp.	4.6	2.6	3.9	4.4	>5.0

risk analysis, whether proliferation and metabolism by pertinent organisms does not exceed previously established acceptable rates. Releasing and packaging of commodities should be done under carefully controlled hygienic conditions so as to avoid later recontamination from the environment.

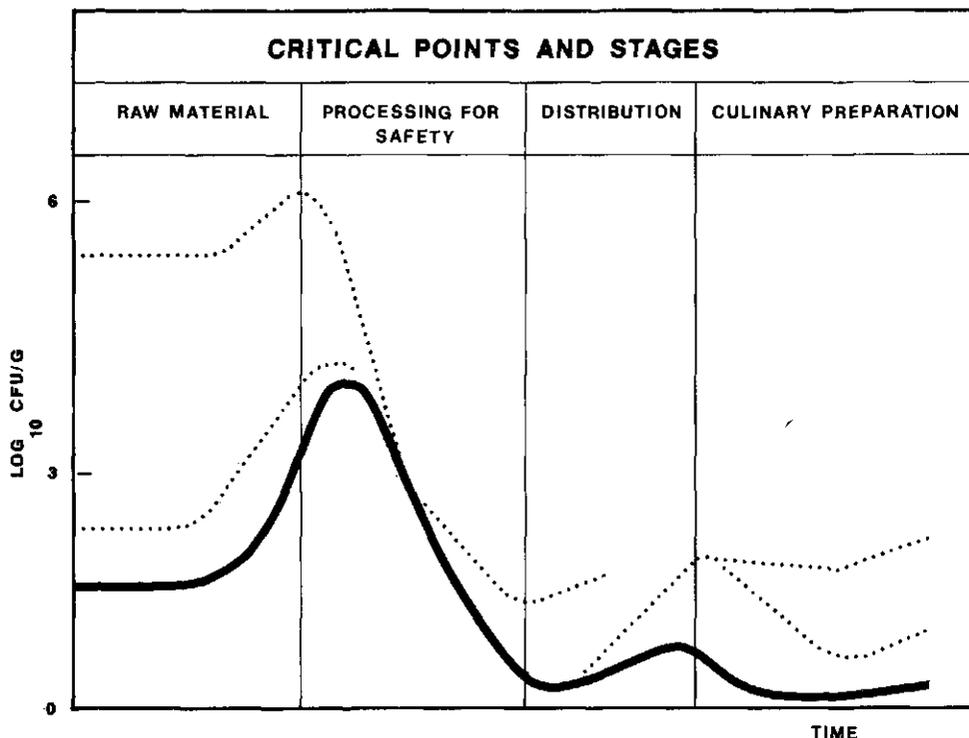


Fig. 1. Holistic Quantitative Risk Analysis (HQRA).  
 \_\_\_\_\_the attainable and maintainable pattern validated by HQRA (Good Manufacturing Practices)  
 -----profiles to be avoided by appropriate measures of intervention

Monitoring procedures developed for validation of safety management

General Aspects

Sampling

The contamination of dried foods bears a sporadic and erratic character. This applies even when careful control of manufacture is exercised.

The drying step may or may not result in some death of non-sporing organisms in delicate vegetable products. Afterwards the products are recontaminated from environmental sources. This occurs incidentally and hence follows no predictable pattern. Upon reaching the food matrix, partial destruction of non-sporing organisms will occur as a result of osmotic shock (Mossel & Koopman, 1965). The reduction in vitality depends

on many factors, including osmolability of the organisms, the  $a_w$  of the food and the environmental temperature. After stabilization of the contaminating microbial population stratification results (Fig. 2). Upon milling and mixing, a certain dispersion of strata occurs, leading to 'nests' of one or more colony-forming units (Fig. 2). In spite of this dispersion, a marked heterogeneity of contamination of the final product remains. Its impact, in the sense of negatively affecting the accuracy of microbial counting procedures made afterwards, can be limited by the choice of proper methods of sampling of consignments.

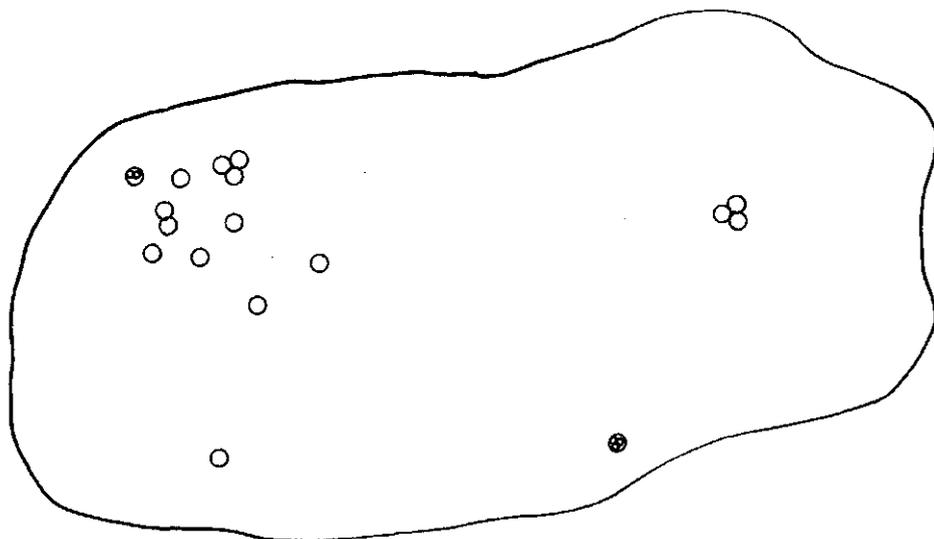


Fig. 2. Heterogeneity of the contaminating microbial population after stabilisation of dried foods.

Increasing the amount of material sampled obviously helps in including contaminated strata in the sample and increases the probability of the detection of contaminants. It has also been demonstrated that the risk of missing contaminants can be markedly diminished by continuously drawing small samples of about 5 g from production streams of dried foods, at a rate of about one sample for every 100 kg (Habraken et al., 1986).

Pooled samples of about 1 kg obtained by this procedure should be carefully divided into the customary smaller subsamples for examination. To avoid diminishing the probability of identifying contaminants the quadrantic diminution procedure (Harrewijn et al., 1972) should be used.

#### Analytical methods

Tests to be used for surveying well managed production and distribution lines have to be and can be simple, giving results as rapidly as possible and leading to readings that are easy to make and that can be interpreted in one sense only. Clearly the precision and accuracy of such tests must

be high. But the main attribute of such in-house procedures is their repeatability.

In this context, it is mandatory to realize that non-sporing organisms routinely encountered in dried foods (Clark et al., 1966; Sheneman, 1973; Mossel & Shennan, 1976; Splitstoesser & Wilkinson, 1981) carry sublethal damage due to exposure to heat, low  $a_w$  and oxygen (Speck et al., 1975; Mossel & van Netten, 1984). They should, consequently, invariably be revitalized by an adequate resuscitation treatment (Mossel & Ratto, 1970; van Doorne & Claushuis, 1979) before detection in selective media be attempted.

Despite these precautions, periodic validation of internal auditing test procedures against methods used by authorities or buyers is prerequisite in all instances. However, small discrepancies with standard procedures can be accepted on three conditions:

- differences must not greatly exceed the usual intrinsic variation of colony-forming units or MPN testing procedures, being of an order of 0.3 and 1.0 on a log<sub>10</sub> scale, respectively (Mossel 1987b);
- the sensitivity of in-house testing must be at least equal to that of the extramural technique, but preferably higher;
- discrepancies between inhouse and extramural testing may depend only on method and may never be affected by commodities (Mossel & van Netten, 1984).

#### Examination for Enterobacteriaceae

Detection of the group and identification of isolates at genus level

In view of the required repeatability and consistency of monitoring data, it needs little justification that tests for the "coli--aerogenes" group will have to be avoided, given their appalling intrinsic variability, i.e. up to 3 orders of magnitude (Pierson et al., 1978; Silliker et al., 1979). They are to be replaced by a search for the entire Enterobacteriaceae group, irrespective of mode of attack on lactose. Such tests are, moreover, ecologically much better founded (Mossel, 1982), show a markedly lower coefficient of variation than corresponding tests for the ill-defined "coli--aerogenes" bacteria (Mossel et al., 1974; 1980) and have a higher sensitivity.

In dried foods, adequately processed for safety, Enterobacteriaceae are scarce. So their detection should rely on a test for presence or absence, rather than on colony counts. The current procedure consists of examining one or two subsamples of 1 g by resuscitation of  $10^{-1}$  macerates for 2 h at room temperature (Mossel & Ratto, 1970; van Doorne & Claushuis, 1979), followed by the addition of an equal volume of double-strength buffered glucose brilliant green bile ("EE") broth and overnight incubation at 30 °C (Mossel et al., 1963). Cultures showing growth, usually accompanied by gas formation are streaked onto MacConkey's violet-red bile lactose agar and again incubated at 30 °C for 18-24 h.

If there is growth, isolates are checked by several tests in sequence. These include in hierarchic order (Mossel et al., 1977):

- attack on lactose made visible on MacConkey agar;
- behaviour in Gram-negative diagnostic tubes (Mossel et al., 1977), indicating oxidase reaction, mode of attack on glucose, motility, production of H<sub>2</sub>S, indole and pigment formation;
- assessment of thermotrophic character (Sheneman 1973) by studying growth on slants of pre-tempered violet-red bile glucose agar at  $42.5 \pm 0.5$  °C (Mossel & Zwart, 1960; Mossel et al., 1986);
- when required, additional examination for assimilation of citrate,

formation of acetylmethylcarbinol, attack on urea, assimilation of malonate, decarboxylation of lysine and  $\beta$ -galactosidase. The identification schemes for lactose-positive and lactose-negative isolates relying on these traits are summarized in Fig. 3 and 4.

All media used in these tests have to be carefully monitored before use, for performance, productivity and any selectivity (Mossel et al., 1983; Visser et al., 1985).

The Enterobacteriaceae most frequently encountered in vacuum-belt-dried speciality foods of pH > 3.8 are presented in Table 3. Not surprisingly, some eight isolates could not be identified given the heterogeneous and not fully investigated taxonomic structure, particularly of the genus *Erwinia* (Verdonck et al., 1987; Beji et al., 1988). Irrespective of these taxonomic limitations, the majority of the isolates was constituted of typical psychrotrophic, epiphytic species of Enterobacteriaceae not pathogenic to man when ingested with food.

Table 3. Enterobacteriaceae types most frequently encountered in vacuum-belt-dried speciality foods of pH > 3.8.

Name of organism	Occurrence	
	number of isolates	proportion of total
<i>Escherichia</i>	41	30
<i>E. coli</i>	(29)	(71)
<i>Klebsiella</i>	29	21
<i>Erwinia</i>	19	14
<i>Enterobacter</i>	17	13
<i>Citrobacter</i>	16	12
<i>Morganella</i>	3	2
<i>Hafnia</i>	2	1
<i>Kluyvera</i>	1	1
Not identifiable	8	6
	136	100

#### Search for Salmonellae

P-A tests for *Salmonella* spp are regularly carried out, mainly to satisfy customers' requirements. We have found a short resuscitation procedure, i.e. about 4 h as recommended by Sveum and Kraft (1981), effective. When this repair treatment is carried out in a good nutrient medium, it allows adequate recovery of sublethally injured cells without, as the authors rightly claim, undue growth of and antagonism by other food bacteria. A prerequisite for the success of this resuscitation technique is, however, that the whole system, and not 1 ml or less, is subsequently subjected to an enrichment procedure. For this purpose, Rappaport-Vassiliadis' MgCl<sub>2</sub> malachite green broth + novobiocin 10 mg/l (RVN) can be used advantageously (van Schothorst et al., 1987). For convenience, the resuscitated macerate may be centrifuged (van Netten et al., 1987) and the pellet cultured in about 200 ml RVN at 43 °C (Mossel et al., 1983). Streaking onto XLD-agar with novobiocin and onto Inoue's mannitol lysine crystal violet brilliant green agar to identify lactose

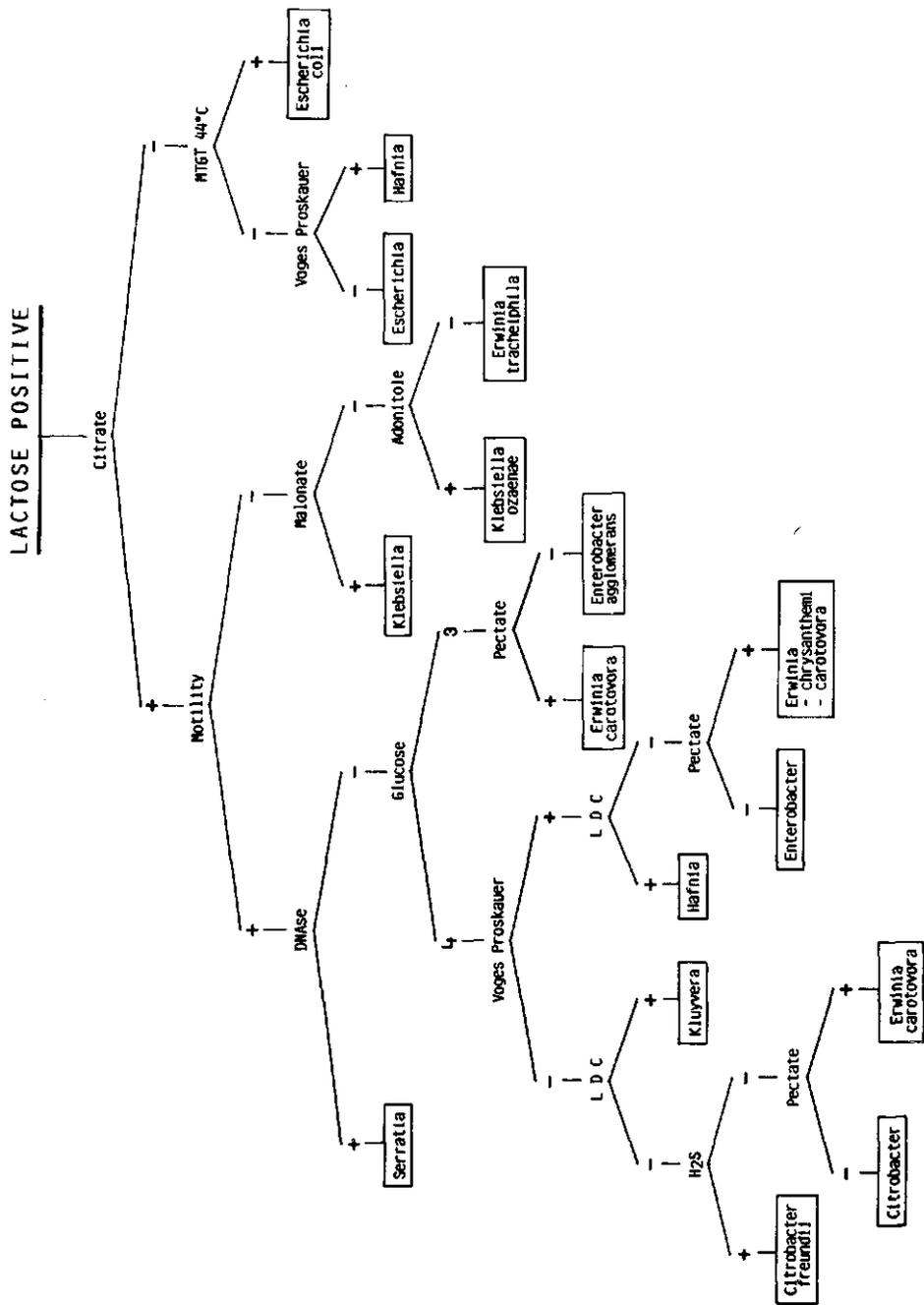


Fig. 3. Identification scheme for lactose-positive Enterobacteriaceae.



positive biotypes (van Schothorst et al., 1987) is the preferred isolation procedure. Identification is done conventionally (Ewing, 1986).

#### Detection and identification of various Clostridium species

As in the previous examinations, a P-A test is used. It relies on the property of the vast majority of Clostridium species to show blackening of media containing sulphite and an iron salt. This is commonly called sulphite reduction (Prévot, 1948), though it may also be cysteine desulfhydration not inhibited by sulphite (Mossel, 1962). Three tests are used in parallel for Clostridium detection (Mossel & de Waart, 1968).

The first one aims at detecting the entire group. Hence use is made of reinforced Clostridium medium (Hirsch & Grinsted, 1954; Barnes & Ingram, 1956) with  $\text{Na}_2\text{SO}_3$  0.5 g/l (Mossel, 1959; Alsina & Lucena, 1988) and polymyxin added at 10 mg/l, the latter to suppress any Enterobacteriaceae. Incubation is at 30 °C (Mossel, 1959). Blackened cultures may be produced by some Bacillus species if anaerobiosis during inoculation and culturing is not absolute (Weenk & Mossel, 1989, in preparation). So in some instances, blackening primary cultures may have to be restabbed into prerduced media and these subcultures further handled under strictly anaerobic conditions. A negative catalase reaction obtained on a dense culture, which will not grow under aerobic conditions, brings final proof of identity.

The second test has Cl. perfringens as a target. It relies on the use of iron sulphite broth with D-cycloserine added at 400 mg/l and incubated at 46 °C (Mossel & Pouw, 1973).

The third P-A test is designed to grow Cl. botulinum selectively. It uses iron sulphite broth with cycloserine, trimethoprim and sulfamethoxazole (Dezfulian et al., 1981; Glasby & Hatheway, 1985; Mills et al., 1985; Silas et al., 1985).

Some variants of Clostridium butyricum have more recently been found to be associated with infantile diarrhoea (Howard et al., 1977; Sturm et al. 1980; Sullivan et al., 1980; Nakamura et al., 1983; Popoff & Ravisce, 1985; Aureli et al., 1976; Babudieri et al., 1986; Fontaine et al., 1986; McCroskey et al., 1986; Thaler et al., 1986; Suen et al., 1988). Because some of the dried speciality foods are incorporated in baby foods, the absence of such clostridia in the commodities has to be guaranteed and validated. A short fermentation spectrum ("gallery") based on earlier collected data on sulphur metabolism (Mossel, 1962) and the latest taxonomic key for carbohydrate fermentation (Cato et al., 1986) was elaborated to allow rapid taxonomic grouping of isolates obtained in any of the enrichment/isolation tests. It is presented in Table 4.

#### Counting of B. cereus and allied toxinogenic bacilli

Because only higher counts of colony-forming units of these organisms (about 100 g<sup>-1</sup> and more) are reason for concern, a direct colony-counting procedure can be used for this element of monitoring. The 10<sup>-1</sup> macerate of the dried food is spread in amounts of 0.5 ml onto dried plates of mannitol egg yolk polymyxin (MYP) agar and incubated overnight at 30 °C. Bacilliiform colonies dissimilating egg yolk but not attacking mannitol are virtually always B. cereus (Mossel et al., 1967; Lancette & Harmon, 1980; Bouwer-Hertzberger & Mossel, 1982).

Large numbers of bacilli resembling B. licheniformis also require attention (Kramer et al. 1982).

Table 4. Short fermentation spectrum ("gallery") for some clostridia.

Strain	Gel- atin	Mot- ility	In- dole	H <sub>2</sub> S formation in			Fermentation of				
				H <sub>2</sub> SC <sup>1</sup>	H <sub>2</sub> ST	H <sub>2</sub> SS	Lac <sup>2</sup>	Sac	Xyl	Man	Lec
<i>C. baratii</i>	-	-	-	+	+	+	+	+	-	-	+
<i>C. bifermentans</i>	+	+	+	+	+	+	-	-	-	-	+
<i>C. butyricum</i>	-	+	-	-	-	-	-	-	-	-	-
<i>C. difficile</i>	+	+	-	+	-	+	-	-	-	+	-
<i>C. parapaperfringens</i>	-	-	-	-	+	+	+	+	-	-	+
<i>C. paperfringens</i>	+	-	-	+	+	+	+	+	-	-	+
<i>C. septicum</i>	+	+	-	-	-	+	+	-	-	-	-
<i>C. sordellii</i>	+	+	+	+	+	+	-	-	-	-	+
<i>C. sporogenes</i>	+	+	-	+	+	+	-	-	-	-	+
<i>C. tyrobutyricum</i>	-	+	-	-	-	-	-	-	-	-	-

1) Composition of sulphur derivative test media used (g/l):

H<sub>2</sub>SC: soya peptone 5.0; NaCl 5.0; tryptone 15.0; iron(III) citrate 0.5; agar 15.0; 1-cysteine hydrochloride 0.5 (dissolved in 20 ml water and filter sterilized); pH = 7.2.

H<sub>2</sub>ST: soya peptone 5.0; NaCl 5.0; tryptone 15.0; iron(III) citrate 0.5; agar 15.0; sodium thiosulphate 0.3; pH = 7.2.

H<sub>2</sub>SS: soya peptone 5.0; NaCl 5.0; tryptone 15.0; iron(III) citrate 0.5; agar 15.0; sodium sulphite 0.5, dissolved in 20 ml water, filter sterilized, and added immediately before use; pH = 7.2.

2) Composition of sugar dissimilation media used (g/l):

Basal medium: soya peptone 5.0; NaCl 5.0; tryptone 15.0; agar 15.0; 1-cysteine hydrochloride 0.3; bromocresol purple 0.01; pH = 7.2.

Carbon sources: 10 g/l added as filter-sterilized solution.

Search of *Listeria monocytogenes*

*L. monocytogenes* has long been identified as an almost ubiquitous rod-shaped non-sporing catalase-positive motile bacterium that, under particular conditions, will attack preferably, though certainly not exclusively, debilitated human hosts. These include pregnant women, neonates, the elderly and other immuno-compromised persons. The disease is serious: it leads to abortion and still birth in pregnant women and septicaemia or meningo-encephalitis in adults and newborn (Schlech, 1986; 1988).

Transmission by foods has been suggested from circumstantial evidence since about 1950 (Urbach & Schabinski, 1955). At least two well investigated outbreaks in the United States (Ho et al., 1986; Linnan et al., 1988) and one in Canada (Schlech et al., 1983) indicate ingestion of soft cheese or vegetables as an aetiological mechanism. The impact of these findings on the food industry has obviously been tremendous, because such a serious extra-enteric disease has never before been linked to a bacterium occurring in food.

The extensive research programmes prompted by this situation have demonstrated that, though interest was mostly focused on dairy products (Fleming et al., 1985; Hird, 1987; Lovett et al., 1987; Breer & Schopfer, 1988; Linnan et al., 1988), a great variety of other foods often carry *L. monocytogenes* (Mossel et al. 1987). These include vegetables (Ho et

al., 1986; Sizmur & Walker, 1988; Steinbruegge et al., 1988), as mentioned before, but also poultry (Gitter, 1976; Pini & Gilbert, 1988; Skovgaard & Morgen, 1988), red meats and meat products (Le Guillou, 1980; Cottin et al., 1985; Nicolas & Vidaud, 1987; Breuer & Prändl, 1988; Buchanan et al., 1988; Schwartz et al., 1988; Skovgaard & Morgen, 1988; Truscott & McNab, 1988) and seafood (Weagant et al., 1988). This abundance is not surprising in view of the widespread occurrence of this bacterium in nature (Welshimer & Donker-Voet, 1971). It calls for surveillance of dried speciality foods of vegetable origin for L. monocytogenes.

The detection of listeriae in foods has long been hampered by lack of an adequately selective culture medium. One of the most effective ones was nalidixic acid tryptaflavin agar of Ralovich et al. (1971). Though it works well for detecting L. monocytogenes in stools, its application to foods, particularly fresh ones, was less successful. This was not surprising because virtually all media used for this purpose, including McBride & Girard's phenylethanol agar (1960) rely only on colony appearance.

For the monitoring of dried speciality foods, both a selective enrichment medium and a reliable selective differential isolation agar are required. For both purposes, we provided the medium with diagnostic traits. These rely on the incorporation of (i) aesculin and ferrous salt leading to blackening if listeriae are present (Rodriguez et al., 1984); (ii) D-mannitol and phenol red, allowing to differentiate Enterococcus species, which are hard to suppress without inhibiting listeriae (van Netten et al., 1988a,b) and which mostly dissimilate aesculin (Mundt, 1986) but generally attack mannitol (Mundt, 1986) whereas most listeriae fail to do so (Seeliger & Jones, 1986). In an extensive investigation (van Netten et al., 1987; van Netten et al., 1988a,b; Perales et al., 1988; van Netten et al., 1989) we established that, in use of selective inhibitors, two different approaches were required, as a result of the different ecological conditions prevailing in liquid enrichment systems as contrasted to the surface of selective solid isolation media.

The application of the elaborated detection methods to dried speciality foods proceeds as follows. An appropriate aliquot of a large well mixed and quadratically diminished (Harrewijn et al., 1972) sample is thoroughly shaken with a ten-fold volume of buffered tryptone soya peptone yeast extract broth and left at ambient temperature for 1½ h to allow resuscitation of the generally sublethally injured Listeria cells (van Netten et al., 1988b). Thereupon an equal volume of double-strength acriflavin phenylethanol phosphomycin mannitol aesculin broth (Perales et al., 1988) is added and the mixture incubated for 40 h at 30 °C. Irrespective of whether blackening of the medium occurs, a loopful enrichment culture is subsequently streaked onto Columbia agar containing aesculin, mannitol, LiCl (Ludlam, 1949) and ceftazidime (Bannerman & Bille, 1988), which is incubated for up to 40 h at 30 °C under microaerobic conditions (van Netten et al., 1988b). Colonies, surrounded by a rather large black halo on a deep cherry-red background are subcultured onto Columbia agar to examine their morphology, motility and positive catalase reaction. If the results indicate that a Listeria sp. has been isolated, search for a weak beta-haemolytic reaction in combination with failure to attack xylose (Seeliger & Jones, 1986) allows to establish whether L. monocytogenes indeed occurred. A positive finding leads to rejection of the consignment. Isolation of a non-monocytogenes type of Listeria points at any rate to a potentially dangerous commodity and further follow-up is required (Mosser, 1982; 1989). Absence of listeriae in subsamples of 100 g of the dried speciality foods is attainable and maintainable if the

GMP outlined is meticulously adhered to.

#### Monitoring for Staphylococcus aureus

Deficiencies in hygienic handling of dried foods may lead to their contamination with Staph. aureus. If temperature abuse occurs during reconstitution (Mosser et al., 1973), the bacteria may proliferate and form enterotoxins which might harm debilitated consumers, even in sub-microgramme amounts (Mosser, 1988). Hence spot checks for Staph. aureus are advisable as a validation procedure for adherence to GMP.

Adequate samples are subjected to the resuscitation procedure outlined in the section on testing for Enterobacteriaceae and subsequently enriched in a liquid modification of Baird--Parker's highly selective medium (van Doorne et al., 1981). Non-blackening cultures are discarded; those showing a pitch-black appearance are subcultured on to Baird--Parker's medium, incubated at 42 °C (van Doorne et al., 1982) and any typical colonies further tested by well established routine procedures (Bouwer-Hertzberger et al., 1982).

#### Interference of Bacillus alvei with colony counts of honey and dried speciality foods derived from honey and its partial control

A curious phenomenon was noted repeatedly and fairly consistently when honey and dried foods derived from it were examined for total aerobic colonization ("plate count"). If a poured-plate procedure was applied, as certain importing countries or users required, very low counts, i.e. of the order  $10^2$  g<sup>-1</sup> were established. On the other hand, if the generally preferred surface-drop-plating technique (Greenwood et al., 1984) was applied, colony counts exceeding  $10^6$  g<sup>-1</sup> were almost invariably obtained after 2 days.

It was, subsequently, observed that surface drop plate counts of colony-forming units did not, as is a rule, depend on the serial dilution plated onto an agar surface. Even one or two colonies of a particular growth type, isolated from honey on such erratic plates lead to crowding, as a result of dramatically spreading on the surface of any nutrient agar. The type of growth could best be described as like a "milky way" (Fig. 5).

The causative organism demonstrated the following traits. It was a Gram-variable rod, irregularly sporing, motile, strictly aerobic, positive for catalase and oxidase, mostly attacking glucose but not mannitol, egg yolk and nitrate, though consistently dissimilating gelatin and casein. Growth stopped just over 45 °C. "Milky way" development was displayed on virtually all media allowing growth, including mannitol egg yolk polymyxin agar. The oligotrophic medium of Schmidt-Lorenz & Jaeggi (1983) or Leclerc et al. (1985), however, mostly lead to normal colonies, particularly when incubated at 42 °C. This procedure unfortunately could not be used for total counts. Short incubation of TSBA plates, i.e. no more than about 15 h (Fig. 5), at 30 °C was, therefore, ultimately used for the examination of honey by surface plating.

The fermentation spectrum of 15 typical "milky way" isolates is summarized in Table 5. The organism is therefore most similar to Bacillus alvei (Smith & Clark, 1938). It was isolated from about half of 25 samples of honey originating from Austria, Central America, Cuba, France, Hungary, Poland, Switzerland, the Soviet Union and Yugoslavia; though never from any vegetable raw material, amongst some 10 000 specimens examined in over 20 years.

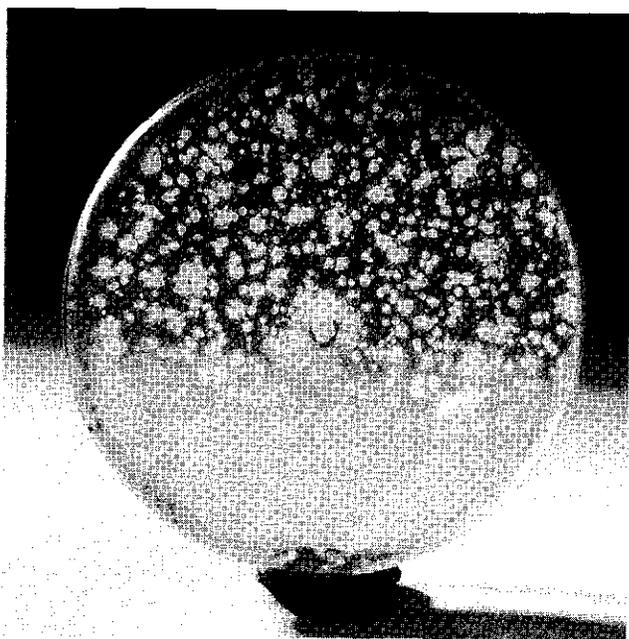
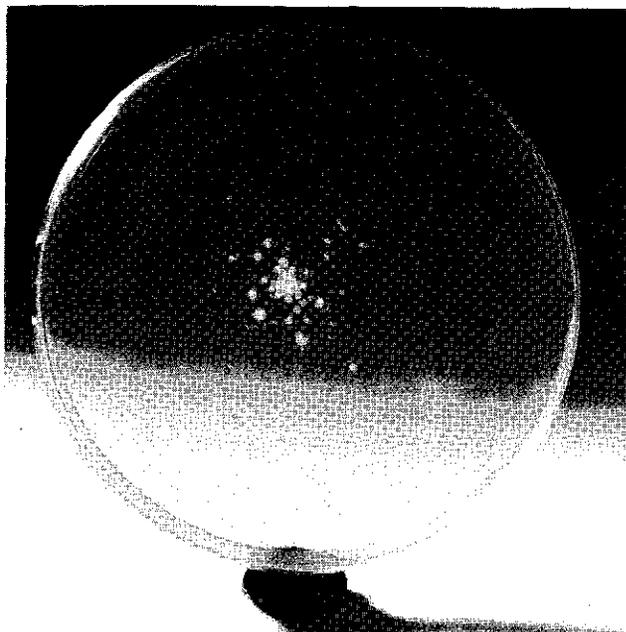


Fig. 5. Appearance of Bacillus alvei and similar species, showing strongly spreading ('milky-way'-like) growth, upon point-inoculation in the center of a solid, appropriate culture medium.  
Top: after 18 h at 30 °C. Bottom: after 24 h at 30 °C.

Table 5. Characteristics of Bacillus alvei-like organisms isolated from honey.

Tests	Results
<u>Gram stain</u>	Gram-variable rod, carrying spores
<u>Growth on</u>	
McConkey agar	-
Kanamycin aesculin azide agar	-
Rogosa agar	-
Mannitol egg yolk polymyxin agar	+
mannitol	-
egg yolk	-
Mannitol salt agar	-
Baird-Parker agar	+ <sup>1</sup>
Milk agar, casein hydrolysis	+
Oligotrophic agar	+
Myxobacterium agar	+
<u>Biochemical attributes</u>	
Gelatinase	+
DNase	-
Nitrate, reduction to nitrite	-
Motility	+
Indole	+
H <sub>2</sub> S from thiosulphate	-
Oxidase	+
Catalase	+
Fermentation of glucose	+
mannitol	-
lactose	+
saccharose	+

<sup>1</sup>) Brown colonies, Egg yolk negative

#### Elaboration and use of microbial reference values for dried speciality foods

##### Precepts

Examination of biological material for pertinent microbiological and chemical attributes makes little sense unless target or reference values (Mossel, 1980) are available, allowing gauging and interpretation of the results. The examination of foods is no exception to this rule. Though chemical, sensory and rheological examination of food samples have presented few problems connected with reference values, microbiological reference values for foods have been amply discussed (Goldenberg et al., 1955; Murray, 1969; Stephens, 1970; Angelotti, 1971; Buyens, 1971; Cranston, 1971; Ingram, 1971; Leininger et al., 1971; Pace, 1975; Briskey, 1976; Mossel, 1980; Garret, 1988; Mossel, 1989). This surprising phenomenon, which for almost four decades marred the microbiological monitoring of foods, is probably due to three factors.

The first is the threat perceived in the terms "standard" or "permitted level", currently used to describe microbiological target values: it sug-

gests "policing action" and "sanctions" when counts are even slightly higher (Bartholomew et al., 1987). The second factor hindering acceptance of microbiological target values is the repeatedly emphasized high intrinsic variability and even inconsistency of microbiological monitoring (Levine, 1961; Ingram, 1961; Bray et al., 1973). The major factor, however, may well be the lack of familiarity with general, or particularly analytical microbiology by scientists who were called upon to pass judgement on microbial limits of acceptability (Mossel & Dijkmann, 1984).

In an attempt to remedy this situation, the following scientific approach was suggested (Mossel, 1980):

1. Adoption of the concept of reference values and particularly reference ranges as used for over half a century in clinical medicine (Gräsbeck, 1976; Scully et al., 1978; Rifkind & Segal, 1983; Silver, 1984; IFCC, 1987);
2. Experimental assessment of reference ranges for foods, as practised in clinical medicine, by surveys on valid specimens, followed by mathematical treatment of survey data as in Fig. 6;
3. Defining "validity" of food specimens as resulting from manufacture, storage and distribution practices previously meticulously validated as the best available, attainable and maintainable ones for every particular commodity;
4. Reference values determined by Procedure 2 have to be validated by holistic quantitative worst-case risk analysis (Mossel, 1988); if necessary, procedures have to be rectified and checked again by risk analysis;
5. Once valid reference values thus assessed being available, basing acceptance plans for consignments of the pertinent food offered for sale, on three-class sampling plans (Bray et al. 1973, Roberts et al. 1986) only;
6. Determining carefully and expertly what the impact of slightly exceeding target values may be for (i) each particular food; (ii) each group of micro-organisms: non-specific counts of colony-forming units, counts of marker organisms, enumeration of spoilage agents, etc. (Mossel, 1983a);
7. Distinguishing sharply and throughout between interpretation of the results of sampling examination expressed in acceptance plans, as defined under Procedure 5 and decisions to be taken about the destination of consignments slightly exceeding reference ranges, taking into account the considerations under Procedure 6.

It has been gratifying to observe during the last twenty years that this approach is being adopted in monitoring-for-acceptance of meat products (Surkiewicz et al., 1972; 1975; Baird-Parker, 1987), dairy products (Shapton, 1987), other manufactured food commodities (Todd et al., 1983; Schwab et al., 1985; Wentz et al., 1985; Warburton et al., 1987; 1988) and meals (Surkiewicz et al., 1979). Where the seven essentials presented above are faithfully followed, the previous endless debates about "standards" in general and acceptability of consignments in particular indicated at the beginning of this section vanish like magic!

#### Special case of reference values for mould propagules

Reasonable reproducibility of results between laboratories of manufacturers and users is generally achieved in reference ranges for bacteria in foods in daily practice. The intrinsic variability in colony counts must be borne in mind. Coefficients of variation are customarily of the order of 5% (Mossel et al., 1980). So with a colony count of the order of 200, the 99% confidence interval is 170-230. The situation is markedly

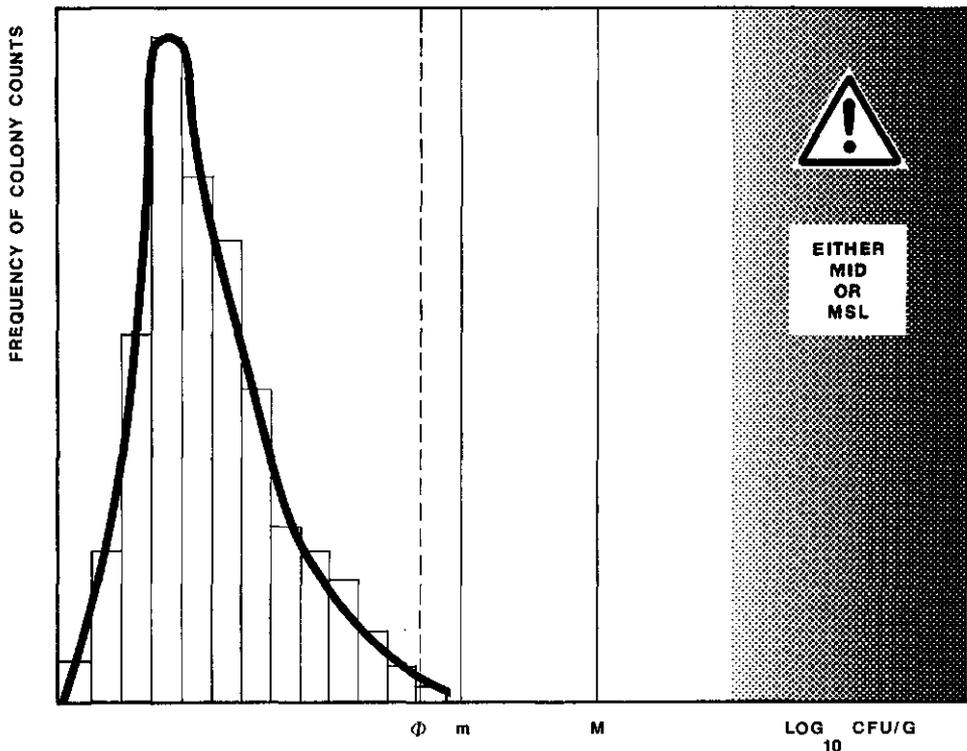


Fig. 6. Distribution plot of the results of microbiological surveys on a given type of food.  $\Phi$ -95th percentile, m-reference value proper, M-maximum count to be expected under conditions of good manufacturing practices, cfu-colony forming units, MID-minimum infectious dose, MSL-minimum spoilage level.

worse for reliability of reference values for moulds.

Many different types of fungi occur in dried foods (Beuchat, 1987; Samson & van Reenen-Hoekstra, 1988). These greatly vary in morphology and consequently the structural units that give rise to colonies on the customarily used spread drop (Mossel, Vega & Put, 1975) plates also show a substantial variation. These mould 'propagules' may consist of single spores, spore agglomerates, complete sporangia and mycelial fragments. The latter can be of diverse size, dependent on many other factors, including whether the mycelium is septate (Fig. 7).

The results of propagule counts thus clearly strongly vary with the following circumstances that can neither be too well controlled, nor predicted to any extent:

- the frequency distribution of types of mould propagules as they occur in the dried food, ready for sale;
- the extent of dispersion of spore agglomerates and mycelial fragments occurring as a result of (i) drawing and quadrantic reduction of samples; (ii) preparation of macerates and decimal dilutions for plating.

Estimation of mould propagules in foods is further marred by the use of a great variety of culture media and incubation conditions (King et al.,



1986). The use of conspicuously inadequate media such as the outdated agars of low pH (Mossel et al., 1962; Koburger, 1970; 1972) has long been abandoned. However, other media, though much better suited for counting of mould propagules, still give heavily discrepant results (Ladiges et al., 1974; Mossel et al., 1975; Zipkes et al., 1981; Banks & Board, 1987).

Manufacturers of dry speciality foods and their customers are, therefore, strongly advised to allow for a much higher variability in mould propagule counts between their respective laboratories, than the one they have come to accept for bacterial colony counts. Successful attempts have been made to limit such discrepancies to a minimum by strictly adhering to a given culture medium, a meticulously standardized inoculation procedure and carefully observed temperature and time of incubation. But even then a marked lack of reproducibility and even of repeatability may sometimes occur. Our experience is that this will nonetheless not damage working relationships and agreement between parties, provided these have been carefully briefed.

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#### References

- Alsina, M.; Lucena, F.; 1988. Evaluacion de medios para sulfitorreductores. *Alimentaria*, Madrid, 25(196)63-67.
- Angelotti, R.; 1971. Catered convenience foods - production and distribution problems and microbiological standards. *J. Milk Food Technol.* 34:227-231.
- Arnon, S.S.; Midura, T.F.; Damus, K.; Thompson, B.; Wood, R.M.; Chin, J.; 1979. Honey and other environmental risk factors for infant botulism. *J. Pediatr.* 94:331-336.
- Aureli, P.; Fenicia, L.; Pasolini, B.; Gianfranceschi, M.; McCroskey, L.M.; Hatheway, C.L.; 1986. Two cases of type E infant botulism caused by neurotoxicogenic *Clostridium butyricum* in Italy. *J. infect. Diseases* 154:207-211.
- Babudierei, S.; Borriello, S.P.; Pantosti, A.; Luzzi, I.; Testore G.P.; Panichi, G.; 1986. Diarrhoea associated with toxigenic *Clostridium* spiroforme. *J. Infection* 12:278-279.
- Badanry, A.S.; Gerba, C.P.; Kelley, L.M.; 1985. Survival of rotavirus SA-II on vegetables. *Food Microbiol.* 2:199-205.
- Baird-Parker, A.C.; 1987. Microbiological criteria for meat and meat products. *J. applied Bacteriol.* 63(6)v.
- Banks, J.G.; Board, R.G.; 1987. Some factors influencing the recovery of yeasts and mould from chilled foods. *Internat. J. Food Microbiol.* 4:197-206.
- Bannerman, E.S.; Bille, J.; 1988. A new selective medium for isolating *Listeria* species from heavily contaminated material. *Applied environm. Microbiol.* 54:165-167.
- Barnes, E.M.; Ingram, M.; 1956. The effect of redox potential on the growth of *Clostridium welchii* strains isolated from horse muscle. *J. applied Bacteriol.* 19:177-178.
- Bartholomew, B.A.; Berry, P.R.; Rodhouse, J.C.; Gilbert, R.J.; Murray,

- C.K.; 1987. Scombrotoxic fish poisoning in Britain - features of over 250 suspected incidents from 1976 to 1986. *Epidemiol. Infection* 99:775-782.
- Bauman, H.E.; 1974. The HACCP concept and microbiological hazard categories. *Food Technol.* 28(9)30-34; 74.
- Beji, A.; Mergaert, J.; Gavini, F.; Izard, D.; Kersters, K.; Leclerc, H.; De Ley, J.; 1988. Subjective synonymy of *Erwinia herbicola*, *Erwinia milletiae* and *Enterobacter agglomerans* and redefinition of the taxon by genotypic and phenotypic data. *Internat. J. system. Bacteriol.* 38:77-88.
- Beuchat, L.R., Editor; 1987. *Food and Beverage Mycology*. Van Nostrand Reinhold, New York.
- Blakey, L.J.; Priest, F.G.; 1980. The occurrence of *Bacillus cereus* in some dried foods including pulses and cereals. *J. appl. Bacteriol.* 48:297-302.
- Bockemühl, J.; Wohlers, B.; 1984. Zur Problematik der Kontamination unbehandelte Trockenprodukte der Lebensmittelindustrie mit Salmonellen. *Zentralbl. Bakt. Hyg. Abt. I, Orig., B* 178:535-541.
- Bouwer-Hertzberger, S.A.; Mossel, D.A.A.; Bijker, P.G.H.; 1982. Quantitative isolation and identification of *Staphylococcus aureus*. In: *Isolation and identification methods for food poisoning organisms*, J.E.L. Corry, D. Roberts, & F.A. Skinner (Editors). Academic Press, London. p. 165-172.
- Bouwer-Hertzberger, S.A.; Mossel, D.A.A.; 1982. Quantitative isolation and identification of *Bacillus cereus*. In: *Isolation and identification methods for food poisoning organisms*, J.E.L. Corry, D. Roberts, & F.A. Skinner (Editors). Academic Press, London. p. 255-259.
- Bray, D.F.; Lyon, D.A.; Burr, I.W.; 1973. Three-class attributes plans in acceptance sampling. *Technometrics* 15:575-585.
- Breer, C.; Schopfer, K.; 1988. *Listeria* and food. *Lancet* 1988(II)1022.
- Breuer, J.; Prändl, O.; 1988. Nachweis von Listerien und deren Vorkommen in Hackfleisch und Mettwürsten in Oesterreich. *Arch. Lebensm. Hyg.* 39:28-30.
- Briskey, E.J.; 1976. Impact of Government regulations on the food industry. *Food Technol.* 30(5)38-43.
- Bryan, F.L.; McKinley, T.W.; Mixon, B.; 1971. Use of time-temperature evaluations in detecting the responsible vehicle and contributing factors of foodborne disease outbreaks. *J. Milk Food Technol.* 34:576-582.
- Buchanan, R.L.; Stahl, H.G.; Bencivengo, M.M.; 1988. Recovery of *Listeria* from fresh retail-level foods of animal origin. *Abstr. Annu. Meet. Amer. Soc. Microbiol.* 281.
- Buyens, H.; 1971. Safeguards for commercially processed and packaged goods. *Quart. Bull. Assoc. Food & Drug Offic. U.S.* 35:276-284.
- Cato, E.P.; George, W.L.; Finegold, S.M.; 1986. Genus *Clostridium* Prazmowski 1880 In: *Bergey's Manual of Systematic Bacteriology*, Volume 2. Williams & Wilkins, Baltimore. p. 1141-1200.
- Christian, J.H.B.; Stewart, B.J.; 1973. Survival of *Staphylococcus aureus* and *Salmonella newport* in dried foods, as influenced by water activity and oxygen. In: *The microbiological safety of food*. B.C. Hoobs, & J.H.B. Christian (Editors). Academic Press, London. p. 107-118.
- Chung, K.C.; Goepfert, J.M.; 1970. Growth of *Salmonella* at low pH. *J. Food Sci.* 35:326-328.
- Clark, W.S.; Reinbold, G.W.; Rambo, R.S.; 1966. Enterococci and coliforms in dehydrated vegetables. *Food Technol.* 20:1353-1356.
- Collins, R.N.; Treger, M.D.; Goldsby, J.B.; Boring, J.R.; Coohon, D.B.; Berr, R.N.; 1968. Interstate outbreak of *Salmonella newbrunswick*

- infection traced to powdered milk. *J. Amer. Med. Assoc.* 203:838-844.
- Cottin, J.; Genthon, H.; Bizum, C.; Carbonelle, B.; 1985. Recherche de *Listeria monocytogenes* dans des viandes prélevées sur 514 bovins. *Sci. Aliments* 5(hors série IV)145-149.
- Cranston, P.M.; 1971. Some practical approaches to microbiological standards. *Food Technol. Australia* 23:340-342.
- Craven, J.A.; 1978. Salmonella contamination of dried milk products. *Victoria Veter. Proceed.* 9:56-57.
- Cunningham, F.E.; 1982. Practical applications of food dehydration: A review. *J. Food Protection* 45:479-483; 491.
- Dezfulian, M.; McCroskey, L.M.; Hatheway, C.L.; Dowell, V.R.; 1981. Selective medium for isolation of *Clostridium botulinum* from human feces. *J. clin. Microbiol.* 13:526-531.
- Doyle, M.P.; Schoeni, J.L.; 1986. Isolation of *Campylobacter jejuni* from retail mushrooms. *Applied environm. Microbiol.* 51:449-450.
- Ewing, W.H.; 1986. Identification of Enterobacteriaceae. Elsevier, Amsterdam.
- Ferreira, M.A.S.S.; Lund, B.M.; 1987. The influence of pH and temperature on initiation of growth of *Salmonella* spp. *Letters applied Microbiol.* 5:67-70.
- Fleming, D.W.; Cochi, S.L.; MacDonald, K.L. et al.; 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *New Engl. J. Med.* 312:404-407.
- Fontaine, O.; Ducluzeau, R.; Raibaud, P.; Chabanet, C.; Popoff, M.R.; Badonal, J.; Gabilan, J.C.; Andremont, A.; 1986. Comparaison entre le nombre et al nature des *Clostridium fécaux* et d'autres facteurs de risque impliqués dans la pathologie intestinale des nouveau-nés. *Annls. Inst. Pasteur Microbiol.* 137 B:61-75.
- Garcia-Villanova Ruiz, B.; Vargas, R.G.; Garcia-Villanova, R.; 1987. Contamination on fresh vegetables during cultivation and marketing. *Internat. J. Food Microbiol.* 4:285-291.
- Garret, E.S.; 1988. Microbiological standards, guidelines and specifications and inspection of seafood products. *Food Technol.* 42(3)90-93; 103.
- Geldreich, E.E.; Bordner, R.H.; 1971. Fecal contamination of fruits and vegetables during cultivation and processing for market. A review. *J. Milk Food Technol.* 34:184-195.
- Gitter, M.; 1976. *Listeria monocytogenes* in "oven-ready" poultry. *Vet. Record* 99:336.
- Glasby, C.; Hatheway, C.L.; 1985. Isolation and enumeration of *Clostridium botulinum* by direct inoculation of infant fecal specimens on egg yolk agar and *Clostridium botulinum* isolation media. *J. clin. Microbiol.* 21:264-266.
- Goldblith, S.; Rey, L.; Rothmayr, W.; 1975. Freeze-drying and advanced food technology. Academic Press, New York.
- Goldenberg, N.; Sheppey, C.G.; Robson, J.N.; 1955. Some tentative bacteriological standards for canned hams. *Annls. Inst. Pasteur Lille* 7:240-248.
- Gräsbeck, R.; 1976. "Normal" and "reference" values for laboratory data. *Lancet* I:244-245.
- Greenwood, M.H.; Coetzee, E.F.C.; Ford, B.M.; Gill, P.; Hooper, W.L.; Matthews, S.C.W.; Patrick, S.; Pether, J.V.S.; Scott, R.J.D.; 1984. The microbiology of selected retail food products with an evaluation of viable counting methods. *J. Hygiene* 92:67-77.
- Habraken, C.J.M.; Mossel, D.A.A.; van den Reek, S.; 1986. Management of *Salmonella* risks in the production of powdered milk products. *Netherl. Milk & Dairy J.* 40:99-116.

- Harrewijn, G.A.; Mossel, D.A.A.; Groote, J. de; 1972. Assessment of the bacteriological safety of lactose. *Netherl. Milk & Dairy J.* 26:141-148.
- Hird, D.W.; 1987. Review of evidence for zoonotic listeriosis. *J. Food Protection* 50:429-433.
- Hirsch, A.; Grinsted, E.; 1954. Methods for the growth and enumeration of anaerobic sporeformers from cheese, with observations on the effect of nisin. *J. Dairy Res.* 21:101-110.
- Ho, J.L.; Shands, K.N.; Friedland, G. et al.; 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. *Archs intern. Med.* 146:520-524.
- Howard, F.M.; Flynn, D.; Bradley, J.M.; Noone, P.; Szawatkowski, M.; 1977. Outbreak of necrotizing enterocolitis caused by *Clostridium butyricum*. *Lancet* 1977(II)1099-1102.
- IFCC (International Federation of Clinical Chemistry); 1987. Approved recommendation (1986) on the theory of reference values. Pt. 1. The concept of reference values. H.E. Solberg. *Clinica Chimica Acta* 165:111-118.
- Ingram, M.; 1961. Microbiological standards for foods. *Food Technol.* 15(2)4-16.
- Ingram, M.; 1971. Microbiological standards for foods. *Food Inds. South Africa*, 24(2)8-13.
- Kautter, D.A.; Lilly, T.; Lynt, R.; 1978. Evaluation of the botulism hazard in fresh mushrooms wrapped in commercial polyvinyl chloride film. *J. Food Protection* 41:120-121.
- Kautter, D.A.; Lilly, T.; Solomon, H.M.; Lynt, R.K.; 1982. *Clostridium botulinum* spores in infant foods: a survey. *J. Food Protection* 45:1028-1029.
- Keoseyan, S.A.; 1971. Incidence of *Clostridium perfringens* in dehydrated soup, gravy and spaghetti mixes. *J. Assoc. Offic. Anal. Chem.* 54:106-108.
- King, A.D., Pitt, J.I., Beuchat, L.R. & Corry, J.E.L. (Editors); 1986. *Methods for the mycological examination of food*. Plenum, New York.
- Koburger, J.A.; 1970. Fungi in foods. I. Effect of inhibitor and incubation temperature on enumeration. *J. Milk & Food Technol.* 33:433-434.
- Koburger, J.A.; 1972. Fungi in foods. IV. Effect of plating medium pH on counts. *J. Milk & Food Technol.* 35:659-660.
- Kramer, J.M.; Turnbull, P.C.B.; Munshi, G.; Gilbert, R.J.; 1982. Identification and characterization of *Bacillus cereus* and other *Bacillus* species associated with foods and food poisoning. In: Isolation and identification methods for food poisoning organisms, J.E.L. Corry, D. Roberts, F.A. Skinner (Editors). Academic Press, London. p. 261-286.
- Ladiges, W.C.; Foster, J.F.; Jorgensen, J.J.; 1974. Comparison of media for enumerating fungi in precooked frozen convenience foods. *J. Milk & Food Technol.* 37:302-304.
- Lancette, G.A.; Harmon, S.M.; 1980. Enumeration and confirmation of *Bacillus cereus* in foods: collaborative study. *J. Assoc. Offic. Anal. Chem.* 63:581-586.
- Leclerc, H.; Mossel, D.A.A.; Savage, C.; 1985. Monitoring non-carbonated ("still") mineral waters for aerobic colonization. *Internat. J. Food Microbiol.* 2:341-347.
- Le Guillou, M.; 1980. *Listeria monocytogenes*. Sa fréquence dans les produits de charcuterie. *Bull. Soc. Vét. Prat. de France* 64:45-53.
- Leininger, H.V.; Shelton, L.R.; Lewis, K.H.; 1971. Microbiology of frozen cream-type pies, frozen cooked-peeled shrimp and dry food-grade gelatin. *Food Technol.* 25:224-226; 229.
- Leniger, H.A.; Bruin, S.; 1977. The state of the art of food dehydration. In: *Food Quality and Nutrition*, W.K. Downey (Editor). Appl.

- Science Publishers, London. p. 265-295.
- Levine, M.; 1961. Facts and fancies of bacterial indices in standards for water and foods. *Food Technol.* 15(11):29-38.
- Li Cari, J.J.; Potter, N.N.; 1970. Salmonella survival during spray-drying and subsequent storage of skim-milk powder. 3. Effects of storage temperature on Salmonella and dried milk properties. *J. Dairy Sci.* 53:877.
- Linnan, M.J.; Mascola, L.; Lou X.D. et al.; 1988. Epidemic listeriosis associated with Mexican-style cheese. *New Engl. J. Med.* 319:823-828.
- Lovett, J.; Francis, D.W.; Hunt, J.M.; 1987. *Listeria monocytogenes* in raw milk: Detection, incidence and pathogenicity. *J. Food Protection* 50:188-192.
- Ludlam, G.B.; 1949. A selective medium for the isolation of *Staphylococcus aureus* from heavily contaminated material. *Monthly Bull. Min. Health & Publ. Health Lab. Serv. London* 8:15-20.
- McBride, M.E.; Girard, K.F.; 1960. A selective method for the isolation of *Listeria monocytogenes* from mixed bacterial populations. *J. Lab. Clin. Med.* 55:153-157.
- McCroskey, L.M.; Hatheway, C.L.; Fenicia, L.; Pasolini, B.; Aureli, P.; 1986. Characterization of an organism that produces type E botulinum toxin but which resembles *Clostridium butyricum* from the feces of an infant with type E botulism. *J. clin. Microbiol.* 23:201-202.
- MacDonald, K.L.; Spengler, R.F.; Hatheway, C.L. et al.; 1985. Type A botulism from sauteed onions: Clinical and epidemiologic observations. *J. Amer. Med. Assoc.* 253:1275-1278.
- Mellor, J.D.; 1978. *Fundamentals of freeze drying*. Academic Press, London.
- Mills, D.C.; Midura, T.F.; Arnon, S.S.; 1985. Improved selective medium for the isolation of lipase-positive *Clostridium botulinum* from feces of human infants. *J. clin. Microbiol.* 21:947-950.
- Mossel, D.A.A.; 1959. Enumeration of sulphite-reducing clostridia occurring in foods. *J. Sci. Food and Agricult.* 10:662-669.
- Mossel, D.A.A.; Zwart, H., 1960. The rapid tentative recognition of psychrotrophic types among *Enterobacteriaceae* isolated from foods. *J. applied Bacteriol.* 23:185-188.
- Mossel, D.A.A.; 1962. Formation d'H<sub>2</sub>S à partir de substrats divers par quelques groupes de bactéries fréquemment rencontrés dans les matières alimentaires périssables. *Annals. Inst. Pasteur* 103:108-111.
- Mossel, D.A.A.; Visser, M.; Mengerink, W.H.J.; 1962. A comparison of media for the enumeration of moulds and yeasts in foods and beverages. *Lab. Practice* 11:109-112.
- Mossel, D.A.A.; Visser, M.; Cornelissen, A.M.R.; 1963. The examination of foods for *Enterobacteriaceae* using a test of the type generally adopted for the detection of salmonellae. *J. applied Bacteriol.* 26:444-452.
- Mossel, D.A.A.; Koopman, M.J.; 1965. Losses in viable cells of salmonellae upon inoculation into dry animal feeds of various types. *Poultry Science* 44:890-892.
- Mossel, D.A.A.; Koopman, M.J.; Jongerius, E.; 1967. Enumeration of *Bacillus cereus* in foods. *Applied Microbiol.* 15:650-653.
- Mossel, D.A.A.; Waart, J. de; 1968. The enumeration of *Clostridia* in foods and feeds. *Annls. Inst. Pasteur, Lille* 19:13-27.
- Mossel, D.A.A.; Ratto, M.A.; 1970. Rapid detection of sublethally impaired cells of *Enterobacteriaceae* in dried foods. *Appl. Microbiol.* 20:273-275.
- Mossel, D.A.A.; Pouw, H.; 1973. Studies on the suitability of sulphite cycloserine agar for the enumeration of *Clostridium perfringens* in food

- and water. Zentralbl. Bakteriologie. Parasitenkunde. Abt. I, Original, A 223:559-561.
- Mossel, D.A.A.; Harrewijn, G.A.; van Sprang, F.J.; 1973. Microbiological quality assurance for weaning formulae. In: The microbiological safety of food. B.C. Hobbs, & J.H.B. Christian (Editors). Academic Press, London. p. 77-87.
- Mossel, D.A.A.; Harrewijn, G.A.; Nesselrooij-van Zadelhoff, C.F.M.; 1974. Standardization of the selective inhibitory effect of surface active compounds used in media for the detection of Enterobacteriaceae in foods and water. Health Labor. Sci. 11:260-267.
- Mossel, D.A.A.; Vega, C.L.; Put, H.M.C.; 1975. Further studies on the suitability of various media containing antibacterial antibiotics for the enumeration of moulds in food and food environments. J. applied Bacteriol. 39:15-22.
- Mossel, D.A.A.; Shennan, J.L.; 1976. Micro-organisms in dried foods: their significance, limitation and enumeration. J. Food Technol. 11:205-219.
- Mossel, D.A.A.; Eelderink, I.; Sutherland, J.P.; 1977. Development and use of single, "polytropic" diagnostic tubes for the approximate taxonomic grouping of bacteria isolated from foods, water and medicinal preparations. Zentralbl. Bakt. Hyg. I, Original, A 238:66-79.
- Mossel, D.A.A.; Drion, E.F.; 1979. Risk analysis as applied to the protection of the consumer against food-transmitted diseases of microbial aetiology. In: Food Microbiology and Technology, B. Jarvis, J.H.B. Christian, & H.D. Michener (Editors). Medicina Viva, Parma. p. 417-430.
- Mossel, D.A.A.; 1980. Assessment and control of microbiological health risks presented by foods. Reference Values ("standards"). In: Food and Health, G.G. Birch & K.J. Parker (Editors). Applied Science Publishers, London. p. 146-150.
- Mossel, D.A.A.; Veldman, A.; Eelderink, I.; 1980. Comparison of the effects of liquid medium repair and the incorporation of catalase in McConkey type media on the recovery of Enterobacteriaceae sublethally stressed by freezing. J. applied Bacteriol. 49:405-419.
- Mossel, D.A.A.; 1982. Marker (index and indicator) organisms in food and drinking water. Semantics, ecology, taxonomy and enumeration. Antonie van Leeuwenhoek 48:609-611.
- Mossel, D.A.A.; 1983a. Essentials and perspectives of the microbial ecology of foods. In: Food Microbiology: advances and prospects. T.A. Roberts & F.A. Skinner (Editors). Academic Press, London. p. 1-45.
- Mossel, D.A.A.; 1983b. Seventy-five years of longitudinally integrated microbiological safety assurance in the dairy industry in The Netherlands. Netherl. Milk Dairy J. 37:240-245.
- Mossel, D.A.A.; Bonants-van Laarhoven, T.M.G.; Ligtenberg-Merkus, A.M.Th.; Werdler, M.E.B.; 1983. Quality assurance of selective culture media for bacteria, moulds and yeasts: An attempt at standardisation at the international level. J. applied Bacteriology 54:313-327.
- Mossel, D.A.A.; Dijkman, K.E.; 1984. A Centenary of academic and less learned food microbiology. Pitfalls of the past and promises for the future. Antonie van Leeuwenhoek 50:644-663.
- Mossel, D.A.A.; van Netten, P.; 1984. Harmful effects of selective media on stressed micro-organisms: Nature and remedies. In: The revival of injured microbes. M.H.E. Andrew & A.D. Russell (Editors). Academic Press, London. p. 329-369.
- Mossel, D.A.A.; van der Zee, H.; Hardon, A.P.; van Netten, P.; 1986. The enumeration of thermotrophic types amongst the Enterobacteriaceae colonizing perishable foods. J. applied Bacteriol. 60:289-295.
- Mossel, D.A.A.; 1987a. An approach to the evaluation of the risk of

- transmission of *Listeria monocytogenes* by foods and its management. *Vet. Med. Hefte (Berlin)* 5:146-170.
- Mossel, D.A.A.; 1987b. The microbiological examination of foods and drinking water in the framework of health protection. In: *Application of Biotechnology to the Rapid Diagnosis of Infectious Diseases*. A.J. Balows, (Editor). Royal Soc. Med., London. Congress & Symp. Series No. 113, p. 45-73.
- Mossel, D.A.A.; van Netten, P.; Perales, I.; 1987. Human listeriosis transmitted by food in a general medical-microbiological perspective. *J. Food Protection* 50:894-895.
- Mossel, D.A.A.; 1988. Impact of foodborne pathogens on today's world and prospects for management. *Animal & Human Health* 1:13-23.
- Mossel, D.A.A.; 1989. *Listeria* in foods. Its detection, enumeration and control. *Internat. J. Food Microbiol.* 8, in press.
- Mujumdar, A.S., Editor; 1987. *Handbook of Industrial Drying*. Marcel Dekker, New York.
- Mundt, J.O.; 1986. Enterococci. In: *Bergey's Manual of Systematic Bacteriology*, Volume 2, Williams & Wilkins, Baltimore. p. 1064.
- Murray, J.G.; 1969. An approach to bacteriological standards. *J. appl. Bacteriol.* 32:123-135.
- Nakamura, S.; Tanabe, N.; Yamakawa, K.; Nishida, S.; 1983. Cytotoxin production by *Clostridium sordellii* strains. *Microbiol. Immunol.* 27:49-5-502.
- Nicolas, J.A.; Vidaud, N.; 1987. Contribution à l'étude des *Listeria* présentes dans les denrées d'origine animale destinées à la consommation humaine. *Rec. méd. vét.* 163:283-285.
- Pace, P.J.; 1975. Bacteriological quality of delicatessen foods: Are standards needed? *J. Milk Food Technol.* 38:347-353.
- Perales, I.; van Netten, P.; Garcia, M.I.; Audicana, A.; Mossel, D.A.A.; 1988. A new medium for the enrichment of *Listeria* spp. from highly contaminated samples (Spanish with English summary). *Revista espan. Microbiol. Clin.* 3:561-567.
- Pierson, C.J.; Emswiler, B.S.; Kotula, A.W.; 1978. Comparison of methods for estimation of coliforms, fecal coliforms and enterococci in retail ground beef. *J. Food Protection* 41:263-266.
- Pini, P.N.; Gilbert, R.J.; 1988. The occurrence in the U.K. of *Listeria* species in raw chickens and soft cheeses. *Internat. J. Food Microbiol.* 6:317-326.
- Popoff, M.R.; Ravisse, P.; 1985. Lesions produced by *Clostridium butyricum* strain CB 1002 in ligated intestinal loops in guinea pigs. *J. med. Microbiol.* 19:351-357.
- Prévot, A.R.; 1948. Recherches sur la réduction des sulfates et des sulfites minéraux par les bactéries anaérobies. *Annls. Inst. Pasteur* 75:571-575.
- Ralovich, B.; Forray, A.; Menő, E.; Malovics, H.; Szazados, J.; 1971. New selective medium for isolation of *L. monocytogenes*. *Zentralbl. Bakt. Hyg. Abt. I, Orig.* 216:88-91.
- Ray, B.; Jezeski, J.J.; Busta, F.F.; 1971. Isolation of salmonellae from naturally contaminated dried milk products. II. Influence of storage time on the isolation of salmonellae. *J. Milk Food Technol.* 34:423-427.
- Rifkind, B.M.; Segal, P.; 1983. Lipid research clinics program reference values for hyperlipidemia and hypolipidemia. *J. Amer. Med. Assoc.* 250:1869-1872.
- Roberts, T.A.; Bryan, F.L.; Christian, J.H.B. et al. (Editors); 1986. *Micro-organisms in Foods. 2. Sampling for Microbiological Analysis-Principles and Specific Applications*. Blackwell, Oxford.
- Rockland, L.B.; Beuchat, L.R. (Editors); 1987. *Water activity. Theory and*

- applications to food. Marcel Dekker, New York.
- Rodríguez, L.D.; Fernandez, G.S.; Fernandez-Garayzabal, J.F. et al.; 1984. New methodology for the isolation of *Listeria* micro-organisms from heavily contaminated environments. *Applied Environm. Microbiol.* 47:1188-1190.
- Rowe, B.; Begg, N.T.; Hutchinson, D.N.; Dawkins, H.C.; Gilbert, R.J.; Jacob, M.; Hales, B.H.; Rae, F.A.; Jepson, M.; 1987. Salmonella ealing infections associated with consumption of infant dried milk. *Lancet* 1987(II)900-903.
- Rude, R.A.; Jackson, G.J.; Bier, J.W.; Sawyer, T.K.; Risty, N.G.; 1984. Survey of fresh vegetables for nematodes, amoebae and *Salmonella*. *J. Assoc. Offic. Anal. Chem.* 67:613-615.
- Samson, R.A.; van Reenen-Hoekstra, E.S.; 1988. Introduction to food-borne fungi. Centraal Bureau voor Schimmelcultures, Royal Netherlands Academy of Arts & Sciences, Baarn & Delft.
- Schlech, W.F.; Lavogne, P.M.; Bortolussi, R.A.; Allan, A.C.; Haldane, E.V.; Wort, A.J.; Hightoner, A.W.; Johnson, S.E.; King, S.H.; Nicholls, E.S.; Broome, C.V.; 1983. Epidemic listeriosis - evidence for transmission by food. *New England J. Med.* 308:203-206.
- Schlech, W.F.; 1986. Listeriosis. New pieces to an old puzzle. *Archs. Intern. Med.* 146:459-460.
- Schlech, W.F.; 1988. Virulence characteristics of *Listeria monocytogens*. *Food Technol.* 42(4)176-178.
- Schmidt-Lorenz, W.; Jaeggi, N.; 1983. Colony counts at 42°C for the evaluation of the hygienic quality of bottled natural uncarbonated mineral water. *Microbiol. Aliments & Nutrition* 1:377-391.
- Schwab, A.H.; Wentz, B.A.; Jagow, J.A.; Swartzentruber, A.; Duran, A.P.; Lanier, J.M.; Barnard, R.J.; Read, R.B.; 1985. Microbiological quality of cream-type pies during processing. *J. Food Protection* 48:70-75.
- Schwartz, B.; Ciesielski, C.A.; Broome, C.V. et al.; 1988. Association of sporadic listeriosis with consumption of uncooked hot dogs and undercooked chicken. *Lancet* 1988(II)779-782.
- Scully, R.E.; McNeely, B.U.; Galdabini, J.J.; 1978. Normal reference laboratory values. *New England J. Med.* 298: 34-45.
- Seals, J.E.; Snyder, J.D.; Edell, T.A.; Hatheway, C.L.; Johnson, C.J.; Swanson, R.C.; Hughes, J.M.; 1981. Restaurant-associated type A botulism: Transmission by potato salad. *Amer. J. Epidemiol.* 113:436-444.
- Seeliger, H.P.R.; Jones, D.; 1986. Genus *Listeria* Pirie 1940, 383<sup>AL</sup>. In: *Bergey's Manual of Systematic Bacteriology*, Volume 2. Williams & Wilkins, Baltimore. p. 1241.
- Shapton, N.F.; 1987. Experiences with the establishment and application of microbiological specifications and criteria for dairy products. *J. applied Bacteriol.* 63(6)v-vi.
- Shapton, N.; 1988. Hazard analysis applied to control of pathogens in the dairy industry. *J. Soc. Dairy Technol.* 41:62-63.
- Sheneman, J.; 1973. Survey of aerobic mesophilic bacteria in dehydrated onion products. *J. Food Sci.* 38:206-209.
- Silas, J.C.; Carpenter, J.A.; Handy, M.K.; Harrison, M.A.; 1985. Selective and differential medium for detecting *Clostridium botulinum*. *Applied Environm. Microbiol.* 50:1110-1111.
- Silliker, J.H.; Gabis, D.A.; May, A.; 1979. ICMSF methods studies. XI. Collaborative comparative studies on determination of coliforms using the most probable number procedure. *J. Food Protection* 42:638-644.
- Silliker, J.H.; Baird-Parker, A.C.; Bryan, F.L. et al. (Editors); 1988. *Micro-organisms in Foods - 4. Application of the Hazard Analysis Critical Control Point (HACCP) System to ensure microbiological Safety and Quality*. Blackwell, Oxford.

- Silver, H.J.; 1984. Laboratory reference ranges for elderly persons. *J. Amer. Med. Assoc.* 252:826.
- Sizmur, K.; Walker, C.W.; 1988. *Listeria* in prepacked salads. *Lancet I*, 1167.
- Skovgaard, N.; Morgen, C.A.; 1988. Detection of *Listeria* spp. in faeces from animals, in feeds and in raw foods of animal origin. *Internat. J. Food Microbiol.* 6:229-242.
- Smelt, J.P.P.M.; Raatjes, G.J.M.; Crowther, J.S.; Verrips, C.T.; 1982. Growth and toxin formation by *Clostridium botulinum* at low pH values. *J. applied Bacteriol.* 52:75-82.
- Smith, N.R.; Clark, F.E.; 1938. Motile colonies of *Bacillus alvei* and other bacteria. *J. Bacteriol.* 35:59-60.
- Speck, M.L.; Ray, B.; Read, R.B.; 1975. Repair and enumeration of injured coliforms by a plating procedure. *Applied Microbiol.* 29:549-550.
- Splittstoesser, D.F.; Wilkinson, M.; 1981. Effect of a repair step on the recovery of coliforms from frozen blanched vegetables. *J. Food Qual.* 4:107-111.
- Steinbruegge, E.G.; Maxcy, R.B.; Liewen, M.B.; 1988. Fate of *Listeria monocytogenes* on ready to serve lettuce. *J. Food Protection* 51:596-599.
- Stephens, R.L.; 1970. Bacteriological standards for foods: A retailer's point of view. *Chem. & Ind.* 7:220-222.
- Sturm, R.; Staneck, J.L.; Stauffer, L.R.; Neblett, W.W.; 1980. Neonatal necrotizing enterocolitis associated with penicillin resistant, toxigenic *Clostridium butyricum*. *Pediatrics* 66:928-931.
- Suen, J.C.; Hatheway, C.L.; Steigerwalt, A.G.; Brenner, D.J.; 1988. Genetic confirmation of identities of neurotoxicogenic *Clostridium baratii* and *Clostridium butyricum* implicated as agents of infant botulism. *J. clin. Microbiol.* 26:2191-2192.
- Sullivan, S.N.; Darwish, R.J.; Schieven, B.S.; 1980. Severe diarrhea due to *Clostridium sphenoides*: A case report. *Canad. Med. Assoc. J.* 123:298.
- Surkiewicz, B.F.; Johnston, R.W.; Elliott, R.P.; Simmons E.R.; 1972. Bacteriological survey of fresh pork sausage produced at establishments under Federal Inspection. *Applied Microbiol.* 23:515-520.
- Surkiewicz, B.F.; Harris, M.E.; Elliott, R.P. et al.; 1975. Bacteriological survey of raw beef patties produced at establishments under Federal Inspection. *Applied Microbiol.* 29:331-334.
- Surkiewicz, B.F.; Campbell, D.F.; Harris, M.E.; 1979. Bacteriological survey of frozen Mexican-style foods produced at establishments under Federal Inspection. *J. Food Protection* 42:46-48.
- Sveum, W.H.; Kraft, A.A.; 1981. Recovery of salmonellae from foods using a combined enrichment technique. *J. Food Sci.* 46:94-99.
- Tamminga, S.K.; Beumer, R.R.; Kampelmacher, E.H.; 1978. The hygienic quality of vegetables grown in or imported into the Netherlands: a tentative survey. *J. Hygiene* 80:143-154.
- Tanaka, N.; 1982. Toxin production by *Clostridium botulinum* in media at pH lower than 4.6. *J. Food Protection* 45:234-237.
- Thaler, M.; Gill, V.; Pizzo, P.A.; 1986. Emergence of *Clostridium tertium* as a pathogen in neutropenic patients. *Amer. J. Med.* 81:596:600.
- Todd, E.C.D.; Jarvis, G.A.; Weiss, K.F. et al.; 1983. Microbiological quality of frozen cream-type pies sold in Canada. *J. Food Protection* 46:34-40.
- Truscott, R.B.; McNab, W.B.; 1988. Comparison of media and procedures for the isolation of *Listeria monocytogenes* from ground beef. *J. Food Protection* 51:626-628; 638.
- Urbach, H.; Schabinski, G.; 1955. Zur Listeriose des Menschen. *Z. Hyg. Infektionskr.* 141:239-248.

- Uzelac, G.; Stille, B.; 1977. Die Ueberlebensfähigkeit von Bakterien fäkalen Ursprungs in Trockenprodukten in Abhängigkeit von der Wasseraktivität. 2. Mitteilung. Eigene Versuche mit *Escherichia coli* und *Streptococcus faecalis*. Deut. Lebensm. Rundschau 73:325-329.
- van Arsdal, W.B.; 1963. Food dehydration. Volume 1. Principles. AVI, Westport, Conn.
- van Arsdal, W.B.; Copley, M.J.; 1964. Food dehydration. Vol. II -Products and technology. AVI, Westport, Conn.
- van Doorne, H.; Claushuis, E.P.M.; 1979. The quantitative determination of Enterobacteriaceae in pharmaceutical preparations. Internat. J. Pharmaceutics 4:119-25.
- van Doorne, H.; Baird, R.M.; Hendriksz, D.T. et al.; 1981. Liquid modification of Baird-Parker's medium for the selective enrichment of *Staphylococcus aureus*. Antonie van Leeuwenhoek 47:267-268.
- van Doorne, H.; Pauwels, H.P.; Mossel, D.A.A.; 1982. Selective isolation and enumeration of low numbers of *Staphylococcus aureus* by a procedure relying on elevated-temperature culturing. Applied environm. Microbiol. 44:1459-1462.
- van Netten, P.; van der Zee, H.; Mossel, D.A.A.; 1984. A note on catalase enhanced recovery of acid injured cells of Gram-negative bacteria and its consequences for the assessment of the lethality of 1-lactic acid decontamination of raw meat surfaces. J. applied Bacteriol. 57:169-173.
- van Netten, P.; Mossel, D.A.A.; van de Moosdijk, A.; 1987. Rapid detection of excessive cfu counts of Enterobacteriaceae, *S. aureus* and yeasts by nitrate reduction/glucose dissimilation (NGD) monitoring in selective media. J. Food Sci. 52:751-756.
- van Netten, P.; van de Ven, A.; Perales, I.; Mossel, D.A.A.; 1988a. A selective and diagnostic medium for use in the enumeration of *Listeria* sp. in foods. Internat. J. Food Microbiol. 6:187-198.
- van Netten, P.; Perales, I.; Mossel, D.A.A.; 1988b. An improved selective and diagnostic medium for isolation and counting of *Listeria* spp. in heavily contaminated foods. Letters appl. Microbiol. 7:17-21.
- van Netten, P.; Perales, I.; van de Moosdijk, A.; Curtis, G.D.W.; Mossel, D.A.A.; 1989. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* in more or less severely contaminated foods: An attempt to reach consensus at the international level. Internat. J. Food Microbiol. 8, in press.
- van Schothorst, M.; Renaud, A.; van Beek, C.; 1987. Salmonella isolation using RVS broth and MLCB agar. Food Microbiol. 4:11-18.
- Verdonck, L.; Mergaert, J.; Ryckert, C.; Swings, J.; Kersters, K.; Ley, J. de; 1987. Genus *Erwinia*: Numerical analysis of phenotypic features. Internat. J. system. Bacteriol. 37:4-18.
- Visser, I.J.R.; Jaisli, F.; Mossel, D.A.A.; 1985. The effect of dried preparations of sulphide iron motility (SIM) agar on the results of motility readings. J. applied Bacteriol. 59:167-170.
- Warburton, D.W.; Weiss, K.F.; Purvis, U.; Hill, R.W.; 1987. The microbiological quality of fermented sausage produced under good hygienic practices in Canada. Food Microbiol. 4:187-197.
- Warburton, D.W.; Weiss, K.F.; Lachapelle, G.; Dragon, D.; 1988. The microbiological quality of further processed deboned poultry products sold in Canada. Canad. Inst. Food Sci. Technol. J. 21:84-89.
- Weagant, S.D.; Sado, P.N.; Colburn, K.G. et al.; 1988. The incidence of *Listeria* species in frozen seafood products. J. Food Protection 51:655-657.
- Welshimer, H.J.; Donker-Voet, J.; 1971. *Listeria monocytogenes* in nature. Applied Microbiol. 21:516-519.
- Wentz, B.A.; Duran, A.P.; Swartzentruber, A.; Schwab, A.H.; McClure,

- F.D.; Archer, D.; Read, R.B.; 1985. Microbiological quality of crabmeat during processing. *J. Food Protection* 48:44-49.
- Wilson, G.S.; 1955. Symposium on food microbiology and public health: general conclusion. *J. appl. Bacteriol.* 18:629-630.
- Wright, C.; Kominos, S.D.; Yee, R.B.; 1976. Enterobacteriaceae and *Pseudomonas aeruginosa* recovered from vegetable salads. *Applied environm. Microbiol.* 31:453-454.
- Young-Perkins, K.E.; Merson, R.L.; 1987. *Clostridium botulinum* spore germination, outgrowth and toxin production below pH 4.6; interactions between pH, total acidity and buffering capacity. *J. Food Sci.* 52:1084-1088; 1096.
- Zipkes, M.R.; Gilchrist, J.E.; Peeler, J.T.; 1981. Comparison of yeast and mould counts by spiral, pour and streak plate methods. *J. Assoc. Offic. Anal. Chem.* 64:1465-1469.

## FOOD ENZYMES: PROSPECTS AND LIMITATIONS

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### Summary

Current applications of enzymes in the main areas of the food industry and new possibilities for their use are discussed in the light of recent developments in basic sciences. Developments in recombinant DNA technology enabling the increased production of specific enzymes and the improvement of enzyme properties, novel purification techniques in the downstream processing of crude enzyme fermentation media and new reactor types for more efficient use of enzymes are briefly dealt with.

Detailed knowledge on the action of enzymes on a molecular level in food systems makes it possible to identify technological relevant enzymes, to improve the composition of enzyme preparations and to acquire better control of enzyme based processes. This is illustrated with recent results of our investigations on plant cell wall modifying enzymes. Descriptors: food enzymes, production, recombinant DNA technology, downstream processing, dairy, starch and sugar, brewing, baking, fruits and vegetables, food ingredients, plant cell wall modifying enzymes, rhamnogalacturonase.

### Introduction

Since ancient times our ancestors have been using enzymes for the preparation of their food without realizing it. The industrial application of enzymes in modern food processing has developed from these traditional sources. Preparation of foods by fermentation-basically enzymic processes - is older than recorded history. Homerus mentioned already the use of the latex of fig trees for the curdling of milk. Extracts of calve stomachs and of papaya were also used for this purpose. Other examples are the tendering of meat by papaya extract, by gastric juices or by just storing meat for a while. Sprouted grains were used for the production of alcoholic beverages. In 1833 Payen and Persoz succeeded to convert insoluble starch to soluble sugars with a cellfree extract of sprouted barley (malt). They established that this extract, which they called diastase, was a heat labile biocatalyst. In 1878 Kühne introduced the term enzyme for such catalysts. The first company for the commercial exploitation of enzymes was founded in 1907 by Röhm and Haas who started to sell proteases extracted from animal pancreas for the tanning of hides. Their preparation was much more attractive than the use of excrements of pigs and dogs which was the common practice in those days (Antibi and Fischlock, 1987).

Through the developments in successively microbiology, physiology and biochemistry, food technologist became more and more aware of the role of enzymes in food production. Enzymes were also recognized as natural constituents of the raw materials of our foods, having many effects on food processing and food quality. The effects of these endogenous enzymes can be undesirable (a.o. browning of fruits and vegetables by polyphenol oxidases, rancidity in milk-, meat-, fish-, cereal- and whey products by

lipases and lipoxygenases, softening of pickles by pectic enzymes and cloud loss of citrus juices by pectin esterases) and must than be prevented or they can be desirable and be used to advantage (a.o. colour and flavour development in tea, dates and cocoa by polyphenol oxidases, aroma development in Roquefort and Camembert cheese by lipases, aroma development in apple juice by lipoxygenases, aroma development in onions and garlic by alliinases, self-clarification of juices by pectin esterases, production of fermentable sugars from starch by malt-amylases). If the latter is the case enzymes can even be supplemented from other sources (Reed, 1975; Schwimmer, 1981; Richardson and Hyslop 1985).

Up to around 1930 industrial enzymes were obtained from sprouted grains, animal glands and tropical plants. Since then they are more and more produced by yeasts, fungi and bacteria. Today about 30 different types of enzyme preparations are produced from pure cultures of selected strains of food grade micro-organisms for use in food production.

#### Advantages and limitations of the use of enzymes

The use of enzymes in food processing has certain advantages: they work at moderate conditions and are energy efficient, they are very specific causing few side reactions and little formation of byproducts, they can readily be inactivated after use but they also have the potential of being reused in immobilized form or in membrane reactors, they cause little pollution and can be considered as natural. In spite of our substantial knowledge of enzymes and their high potential for applications their actual use in food industry is relatively small. Limiting factors for their application are enzyme- and process costs, undesirable side activities, operational incompatibilities, lack of control and lack of knowledge of the molecular mechanism of the action of enzymes on complex substrates in food systems and how the substrate modifications relate to processes, functional properties of food ingredients and quality of food products. Food technologists have an important task in providing this knowledge which is necessary for the development of better enzyme preparations and new enzyme applications.

#### Improvement of technical enzyme preparations

##### Purification

Commercial enzyme preparations are generally crude and contain many side activities. Microbial preparations are often just precipitates of the fermentation liquor and contain all of the enzymes excreted by the organisms as well as inert proteins. In many applications these side activities do not interfere with the desired enzyme action, however when they do either other micro-organisms must be selected which do not accumulate the undesirable enzymes or the undesirable enzymes must be removed by selective inactivation or preferential adsorption (Reed, 1975; Meyrath and Volavsek 1975). Lately novel purification techniques have been developed in the downstream processing of crude enzyme fermentation media like liquid-liquid extraction (aqueous two-phase extractions and reversed micellar extraction) and affinity chromatography (Somers et al. 1989). For the isolation of endo-polygalacturonase (PG) Rozie et al., (1988), prepared calcium alginate beads by complexing alginate with calcium ions. PG was found to bind by biospecific and nonspecific ionic interactions to these inert beads. Adsorption characteristics could be influenced by changing the pH and ionic strength of the system. The beads

could be used extensively (more than 100 times) in a fluidized bed reactor without loss of binding capacity. The enzyme was purified by a factor 3 and could be recovered completely (Figure 1, Somers et al., 1989).

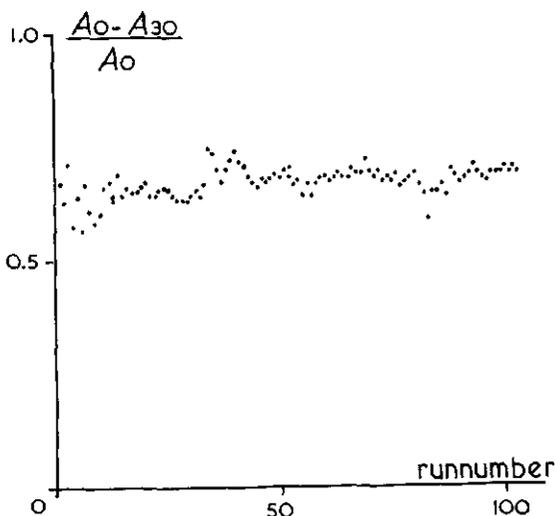


Fig. 1. Repeated adsorption and desorption of endo-polygalacturonase on alginate beads. A<sub>0</sub>, enzyme activity in solution before adsorption; A<sub>30</sub> enzyme activity remaining in solution after 30 min of adsorption (from Somers et al., 1989).

#### Increasing enzyme production and improve enzyme properties

The conventional techniques to improve enzyme production are classical mutagenesis in combination with selection and by optimization of culture parameters such as pH, agitation, temperature and nutrient requirements. Recombinant DNA technology offers a more rational approach and has far reaching potential for boosting the production of specific enzymes (genetic purification) and for improving properties of enzymes. The ability to selectively insert genes of one organism into another will allow the transplantation of for instance thermostable enzymes from a thermostable non-food grade micro-organism in a food grade micro-organism (Wasserman, 1984; White et al., 1984; Meade et al., 1987). Also enzymes from plant or animal sources can be cloned and then expressed in already tailored industrial micro-organisms. Examples of such developments are the cloning of chymosin, the milk clotting enzyme by choice, in yeasts and fungi which allows its production by fermentation (Scott et al., 1987; Poldermans, 1989; van den Berg et al., 1989) and the cloning of a specific  $\alpha$ -galactosidase of plant origin which is able to split galactosyl residues from the galactomannan guar to improve its functional properties (McCleary et al., 1984; Dea, 1987), in a Saccharomyces cerevisiae strain (Unilever, 1988). Many amylase and cellulase genes have also been cloned and expressed in many other micro-organisms (Meade et al., 1987). Heim et al. (1988) were successful in increasing the production of a pectin lyase more than 20-fold by isolating the gene for

pectin lyase production and bringing it back in the original fungus strain in a multycopy form.

Detailed knowledge of the structure-function relations in enzymes makes it possible to specifically alter the nucleotide sequence of a particular gene (site-directed mutagenesis). After bringing this modified gene to expression in a proper host enzymes adapted to the desired conditions can be obtained (Scott et al., 1987). Perry and Wetzel (1984) succeeded not only in cloning a T4 lysozyme into Escherichia coli but also in improving the thermal stability of the expressed enzyme by replacing a specific amino acid in the peptide chain with cysteine through site-directed mutagenesis which enabled the alteration of the protein to a more stable form by formation of an extra disulfide bond.

The possibilities of recombinant DNA technology appear unlimited. An uncertain factor is the acceptance of such enzymes by the public. Recently, genetically engineered chymosin has been permitted for industrial application in cheese production in Switzerland.

### Food industries and their enzymes

Table 1 gives an overview of the applications of enzymes in the main areas of the Food Industry. From the enzymes used in the dairy industry animal rennets for the production of cheese are by far the most important enzymes. For Dutch cheeses calf's rennet is preferred although pepsin from cows appears to be a good alternative when used at the proper pH (Aalbersberg and Folstar, 1986). Chymosin is the main enzyme in calf's rennin which splits the peptide bond between phenylalanine and methionine (positions 105 and 106) in K-casein, a glycoprotein composed of 169 amino acids and present in the outer layers of the casein micelles. K-casein is considered to stabilize these micelles in milk. As a result of the splitting of the peptide bond ca 1/3 of the K-casein goes in solution, the remaining part of the casein micelle precipitates forming curd and whey. During ripening of the cheese the chymosin enclosed in the curd remains active and slowly degrades the casein components in an aspecific way and thus contributes to structure and flavour. Rennets from other sources (cow-, pig-, chicken stomachs, fig leaves, Mucor miehei) give different proteolysis patterns than chymosin and this may lead to taste and structure deficiencies in older cheeses (Aalbersberg and Folstar, 1986; Ducroo, 1982). The performance of Mucor miehei rennet could be improved by an oxydation treatment which reduced the aspecific action and made the enzyme more heat-labile and herewith also more suited for further processing of the whey (Scott et al., 1987). The most important development in this field is the production of calf chymosin by fermentation of genetically engineered micro-organisms.

Lactase, or  $\beta$ -galactosidase, which hydrolyses lactose in glucose and galactose, is used to prevent crystallization of lactose during concentration of milk products, to reduce the lactose content in milk to be consumed by people who suffer from lactose-intolerance and for treatment of whey to make it sweeter and to enable concentration. There are two main sources of  $\beta$ -galactosidases; the enzyme obtained from yeast is optimally active at 35-40°C and at the pH of milk (6.5), while the fungal enzyme shows optimal activity at 60°C and pH 4.7 and is therefore more suited for acid whey (Coughlin and Charles, 1980).

Table 1. Food Industries and their enzymes\*.

ENZYMES	APPLICATIONS
<u>Dairy Industry:</u> (80x 10 <sup>6</sup> \$ US) <sup>a</sup>	
Proteases: (Animal/Microbial rennets)	Curd formation/Ripening
Lactase*	Lactose conversion
Lipase	Flavour formation
Lysozyme	Suppress late blowing cheese
<u>Starch and Sugar Industry:</u> ( 110x 10 <sup>6</sup> \$ US) <sup>a</sup>	
Alpha-amylase	Converted Starches
Beta-amylase	Malto/limit dextrins, Maltose syrup
Gluco-amylase <sup>b</sup>	Glucose syrup
Isomerase <sup>c</sup>	Isomerase syrup
Pullulanase	Glucose/maltose syrup
Isoamylase	Oligo-amylases
CycloGlucosylTransferase	Malto-oligosaccharides
Xylanases	β Cyclodextrins Starch-Gluten separation wheat
<u>Brewing Industry:</u> (25x 10 <sup>6</sup> \$ US) <sup>a</sup>	
Alpha-amylase	Starch Liquefaction
Beta-glucanases	Filtration
Protease (Bacillus)	Soluble amino compounds
Protease (papain)	Stabilization
Gluco-amylase	Light beer
Pullulanase	Light beer
Xylanase	Filtration
<u>Baking Industry:</u> (17x 10 <sup>6</sup> \$ US) <sup>a</sup>	
Alpha-amylase	Baking volume, fermentable sugars
Xylanase	Baking volume, water binding
Protease (fungal)	Baking volume, dough handling
Protease (Bacillus)	Biscuits, crackers, wafers.
Lipoxygenase	Bleaching
Phospholipase A and D	Activated gluten
<u>Fruit and vegetable Industry:</u> (16x 10 <sup>6</sup> \$ US) <sup>a</sup>	
Pectinesterase	Juice clarification,
Polygalacturonase	Maceration, Citrus juice stabilization/visc. reduction
Pectinesterase + Polygalacturonase and/or Pectin lyase	Juice clarification, juice/oil extraction, Citrus peel oil, citrus pulp wash
Pectin lyase; Pectate lyase	Maceration
Arabinanases	Juice clarification
Pectinesterase/Polygalacturonase/ Pectin lyase + (Hemi-) Cellulases	Liquefaction, clear/cloudy juices Enhance natural product extraction Valorization biomass/feed

Table 1. Food Industries and their enzymes\* (continued).

ENZYMES	APPLICATIONS
<u>Food Ingredient Modification, new developments:</u>	
Lipases	Fatty acid production Interesterification Ester synthesis
Proteases	Protein functionality Protein hydrolysates
Phosphorylase	Starch, chain elongation
Branching enzyme	Starch functionality
Pectinesterase	Pectin modification
Polygalacturonase	Pectin modification
Alpha-galactosidase	Galactomannan modification
<u>Miscellaneous applications, new developments:</u>	
Papain/Bromelin	Meat tenderizers
Phospholipase A <sub>2</sub>	Mayonnaise stability
Invertase	Confectionery
Glucose oxydase	Sugar/ O <sub>2</sub> removal
Phytase	Cereal fibre
Naringinase	Bitterness grape fruit juice
Nucleases	Taste enhancer
Beta-glucosidase	Aroma wine

\* estimated value 1987; <sup>b</sup> also in immobilized form; <sup>c</sup> also in immobilized, regenerable form.  
 \* References: Birch et al., 1981; Drapron and Godon, 1987; Ducroo, 1982; Dupuy, 1982; Poldermans, 1989; Reed, 1975; Richardson and Hyslop, 1985; Ruttloff, 1987; Stanton, 1988; Voragen and Pilnik, 1989; West, 1988.

Lipases are used for the production of certain cheeses (Provolone, Romano) where they contribute to the characteristic . Mixtures of lipases and proteases are used for the production of cheese flavours. There is a need for proteases which can speed up the ripening of cheese without causing taste and stucture defects (Aalbersberg and Folstar, 1986; Scott et al., 1987).

The starch and sugar industry is at present almost completely enzyme based. Figure 2 shows the sites of attack of the various amylases on amylopectin. Starch degrading enzymes can be of plant, animal or microbial origin. Alpha-amylases split 1,4 linkages at random, producing maltodextrins as shown at the top. Eventually oligosaccharides, maltose and glucose are obtained. There are several exo-amylases; gluco-amylases split off glucose from the non-reducing chain end. Next to 1,4 linkages these enzymes are also able to split 1,6 branch points, however at a much lower rate. Beta-amylases split off the dimer maltose but stop close to the 1,6 branch point, leaving  $\beta$ -limit dextrin. Oligosaccharide forming amylases produce maltotriose, -tetraose, -pentaose etc. also leaving limit dextrans (Nakakuki and Kainuma, 1984). There are two types of debranching enzymes splitting only 1,6 linkages of which pullulanase is more active on low molecular weight substrates and iso-amylase on the

high molecular weight substrates forming short chain amyloses (Kainuma et al., 1978; Jensen and Norman, 1984).

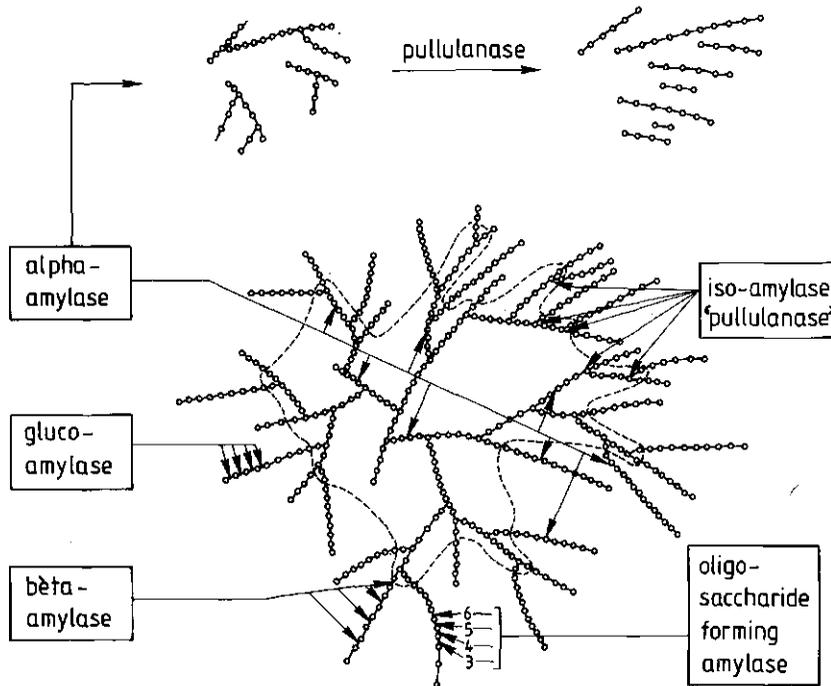


Fig. 2. Schematic structure of amylopectin and the sites of attack of the various amylases.

There are also transferase enzymes which are able to increase the branching of amylopectin of the normal 4-5 % to ca. 10 % and thus give new properties to the starch preparation (Spa et al., 1978; Okada et al., 1984). Amylases are available from many sources and have different pH and temperature characteristics, the  $\alpha$ -amylase from *Bacillus licheniformis* for example is still active at temperatures over 100 °C and is therefore the enzyme by choice for liquefaction of starch (Ducroo, 1982). The enzymes discussed here show only poor activity on native granular starch, recently however a number of enzymes have been isolated which can degrade native starch (Ueda et al., 1984; Abe et al.; 1988).

Amylases are used in the starch industry to produce modified starches. A new development is the production of maltodextrins which can be used as a fat replacer or in combination with emulsifiers as structure improvers (Karper and Gruppen, 1986). Amylases, in various combinations, are also used to produce tailor-made starch syrups including isomerase using immobilized glucose-isomerase (one of the first successes of Biotechnology). Finnsugar has developed a matrix with immobilized glucose-isomerase which can be regenerated when the enzyme is exhausted (Scott et al., 1987). Also immobilized gluco-amylase has been introduced in this industry. A recent development in this field is the use of pullulanase preparations with better pH and temperature characteristics in combination with gluco-amylase or  $\beta$ -amylase to improve glucose and

maltose production (Ducroo, 1982). Another enzyme which is getting more attention is cyclodextrin-glucosyl-transferase for the production of cyclodextrins. These cyclodextrins gain more and more interest in the food industry for molecular encapsulation of compounds to protect them from degradation by light or heat, from oxidation and from evaporation or to mask their taste or smell (Pszczola, 1988; Patington, 1987; Szejtli, 1984, 1988; Figure 3).

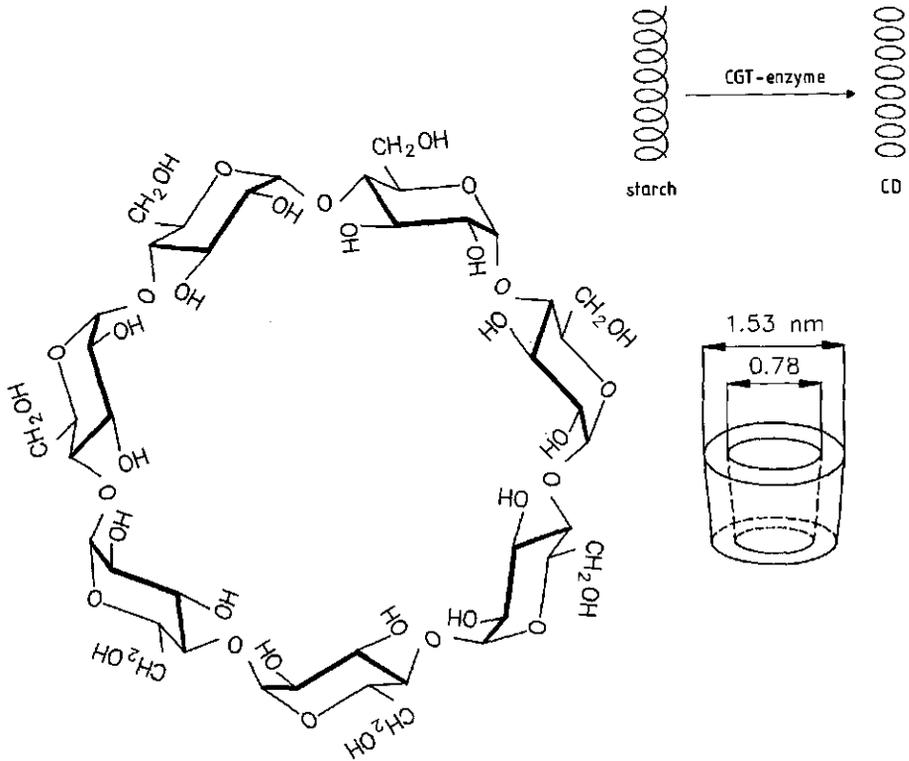


Fig. 3. Formation of cyclodextrins from starch with cyclodextrin-glucosyl-transferase (CGT); structure of  $\beta$ -cyclodextrin and its molecular dimensions.

In western countries beer is generally made with the enzymes present in malt. Papain is already being used for a long time to make the beer chillproof i.e. prevent haze formation as a result of complexation of oxidized polyphenols with proteins. When so called adjuncts are used in large quantities suppletion of the malt enzymes with  $\alpha$ -amylase and  $\beta$ -glucanases may be necessary (Ducroo, 1982). In third world countries expensive, imported malt is more and more substituted with domestic starch sources like corn, manioc, sorghum or rice. To improve the accessibility of the starch for conversion to fermentable sugars and to provide amino nutrients for the yeast there is a need for a brewing enzyme containing  $\alpha$ -amylase,  $\beta$ -glucanases, xylanase (Ducroo, 1988) and protease. At present this area is subject of intensive research. For production of

light or low calory beer gluco-amylase (or amyloglucosidase) is added to convert the limit dextrins in the wort to fermentable sugars. Since pullulanases are available with better pH and temperature characteristics these enzymes are recommended for use in combination with gluco-amylase (Olesen et al., 1984; Ducroo, 1982).

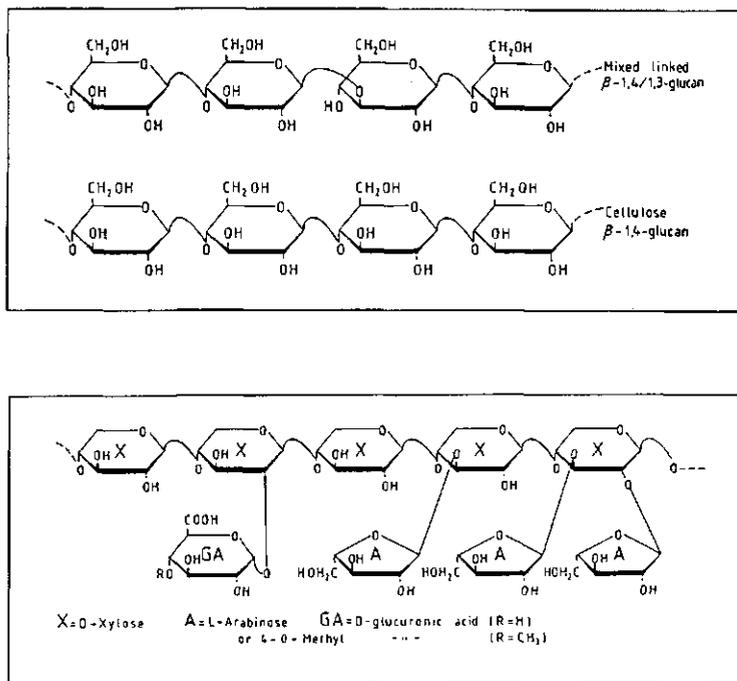


Fig. 4. Structure of arabinoxylans and  $\beta$ -glucans in cereals.

Exogenous enzymes may enable a better control of the baking process particularly when the level of endogenous  $\alpha$ -amylase in the flour is low in relation to the endogenous  $\beta$ -amylase activity resulting in insufficient formation of fermentable maltose from the damaged starch granules. By the addition of  $\alpha$ -amylase in the form of malt or preferably of thermo-labile fungal  $\alpha$ -amylase the formation of fermentable sugars can be regulated without liquefaction of starch taking place. Fungal proteases with a slight activity on gluten may be added to improve the gluten elasticity which can be of advantage in the baking process. Proteases which degrade the gluten proteins to a higher extent are used for the baking of biscuits, crackers and wafers where they facilitate the process and improve the organoleptic quality (Ducroo, 1982; Drapron and Godon, 1987; Haseberg, 1988). The last years it has become evident that the non-starch polysaccharides (predominantly arabinoxylans and  $\beta$ -glucans; Figure 4) in cereal flours have an important influence on the baking process. Xylanases, present as side activity in crude  $\alpha$ -amylase preparations, have been shown to have a positive effect on the baking quality of flours and on the separation of wheat starch and gluten (Ducroo, 1982; Haseberg, 1988). The mechanism of their role is poorly understood and therefore difficult to control. In the literature also the use of phospholipases is mentioned which modify the phospholipids present in the flour and improve

in this way the performance of the gluten. They also may act on soy lecithin present in bread improvers, the modified lecithin than interacts with the gluten resulting in improved baking quality (Scott et al., 1987; Figure 5).

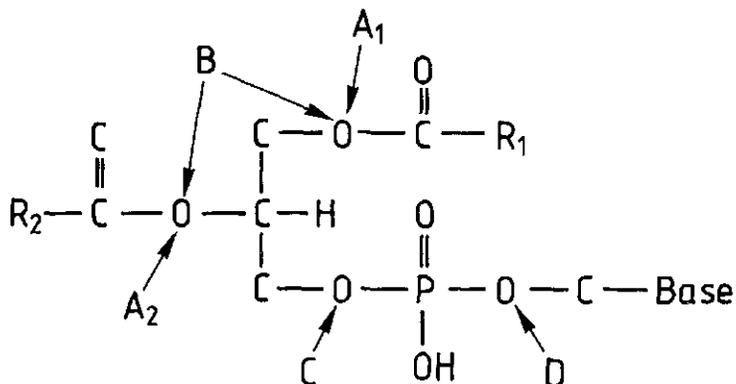


Fig. 5. Schematic structure of phospholipids and sites of attack of various phospholipases.

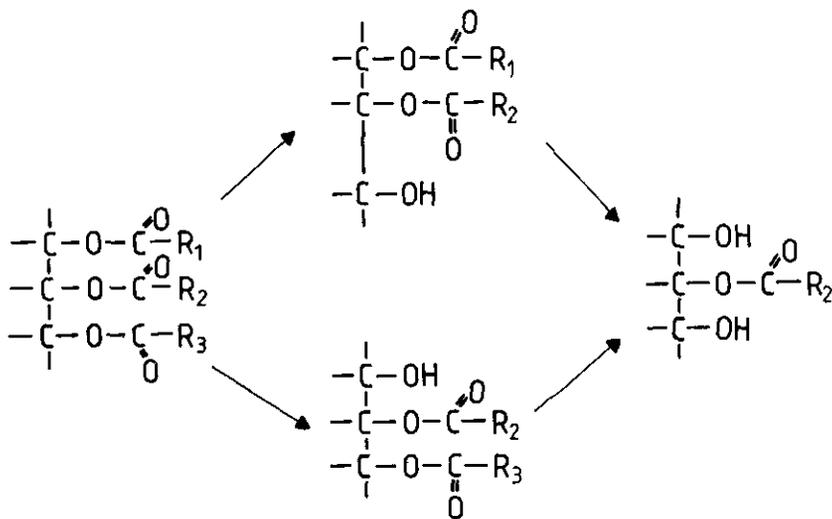


Fig. 6. The action of triacylglycerol hydrolases (lipases). The remaining acyl residue is cleaved by pancreas lipase only after acyl migration.

In the area of food ingredient modification (Table 1) lipases are recently finding great interest for their use to hydrolyse fats into the constituent fatty acids and glycerol (Figure 6) (Bühler and Wandrey, 1988; Pronk et al., 1988), for interesterification of fats and for synthesis of desired surfactants and emulsifiers in low-water systems. Micro-

bial enzymes seem more suitable for this purpose than animal lipases because they are much less specific for fatty acid position and chain length. In comparison to other industrial enzymes lipases are expensive and the costs of their use can be reduced by re-using them in membrane reactors or in immobilized form (West, 1988; Hoq et al., 1985). Also enzymic modification of industrial proteins gains renewed attention. There is a need for balanced mixes of endo- and exopeptidases to prepare protein hydrolysates for dietary purposes (not allergenic, not bitter) or as a base for flavour compounds. Such mixtures might replace chemical hydrolysis which has some serious drawbacks like for instance formation of toxic dichloropropanol. Limited hydrolysis with specific endopeptidases may introduce desired functionalities to proteins like foaming and emulsifying properties. This often leads to loss of stability of the modified proteins but this can be restored by compounding with proteins with high stability (Ruttloff, 1987; Phillips, 1977).

Modifications of pectins can be obtained by treatment with fungal pectin esterase for the manufacture of low-ester pectins with reduced calcium sensitivity (Ishii et al., 1979) and by treatment with endopolygalacturonase for the manufacture of pectins with reduced viscosity and reduced calcium sensitivity (De Vries, 1989).

A number of the enzymes mentioned under miscellaneous applications (Table 1) are already known for a long time. New developments of interest are: - the potential application of  $\beta$ -glucosidases for the hydrolysis of monoterpene-glucosides to enhance the aroma of certain grape juices and wines through the release of volatile monoterpenes (Aryan et al., 1987; Figure 7), - the specific modification of phospholipids in egg yolk with phospholipase A<sub>2</sub> to improve their emulsifying properties and to make them more heat stable (Unilever, 1988), - the reduction of the phytate level in whole wheat flour, cereal fibers and legumes by hydrolysis of the inositol-hexa-phosphate with phytase. This eliminates the anti-nutritional effect of phytate which is based on its metal chelating action resulting in reduced mineral availability (Poldermans, 1989; Figure 8).

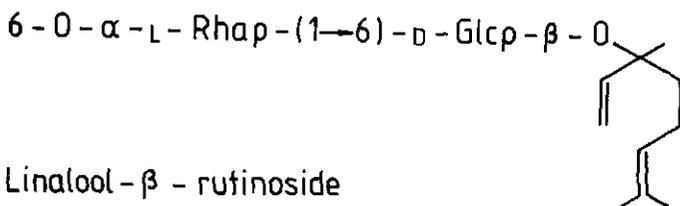


Fig. 7. Structure of Linalool- $\beta$ -rutinoside, a monoterpene glycoside.

The applications of enzymes in fruit and vegetable processing have developed from the original use of pectic enzymes for treatment of soft fruit to ensure high yields of juice and pigments upon pressing and for the clarification of raw press juices. Technical enzyme preparations in use for these processes contain pectin esterases, polygalacturonases and pectin lyases in varying amounts along with other enzymes such as arabinanases, galactanases, xylanases,  $\beta$ -1,4-glucanases, glycosidases and proteases. It has been shown that through the combined activity of pectin esterase and polygalacturonase the pectin dissolved in the juice, as well as the negatively charged pectin coating of the suspended particles are degraded and the positively charged proteinaceous nuclei are exposed.

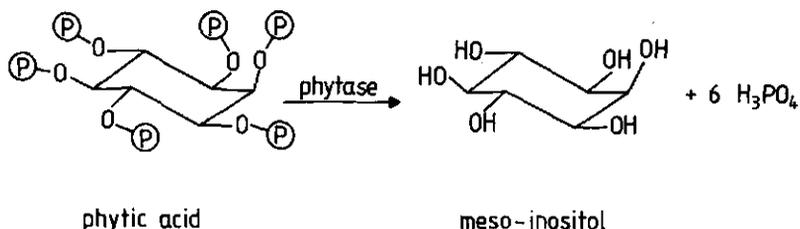


Fig. 8. Conversion of phytic acid to meso-inositol.

Subsequently the destabilized particles flocculate through electrostatic interaction and precipitate. (Endo, 1965; Yamasaki et al., 1967). Apple juice clarification is also possible with pectin lyase for which the highly esterified apple pectin is an ideal substrate (Ishii and Yokotsuka, 1973). Also a commercially available fungal pectin esterase has been introduced for clarification of cider and lemon juice (Baron and Drilleau, 1982). Clarification and complete depectinization enables concentration of fruit juices and avoids gelling of the concentrates.

The use of enzymes to facilitate pressing of soft fruits (pulp enzyming) was adapted by de Vos and Pilnik (1973) for juice recovery from apples which were difficult to press because of variety or prolonged storage. This is now an established process in the apple juice industry and has also found application in grape juice processing. For apples it was shown that enzyme mixtures capable of rapidly degrading highly methylated pectin perform well (Voordouw et al., 1974).

Fungal pectinase preparations containing predominantly endopolygalacturonase and free of pectin esterase are used successfully as macerating enzymes for the production of pulpy nectars which have a more smooth consistency and have higher contents in soluble solids, pigments and nutrients than products prepared by a mechanical-thermal process (Gramp, 1972). These suspensions of loose cells from fruit and vegetable tissues are obtained by weakening the cell cohesion by a limited pectin breakdown particularly in the middle lamella. Enzyme preparations which have only one depolymerase system are chosen (Pilnik, 1982). The presence of pectin esterase can easily transform the macerating activity of a pure polygalacturonase into cell disintegration activity because of the general depolymerizing activity of the pectin esterase/polygalacturonase combination. Blanching is therefore indicated when endogenous pectin esterase is present. Maceration can also be achieved with endopectin lyase (Ishii, 1976) or with bacterial pectate lyase (Wegener and Henniger, 1987). Rombouts (1981) explained the potential of pectate lyases for maceration of potatoes and vegetables by their optimal activity on intermediate esterified pectins.

Through the use of pectic and cellulolytic enzymes the cell walls of fruit pulps can be degraded to the stage of almost complete liquefaction which has led to the introduction of the enzymic liquefaction process for (tropical) fruit and vegetables (Pilnik et al., 1975). With this process clear, cloudy and pulpy fruit juices can be obtained in yields exceeding 90%. As a result of the solubilization of cell wall polysaccharides, the dry matter content of the juices is also sensibly increased. The presence of both endo- and exo-  $\beta$ -1,4 glucanases (cellulases) next to pectic enzymes is essential (Renard et al., 1989). New brands of pectic enzymes with broader spectra of cellulolytic and hemi-cellulolytic activities

(arabinanases, galactanases) have been introduced. The liquefaction process is finding more and more applications.

It is easy to understand that the 'pulp enzyming' process makes different demands to enzyme-formulations to be used than the 'liquefaction process'. It is also evident that the juices obtained with these processes have different characteristics.

#### Basic studies of enzymic cell wall modification

Basic studies of the composition and structure of plant cell wall polysaccharides and of the degradation of cell wall models with a whole range of pure, well characterized enzymes by detailed analysis of the cell wall polysaccharide fragments released from cell wall models and native plant tissues, enabled us to identify technologically relevant enzymes, to understand their action on a molecular level and to improve enzyme formulations and applications. These studies also led to the discovery of a novel pectic enzyme which is specific for hairy regions of pectins.

The contribution of Catherine Renard et al (1989) demonstrates this for the enzymic degradation of apple cell walls. It shows that solubilization of pectins rich in associated arabinans and galactans, which were firmly embedded in apple cell walls is most effective with enzyme formulations which are highly active on high esterified pectins. This results in disintegration of the cell walls and a drastic increase in their water holding capacity. The hemi-cellulases tested hardly contributed to the solubilization of pectin. High degrees of liquefaction can be observed when combinations of endo- $\beta$ -1,4-glucanases with

Table 2. Galacturonide content and amounts and characteristics of Polysaccharides isolated from apple juices prepared by different processes and different enzymes (Voragen and Pilnik, 1989; Voragen et al., 1986a).

Process: Enzyme preparation:	<u>Pressing</u>	<u>Pulp Enzyming</u>			<u>Liquefaction</u>	
		A	B	C	C	D
Galacturonides:						
Content (mg/ml juice)	0.6	2.5(1,6)*			4.3	
Polysaccharides:						
Content (mg/kg pulp)	155	1075	1860	1075	3000	4300
Sugar Composition: Mole %						
Rhamnose	3	5	3	5	4	4
Arabinose	21	38	6	52	43	54
Xylose	2	5	2	5	6	8
Galactose	19	9	4	7	6	8
Glucose	2	1	1	1	1	0
Anh.galacturonic acid	53	42	84	30	40	20
Degree of methylation	52	72	76	31	59	39
Degree of acetylation <sup>a</sup>	15	18	6	13	28	29

a) Degree of acetylation calculated on anh.galacturonic acid content.

\* After clarification with bentonite and gelatine.

formulations highly active on high esterified pectin were used, the liquefaction was further enhanced by the addition of  $\text{exo-}\beta\text{-1,4-galacturonidase}$ .

Treatment of apple pulp with such enzymes causes the release of cell wall fragments in the juice as mono-, oligo- and polysaccharides. Table 2 shows the overall galacturonide content and amounts and composition of high molecular weight polysaccharide fragments present in apple juices prepared by conventional pressing, pulp enzyming and enzymic liquefaction using technical enzyme preparations which differed in level and type of pectic, hemi-cellulolytic and cellulolytic activities.

The polysaccharide fractions were isolated as freeze-dried dialysis retentates from raw juices clarified with bentonite, gelatin and Klar-Sol 30. The galacturonide content was determined in the raw juice. In the enzyme treated pulps the solubilized pectic material occurred mainly as monomeric and oligomeric galacturonides. About 5% of the dimers and trimers, and 20-25% of the trimers were found to be present in unsaturated form (figure 9), they are typical reaction products of pectin lyase action (Voragen et al., 1986b).

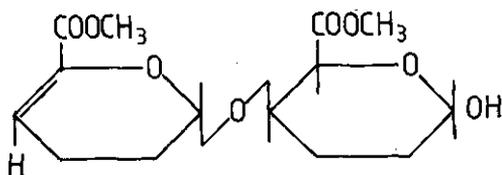


Fig. 9. Structure of unsaturated dimethyldigalacturonate.

Oligogalacturonic acids and in particular unsaturated oligogalacturonides were found to be very reactive precursors for browning reactions, also the browning of fructose containing models was strongly enhanced by the presence of unsaturated galacturonides and amino acids. These reactions will not create a problem when the juices are handled properly (Voragen et al., 1988).

The effect of enzyming on the release of polysaccharide fragments is unequivocal, the largest amounts were released in the liquefaction process. The amounts and compositions of the polysaccharide fractions also depended from the technical preparations used. Distinct variations in arabinose, galactose, galacturonic acid and xylose contents were observed and fragments with increased acetyl contents accumulated in the liquefaction juices. The polysaccharide fraction released during the liquefaction process by preparation D was shown to consist of an almost pure, linear arabinan fraction and a pectin fraction rich in arabinose side chains (Voragen et al., 1986b). The structural features of the pectic fraction were studied by establishing its sugar and glycosidic linkage composition, its degradability by alkali, acid, 4-methylmorpholine-N-oxide and with pure, well defined enzymes including structural characteristics of the reaction products formed, and its molecular weight distribution (Schols et al., 1989a). The pectin fraction was characterized as a highly branched rhamnogalacturonan, its hypothetical structure is shown in figure 10. It resembles the structure of hairy regions of pectins as described by De Vries et al. (1986), its lower galactose content can be explained by the galactanase activity present in D. The fraction is rich in arabinose as is also the case for fragments released by C.

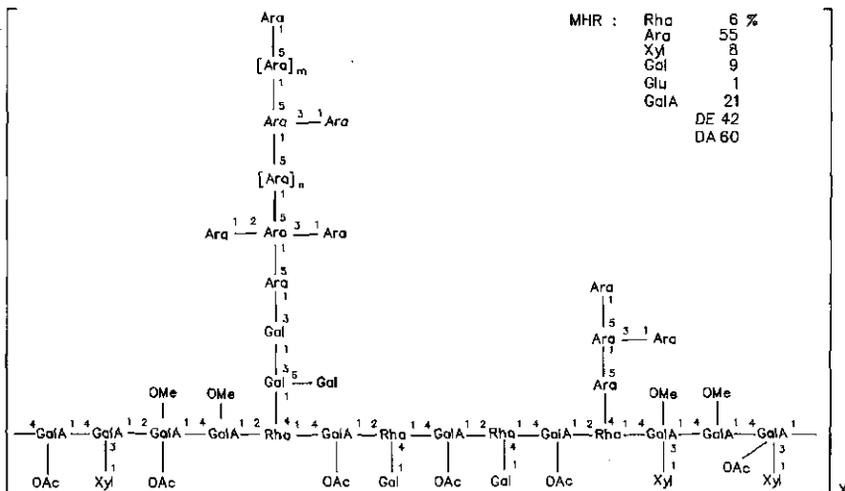


Fig. 10. Tentative structure of modified hairy regions (MHR; Schols et al., 1989a).

Preparation B on the other hand released fragments low in arabinose, this preparation was however shown to contain the complete spectrum of arabinolytic enzymes. We isolated 3 enzymes which together can effectively degrade native arabinans to arabinose monomers. Two of them are arabinofuranosidases, both active on p-nitrophenyl-arabinofuranoside, type I is also active on oligoarabinosides but not on polymeric arabinans, type II readily debranches arabinans but can also split of arabinose from linear  $\alpha$ -1,5-arabinan starting from the non-reducing chain end. The third enzyme is an endo- $\alpha$ -1,5-arabinanase which is only active on linear regions of arabinans, it can only degrade branched arabinans completely when arabinofuranosidase activity is present (Figure 11).

Arabinans and arabinan-rich pectins solubilized in the juice are potential precursors for the formation of hazes in apple and pear juice concentrates. This is particularly the case when they are linearized by exposure to arabinofuranosidase type II activity (in the absence of endoarabinanase) and/or chemically debranched by heat treatment as a result of juice acidity (Voragen et al., 1987).

These linear arabinans are sensitive to chain association and haze formation. The use of enzymes with sufficient levels of endo-arabinanase and arabinofuranosidase II activity is therefore the best solution for the arabinan haze problem.

Rhamnogalacturonase: a novel enzyme degrading highly branched rhamnogalacturonan regions

The hairy pectin fraction isolated from apple juice prepared with enzyme D (MHR, modified hairy regions) was found to be resistant to degradation by the enzyme systems present in most of the technical

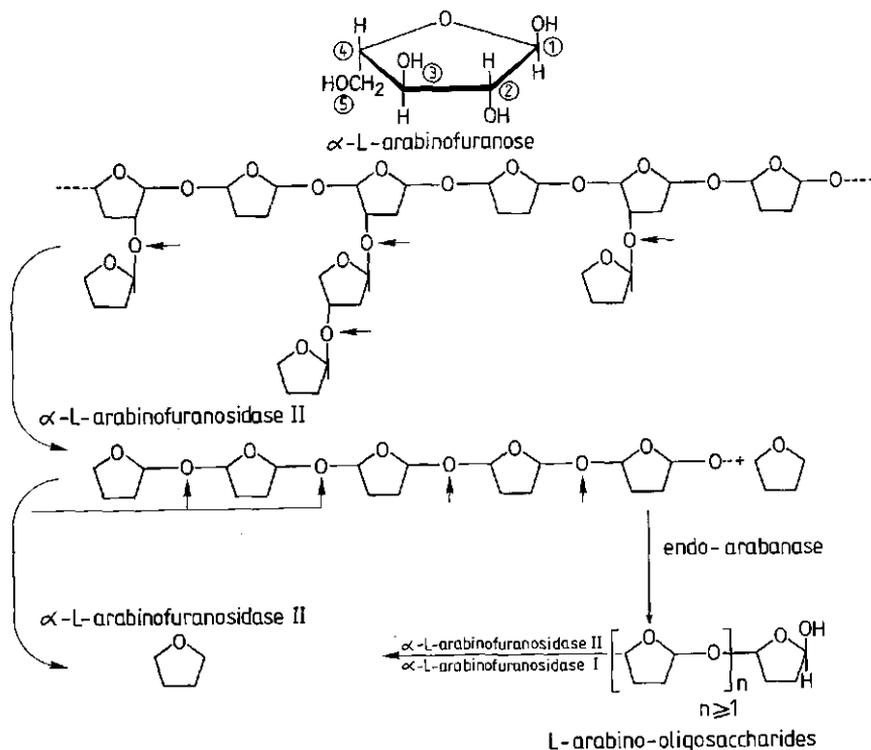


Fig. 11. Schematic structure of arabinan and sites of attack of arabinan degrading enzymes isolated from *Aspergillus niger* (Rombouts et al., 1988).

preparations. So far, from ca 40 technical enzyme preparations tested on this substrate only Ultra SP (Novo Ferment AG, Basel, Switzerland, enzyme C) was found to be able to depolymerize the rhamnogalacturonan backbone of these fragments. This activity could only be made manifest by measuring the shift in the molecular weight distribution as shown in Figure 12.

By an involved purification procedure as outlined in Figure 13, we were able to isolate an electrophoretically pure enzyme which was particularly active on de-esterified MHR (treated with cold 0.05 N sodium hydroxide, MHR-S) or acid treated MHR to remove arabinan side chains (pH 0.3, 2 h, 80 °C, MHR-HCL) (Schols et al., 1989b). Apparently the enzyme activity is enhanced by removal of methoxyl and/or acetyl groups and arabinan side chains. The enzyme showed no activity on high methoxyl pectin, polygalacturonic acid, arabinan, xylan, galactan, carboxymethylcellulose and various para-nitrophenyl glycosides. The enzyme had a molecular weight of 63.000 D and was optimally active in the pH range 3 to 6 temperature range 30 to 50 °C. From a digest of MHR-S with the purified enzyme we isolated oligomeric fragments by gelpermeation chromatography on a Sephadex G 50 column. The elution profile and the sugar composition of the pooled fractions are shown in Figure 14.

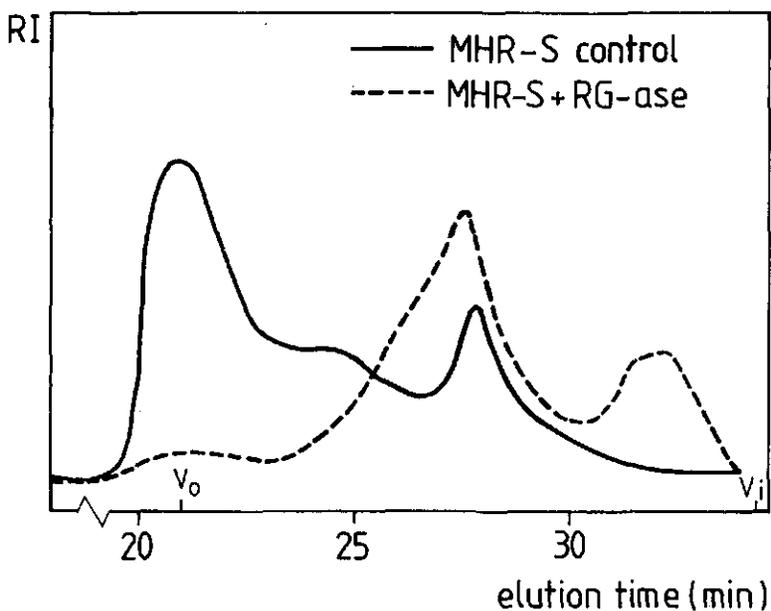


Fig. 12. Shift in molecular weight distribution of saponified MHR (MHR-S) as measured with high performance gel permeation chromatography (HPGPC) after treatment with enzyme isolated from Ultra SP (24 h, 30 °C). HPGPC conditions: BioGel TSK 40XL, 30XL, 20XL in series, eluted with 0.4 M NaAc, pH 3.4 at 30 °C.

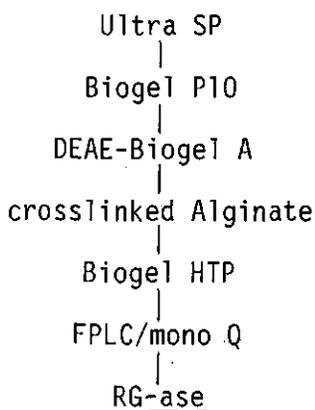


Fig. 13. Purification procedure used for the isolation of rhamnogalacturonase.

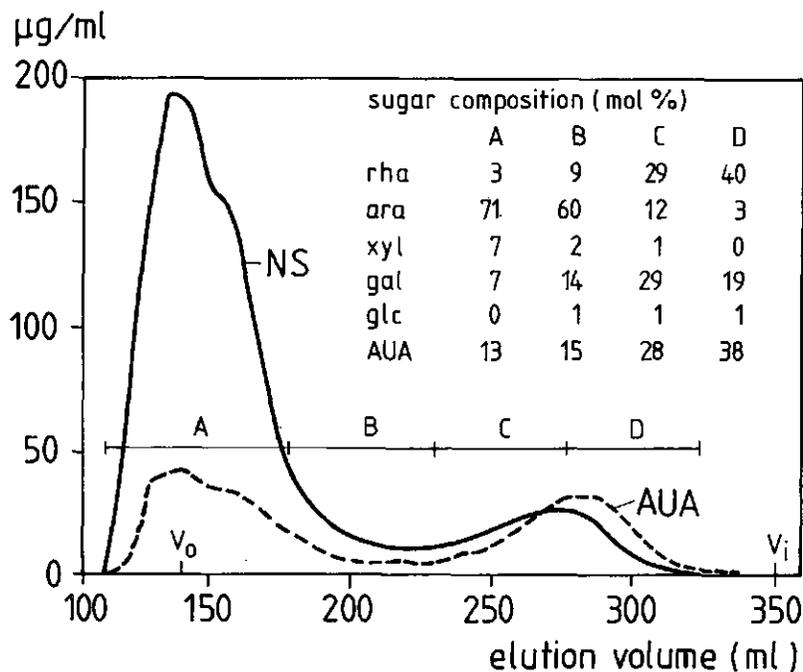


Fig. 14. Elution profile of MHR-S treated with rhamnogalacturonase (24 h, 30 °C) obtained by gelpermeation chromatography over Sephadex G50 and sugar composition in mole % of fractions pooled as indicated. AUA is anhydrogalacturonic acid concentration in eluate (---), NS is concentration of neutral sugars (—), rha = rhamnose, ara = arabinose, xyl = xylose, gal = galactose, glc = glucose.

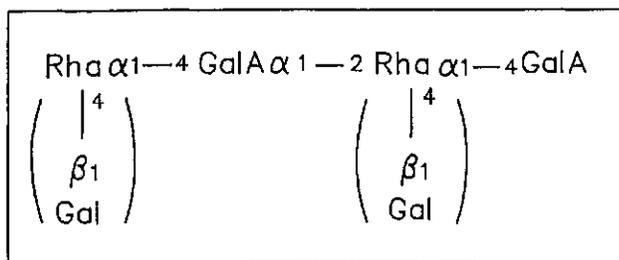


Fig. 15. Schematic structures of oligomers produced from MHR-S by rhamnogalacturonase. Tetramer = Rh<sub>2</sub>GalA<sub>2</sub>; pentamer (2 types) = Rh<sub>2</sub>GalA<sub>2</sub>Gal<sub>1</sub>; hexamer = Rh<sub>2</sub>GalA<sub>2</sub>Gal<sub>2</sub>. Rha = rhamnose, GalA = galacturonic acid, Gal = galactose.

From methylation analysis and NMR studies we were able to identify the composition and structure of the oligomers present in fraction D (Colquhoun et al., 1989). This fraction was found to be a mixture of a tetramer (Rha<sub>2</sub>Gal<sub>2</sub>), 2 types of pentamers (Rha<sub>2</sub>Gal<sub>2</sub>Gal<sub>1</sub>) and a hexamer (Rha<sub>2</sub>Gal<sub>2</sub>Gal<sub>2</sub>), their structures are schematically shown in figure 15. The hypothetical structure of MHR shown in figure 10 takes account of the structures of these oligomers. From these results we conclude that the enzyme isolated from Ultra SP is able to split glycosidic linkages in the rhamnogalacturonan backbone of (apple) pectins producing, next to other not yet fully identified reaction products, a range of oligomers composed of galacturonic acid, rhamnose and galactose with rhamnose at the non-reducing end. We therefore propose the name rhamnogalacturonase (RG-ase) for this novel enzyme. RG-ase is a valuable, analytical tool for revealing the structure of complex pectic substances and might play a role in the degradation of plant tissues, in particular in combination with other enzymes. Some preliminary results are shown in the contribution of Catherine Renard et al. (1989).

At present we are following the same approach for studying the structural polysaccharides of other fruits, vegetables, cereals and raw materials for feed, to be able to optimize their enzymic modification leading to new processes and new products.

I expect that food enzymes will continue to gain in importance, their use in food systems will increase only gradually because of the complexity of these systems, their use for the preparation of food ingredients will probably increase faster. New applications will be developed just like more specific and better tailored enzymes. These developments will be greatly accelerated when genetically engineered enzymes get more and more accepted.

#### References

- Aalbersberg, W.I.J.; Folstar, P.; 1986. Biotechnologie bij de zuivelbereiding. *Biotechnologie in Nederland* 3(6): 269-272.
- Abe, J.; Nakajima, K.; Nagano, H.; Hizukuri, S.; Obata, K.; 1988. Properties of the raw-starch digesting amylase of *Aspergillus* SP.K-27: a synergistic action of glucoamylase and alpha-amylase. *Carbohydrate Research* 175: 85-92.
- Antébi, E.; Fishlock, D.; 1987. *Biotechnologie: Een nieuwe industriële revolutie. Natuur & Techniek*, Maastricht/Brussel. Dutch edition of *Le Génie de la Vie*, Editions Hologramme, Neuilly-sur-Seine, France.
- Aryan, A.P.; Wilson, B.; Strauss, C.R.; Williams, P.J.; 1987. The properties of glycosidases of *Vitis vinifera* and a comparison of their B-glucosidase activity with that of exogenous Enzymes. An assessment of possible applications in enology. *Am. J. Enol. Vitic.*, 38(3): 182-188.
- Baron, A.; Drilleau, J-F.; 1982. Utilisation de la pectinesterase dans les industries cidricoles. In: P. Dupuy (Ed.): *Use of Enzymes in Food Technology. Technique et Documentation Lavoisier*. Paris. p. 471-476.
- Berg van den, J.A.; Ooijen van, A.J.J.; Rietveld, K.; 1989. *Kluyveromyces* as a host strain. European patent application EP-A-0301670.
- Birch, G.G.; N. Blakebrough & K.J. Parker (Eds.): 1981. *Enzymes and Food Processing*. Applied Science Publishers, London.
- Bühler, M.; Wandrey, C.; 1988. Enzymatische Fettspaltung. *Alimenta* 2: 22-25.

- Carasik, W.; Carroll, J.O.; 1983. Development of immobilized enzymes for production of high fructose corn-syrup. *Food Technology* 37(10): 85-91.
- Colquhoun, I.J.; Ruiter de, G.A.; Schols, H.A.; Voragen, A.G.J.; 1989. 2-D NMR studies of rhamnogalacturonan oligomers of an apple pectin. Submitted for publication.
- Coughlin, R.W.; Charles, M.; 1980. Applications of lactase and immobilized lactase. In: W.H. Pitcher (Ed.): *Immobilized enzymes for food processing*. CRC Press Inc, Boca Raton, Florida. p. 154-173.
- Dea, I.C.M. 1987. The role of structural modification in controlling polysaccharide functionality. In: M. Yalpani (Ed.): *Industrial Polysaccharides: Genetic Engineering, Structure/Property Relations and Applications*. Elsevier Science Publishers B.V., Amsterdam, The Netherlands. p.207-216.
- Drapron, R.; Godon, B.; 1987. Role of enzymes in baking. In: J.E. Kruger; D. Lineback; C.E. Stauffer (Eds.): *Enzymes and their role in cereal technology*. American Association of cereal chemists. p.281-324.
- Ducroo, P.; 1988. Production of beer. United States Patent 4,746,517.
- Ducroo, P.; 1982. Utilisation industrielle des enzymes. *Ind. Aliment. Agric.* 99: 401-415.
- Dupuy, P.(Ed.): 1982. Use of enzymes in food technology. *Technique et Documentation Lavoisier*, Paris.
- Endo, A.; 1965. Studies on pectolytic enzymes of molds. Part XVI. Mechanism of enzymatic clarification of apple juice. *Agric. biol. Chem.* 29: 229-233.
- Grapp, E.; 1972. Die Veränderung der Technologie am Beispiel der Obst- und Gemüseverarbeitung mit einem speziellen Pektinase präparat. *Dechema Monographien* 70: 175-186.
- Haseberg, ter E.; 1988. Enzymenanwendung in der Mühle. *Alimenta* 1: 2-10.
- Heim, J.; Gijssler, C.; Visser, J.; Kester, H.C.M.; 1988. European Patent Application 4-16317/+.
- Hoq, M.M.; Tagami, H.; Yamane, T.; Shimizu, S.; 1985. Some characteristics of continuous glyceride synthesis by lipase in a microporous hydrophobic membrane bioreactor. *Agric. biol. Chem.* 49:335-342.
- Hultin, O.H.; 1983. Current and potential uses of immobilized enzymes. *Food Technology* 37(10): 66-82.
- Ishii, S.; Yokotsuka, T.; 1973. Susceptibility of fruit juice to enzymatic clarification by pectin lyase and its relation to pectin in fruit juice. *J. agric. Food Chem.* 20:265-272.
- Ishii, S.; 1976. Enzymatic maceration of plant tissues by endo-pectin lyase and endo-polygalacturonase from *Aspergillus japonicus*. *Physiology and Biochemistry* 66: 281-289.
- Ishii, S.; Kiho, K.; Sugiyama, K.; Sugimoto, H.; 1979. Ger. P Appln. 2843351.
- Jensen, B.F.; Norman, B.E.; 1984. *Bacillus acidopulluliticus* pullulanase: application and regulatory aspects for use in the food industry. *Process Biochemistry* 19(8): 129-134.
- Kainuma, K.; Kobayashi, S.; Harada, T.; 1978. Action of *Pseudomonas* isoamylase on various branched oligo- and poly-saccharides. *Carbohydrate Research* 61:345-357.
- Karper, F.S.; Gruppen, H.; 1987. Replace oil and fat with potato-based ingredient. *Food Technology* 41(3):112-113.
- McCleary, B.V.; Dea, I.C.M.; Windust, J.; Cooke, D.; 1984. Interaction properties of D-galactose depleted guar galactomannan samples. *Carbohydrate Polymers* 4:253-294.

- Meade, J.H.; White, J.; Shoemaker, S.P.; Gelfand, D.H.; Chang, S.; Innis, M.A.; 1987. Molecular cloning of carbohydrases for the food industry. In: D. Knorr (Ed.): Food Biotechnology. Marcel Dekker, Inc. New York. p. 393-411.
- Meyrath, J.; Volavsek, G.; 1975. Production of microbial enzymes. In: G. Reed (Ed.): Enzymes in Food Processing. Academic Press, New York. p. 255-300.
- Nakakuki, T.; Kainuma, K.; 1984. Present and future of development of malto-oligosaccharides. Chemical Economy & Engineering Review 16(7-8):23-30.
- Okada, S.; Kitahata, S.; Yoshikawa, S.; Sugimoto, T.; Sugimoto, K.; 1984. Process for the production of branching enzyme, and a method for improving the qualities of food products therewith. United States Patent, 4,454,161.
- Olesen, T.; Pommer, K.; Stentebergjerg-Olesen, P.; 1984. New enzymes for brewing: Promozyme, a debranching enzyme and SP-249, a glucanase. Journal Am. Soc. Brew. Chem., 42: 85-89.
- Pagington, J.S.; 1987. Beta-Cyclodextrin: the succes of molecular inclusion. Chemistry in Britain (5):455-458.
- Perry, J.L.; Wetzal, R.; 1984. Disulfide bond engeneered into T4 lysozyme: Stabilization of the protein toward thermal inactivation. Science 226:555-557.
- Phillips, M.C.; 1977. The conformation and properties of proteins at liquid interfaces. Chemistry and Industry. p. 170-176.
- Pilnik, W.; Voragen, A.G.J.; Vos de, J.; 1975. Enzymatische Verflüssigung von Obst und Gemüse. Flüssiges Obst 42(11): 448-451.
- Pilnik, W.; 1982. In: P. Dupuy (Ed.): Use of Enzymes in Food Technology. Technique et Documentation Lavoisier. Paris. p. 425-450.
- Poldermans, B.; 1989. Enzymen en voedingsmiddelen. Accepted for publication in Voedingmiddelentechnologie.
- Pszczola, D.E.; 1988. Production and potential food applications of Cyclodextrins. Food Technology 42(1):96-100.
- Reed, G. (Ed.): 1975. Enzymes in Food Processing. Academic Press, New York.
- Reed, G.; 1975. The nature of enzymatic reactions. In: G. Reed (Ed.): Enzymes in Food Processing. Academic Press, New York. p. 7-13.
- Renard, C.M.G.C.; Voragen, A.G.J.; Schols, H.A.; Searle-van Leeuwen, M.J.F.; Thibault, J.F.; Pilnik, W.; 1989. Apple protopectin: preliminary study of enzymic extraction. In: J.P. Roozen, F.M. Rombouts, A.G.J. Voragen (Eds.): Food Science: Basic Research for Technological Progress. PUDOC, Wageningen, The Netherlands.
- Richardson, T.; Hyslop, D.B.; 1985. Enzymes. In: O.M. Fennema (Ed.): Food Chemistry. Marcel Dekker, Inc., New York and Basel. p.371-476.
- Rombouts, F.M.; 1981. In: C.G. Stewart, M. Moo-Young, I. Russell (Eds.): Current developments in yeast research-proceedings of the fifth International Symposium on yeasts. Pergamon Canada Ltd., Willowdale, Ont., Canada. p. 585-592.
- Rombouts, F.M.; Voragen, A.G.J., Searle-van Leeuwen, M.J.F.; Geraeds, C.C.J.M.; Schols, H.A.; Pilnik, W.; 1988. The arabinanases of Aspergillus niger. Purification and characterization of two  $\alpha$ -L-arabinofuranosidases and an endo-1,5- $\alpha$ -L-arabinanase. Carbohydrate Polymers 9: 25-47.
- Rozie, H.; Somers, W.; Bonte, A.; Visser, J.; Van 't Riet, K.; Rombouts, F.M.; 1988. Adsorption characteristics of endopolygalacturonase on alginate beads. Biotechnology and Applied Biochemistry 10:346-358.

- Ruttloff, H.; 1987. Impact of biotechnology on food and nutrition. In: D.Knorr (Ed.): Food Biotechnology. Marcel Dekker Inc. New York. p. 37-94.
- Schols, H.A.; Posthumus, M.A.; Voragen, A.G.J.: 1989a. Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. Submitted for publication.
- Schols, H.A.; Geraeds, C.C.J.M.; Kormelink, F.J.; Searle-van Leeuwen, M.J.F.; Voragen, A.G.J.: 1989b. Rhamnogalacturonase: a novel enzyme degrading pectin hairy regions. Submitted for publication.
- Schwimmer, S. 1981. Source Book of Food Enzymology. AVI Publishing Co, Westport, Connecticut, USA.
- Scott, D.; Hammer, F.E.; Szalkucki, T.J.; 1987. Bioconversions: Enzyme Technology. In: D. Knorr (Ed.): Food Biotechnology. Marcel Dekker, Inc. New York. p. 413-440.
- Somers, W.; Visser, J.; Rombouts, F.M.; Riet van 't, K.; 1989. Developments in downstream processing of (poly)saccharide converting enzymes. Accepted in Journal of Biotechnology.
- Somers, W.; Rozie, H.; Rombouts, F.M.; Visser, J.; van 't Riet, K.; 1989. Isolation and purification of endo-polygalacturonase by affinity chromatography in a fluidized bed reactor. The Biochem. Engineer. Journal 40:B7-B19.
- Spa, H.A.; Kaper, F.S.; Hokse, H.; 1978. Enzymatische reakties aan zetmeel. Procestechniek 33: 318-321.
- Stanton, R.: 1988. The use of enzymes in the food industry. Food Science and Technology Today 2(3): 181-189.
- Szejtli, I.J.; 1984. Industrial applications of cyclodextrins. In: J.L. Aitwood; J.E.D. Davies; D.D. Macnicol (Eds.): Inclusion Compounds. Academic Press, London, p. 331-389.
- Szejtli, J.; 1988. Cyclodextrin Technology. Kluwers Academic Publishers. Dordrecht, The Netherlands.
- Ueda, S.; Saha, B.S.; Koba, Y.; 1984. Direct hydrolysis of raw starch. Microbiological Sciences 1(1):21-24.
- Unilever; 1988. Biotechnology: Van Wetenschap naar Winkelschap. Unilever Research Laboratorium Vlaardingen, The Netherlands.
- Voragen, A.G.J.; Pilnik, W.; 1989. Pectin degrading enzymes in fruit and vegetable processing. In: J.R. Whitaker; P. Sonnet (Eds.): Biocatalysis and Biomimetrics: Aspects of enzyme chemistry for agriculture. ACS Symposium Series, American Chemical Society, Washington, DC, in press.
- Voragen, A.G.J.; Rombouts, F.M.; Searle-van Leeuwen, M.J.F.; Schols, H.A.; Pilnik, W.; 1987. The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from Aspergillus niger. Food Hydrocolloids 1: 423-437.
- Voragen, A.G.J.; Schols, H.A.; Pilnik, W.; 1988. Non-enzymatic browning of oligogalacturonides in apple juice models. Zeitschrift Lebensmittel Untersuchung und Forschung 187: 315-320.
- Voragen, A.G.J.; Schols, H.A.; Siliba, H.A.I.; Pilnik, W.; 1986b. Enzymic lysis of pectic substances in cell walls. Some implications for fruit juice technology. In: M. Fishman, J. Jen (Eds.): Recent advances in the chemistry and functions of pectins. ACS Symposium Series, American Chemical Society, Washington, DC. 310: 230-247.
- Voragen, A.G.J.; Wolters, H.; Verdonschot-Kroef, I.; Rombouts, F.M.; Pilnik, W.; 1986a. Effect of juice-releasing enzymes on juice quality. In: Berichte Wissenschaftliche Technische Kommission, Internationale Fruchtsaft-Union, Proceedings of the XIXth International Fruit Juice Symposium, The Hague, The Netherlands. Juris-Verlag, Zürich, Switzerland. p. 453-462.

- Vos de, L.; Pilnik, W.; 1973. Pectolytic enzymes in apple juice extractions Process Biochem. 8(8): 18-19.
- Voordouw, G.; Voragen, A.G.J.; Pilnik, W.; 1974. Apfelsaftgewinnung durch Maischefermentierung. III. Der Einfluss spezifischer Enzymaktivitäten. Flüssiges Obst, 41(7): 282-284.
- Vries de, J.; 1989. Polysaccharide chemistry: Three examples of basic research for technological progress in the food area. In: J.P. Roozen, F.M. Rombouts, A.G.J. Voragen (Eds.), Food Science: Basic Research for Technological Progress. PUDOC, Wageningen, The Netherlands.
- Vries de, J.; Voragen, A.G.J.; Rombouts, F.M.; Pilnik, W.; 1986. Structural studies of apple pectins with pectolytic enzymes. In: N. Fishman, J. Jen (Eds.): Recent advances in the chemistry and functions of pectins. ACS Symposium Series, American Chemical Society, Washington, DC. 310: 38-48.
- Wasserman, B.P.; 1984. Thermostable enzyme production. Food Technology 38(2): 78-89.
- Wegener, Ch.; Henniger, H.; 1987. Charakterisierung und Einsatzmöglichkeiten einer Pektatlyase aus Erwinia carotorera. Nahrung 31: 297-303.
- West, S.I.; 1988. Enzymes in the food processing industry. Chemistry in Britain (12) p. 1220-1222.
- White, T.J.; Meade, J.H.; Shoemaker, S.P.; Kothe, K.E.; Innis, M.A.; 1984. Enzyme cloning for the food fermentation industry. Food Technology 38(2): 90-98.
- Yamasaki, M.; Kato, A.; Chu, S.Y.; Arima, K.; 1967. Pectic enzymes in clarification of apple juice. Part II: The mechanism of clarification. Agric. biol. Chem. 31:552-560.

## PROGRESS IN FOOD PRESERVATION PROCESSES

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### Summary

The food industry is challenged to meet current consumers' wishes with respect to safety, wholesomeness, taste, appearance and fresh or fresh-like character of food products. Nowadays a wide choice of physical preservation methods has become available. It includes existing methods based on (1) application of heat like pasteurization and sterilization, (2) removal of water like evaporation and drying, (3) reduction of the food temperature and (4) centrifugation and filtration. Newer methods dealt with include microwave heating and Ohmic heating. Potential benefits of the different methods as well as potential deleterious effects on the structure and the taste of food are discussed.

Descriptors: pasteurization, sterilization, evaporation, drying, refrigeration, centrifugation, filtration, microwaves, Ohmic heating.

### Introduction

The aim of modern food processing is to produce food at a specified, constant quality level at lowest possible costs. Quality requirements include first of all the microbiological and toxicological safety of the product at a certain shelf-life period, whereas increasing emphasis is laid upon the nutritional properties of the food as well as on the sensoric characteristics of the product. Nowadays observed consumers' interests are highly focused on fresh or fresh-like properties of products. With this trend on the market today, many thermally processed foods are experiencing a decrease in popularity and in sales volumes. As a result the processor will lay emphasis on minimizing the disruption of desired quality properties as for instance natural taste, smell and consistency. Moreover, maintaining the nutritional quality or even enhancing it is considered to be of great importance.

These quality requirements can be considered as the driving force that has led to several interesting developments in food preservation techniques. In this article these developments are reviewed, both with respect to existing technologies as well as for new techniques which may become of greater commercial interest in the near future.

Methods of preservation can roughly be divided into physical and (bio)chemical methods. This survey is restricted to the physical preservation methods, such as methods based on (1) application of heat (blanching, pasteurization, sterilization), (2) removal of water (evaporation, drying), (3) reduction of the temperature of the food (refrigeration, freezing), (4) separation (centrifugation, filtration) (5) application of electric energy (Ohmic heating) and (6) radiation (gamma rays, infrared, ultraviolet, microwaves).

## Application of heat

During thermal processing many different changes occur in the quality of food. In those cases where heat is applied as a preservation technique only, these changes are often undesirable; examples are loss of natural taste, smell and structure as well as reduction of nutritional value or discoloration. Sometimes application of heat is not only used for preservation, but also in order to achieve desired changes such as softening of the texture, development of cooked food aroma, destruction of antidigestive factors as trypsin and amylase inhibitors or improvement of the bio availability of proteins and carbohydrates (I.F.T., 1986). At some stage of the heat treatment an optimal thermal treatment or cook is reached, at which point the different food quality attributes are at their best. This is of course the heat treatment that should be strived for in the design of processes in terms of time-temperature combinations.

Usually the z-values (which are defined as the increase in temperature in order to accelerate a reaction 10 times) of quality changes (as Maillard-product formation or break-down of texture) are twice to three times as high as the z-value of bacteriological inactivation. In spite of this, undesired quality changes often occur. This is due to the problem that the desired F-value in the centre of the food cannot be reached fast enough, which results in very long processing times. Various techniques have been developed in order to overcome these problems. In fact these techniques are usually working under UHT or HTST conditions.

In a number of cases it appeared to be possible to use the direct contact heating technique, either by steam injection (treatment of the product in a closed vessel with super-heated steam) or by steam infusion (transporting the liquid food continuously through an atmosphere of pure steam). Sterilization by direct steam injection is used as a pretreatment preceding evaporation.

Nowadays, the majority of sterilization plants have indirect systems. In fact three different types of heat exchangers can be used: the tube-type, plate type, or scraped surface heat exchanger. It is almost needless to say that the choice of the type of heat-exchanger largely depends on the properties and the desired quality of the final product.

At the present there is also much interest in optimizing sterilization treatments. This may be done by using cook values C to measure quality changes during thermal processing. The C-value is defined in a similar way to the F-value, used for evaluation of the sterilization efficiency and selection of the correct process. Ohlsson recently presented a study on C-values (Ohlsson, 1988). He concluded that only for true HTST processes such as aseptic processing or microwave sterilization the cook-values are in the same range as the optimal values.

The empirical models based on z and C-curves are most widely used at the moment. New techniques and developments are calling for more accurate calculation methods, i.e. the Arrhenius model. This is also favoured by modern calculation techniques (Hallström, 1988).

Another system was recently presented by Stork, the so-called "In-flow" FSTP system (= Fraction Specific Thermal Processing) of liquid foods containing particles (Hermans, 1988). The system is characterized by the presence of one or two selective holding sections in a continuous in-line sterilization system; in the holding section the particles are separated from the liquid; the particles remain in the holding section during a certain time while the liquid is flowing through. Essentially,

the residence time of the particles in the sterilizer is longer than the residence time of the liquid, thus creating optimal F and C-values for particles and liquid separately. The system was presented as a pilot-plant system; further commercialization in the food industry will have to be awaited.

### Removal of water

#### Evaporation

Falling film evaporators are widely used in the food industry; either to produce consumer products like fruit concentrates and condensed milk or as

a preconcentration step before drying. In the dairy industry much effort used to be directed towards reduction of energy consumption. This resulted in a gradual replacement of 3 to 4-stage evaporators by 5, 6 or even 7-stage evaporators. Today there is an increasing interest in the effect of evaporator-design and processing-conditions on the quality of the concentrate to be produced. Even a quite superficial survey of the literature shows that optimal evaporator-design and optimal process conditions are largely product dependent. In order to clarify this statement, two examples will be mentioned.

The first one refers to evaporated milk. It is well-known that the total time-temperature treatment during preheating and evaporation influences the stability and quality of the end product. The consumer expects a stable product without formation of deposit. According to De Wit (1977) the formation of such a deposit may occur during sterilization of the concentrate and during storage, as a result of improper sterilization conditions; it depends on the denaturation of whey proteins in combination with calcium phosphate salts that have become insoluble (De Wit & Klarenbeek, 1989). It occurs especially in the concentrate, as its protein and salt percentage is very high. In order to avoid formation of deposits, the total time-temperature treatment during preheating of the milk and the subsequent evaporation process is extremely important. During preheating and evaporation denaturation of whey proteins may occur, in fact denaturation starts above 70 °C. It has been established that formation of deposits in the concentrate during sterilization can be avoided by a pretreatment of the milk of approximately 3 minutes at 115 - 120 °C. Due to that treatment a part of the whey proteins denaturates in the milk; the denaturated molecules form a deposit of the casein micels and the fat-droplets; as a result of this mechanism aggregation of denaturated whey proteins both in milk as well as in the concentrate can be avoided. Obviously, a part of the whey proteins denaturates during preheating and evaporation. The remaining part contributes to the viscosity of the concentrate. Therefore, both preheating treatment and evaporation are critical steps: time-temperature treatment should be sufficient to assure that a part of the whey proteins will denaturate in order to avoid formation of deposits during sterilization of the concentrate whereas the remaining part of undenaturated proteins should be large enough to take care of the required viscosity level of the concentrate.

The second example refers to tomato-paste. In the tomato industry it is common to concentrate tomato-juice of 5 % soluble solids six or even eight times by means of two- or three-stage evaporators. The gross-viscosity of the tomato-paste is considered to be one of the most important quality parameters. Investigations by Heutink (1986) showed

that the gross-viscosity of paste does not depend on the type of evaporator used or the temperatures employed during the evaporation process. The viscosity, plotted as a function of the percentage of insoluble solids for different evaporators under various processing conditions showed a linear relationship. Further, it was observed that the percentage of dilution-loss, which is defined as the difference between the viscosity of the juice before evaporation and the viscosity of diluted paste (measured as single strength juice) was not temperature-dependent, but shear-dependent. Heutink found a linear correlation between the percentage of evaporated water and the percentage of dilution-loss. This correlation did not depend on the temperature treatment in the evaporator.

The conclusion from a comparison between the two examples can be formulated as follows: each product to be concentrated requires its specific design of equipment and tailored processing-conditions. Therefore, it is essential to know whether the product quality is shear- or heat-dependent. This sets new challenges to research on evaporation, provided that knowledge on physical properties and composition of the product will be fruitfully combined with expertise in engineering and design of equipment.

#### Reversed osmosis

In connection with the subject of water removal by evaporation it should be mentioned that in some cases it was found to be useful to remove a part of the water by reversed osmosis. This preconcentration step is known for milk, whey, sugar solutions and fruit juices. Usually, the main reason for selecting this technique is its inherent low costs of water removal (Hanemaaijer, 1985; Versteeg, 1985).

#### Freeze-concentration

A technique of water removal which will leave the quality of the food relatively unchanged is freeze-concentration. In a recent summary Van Pelt (1988) mentioned that during the last few years much effort has been paid to take away the original disadvantages of the process. It was shown that reduction of investment costs and energy requirements could be achieved by upscaling the installations. Industrial applications are found for fruit juice, beer, vinegar and coffee.

#### Drying

In the area of drying, progress has been made by the introduction of new types of dryers, the multi-stage spray dryer (MSD) and the so-called Filtermat dryer, both presented in the late seventies and early eighties. Driving force behind these developments was not only the need for reduction of energy consumption; there was also interest in the improvement of product quality and the creation of additional manufacturing techniques supporting new product development. Such a product development area is the production of agglomerated powders. In Figure 1 a flow diagram of the MSD is presented. It is a diagram of the dryer in the pilot plant of NIZO.

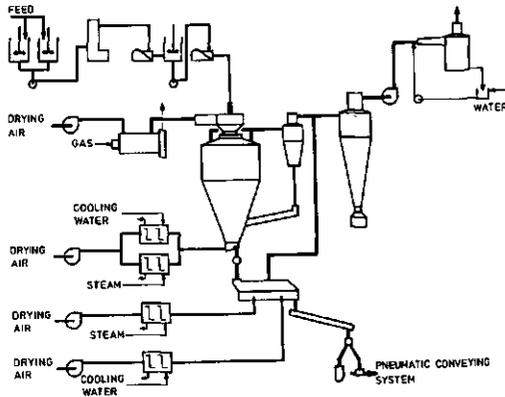


Figure 1. Integrated fluid bed spray dryer MSD.

By using a high pressure feed pump concentrate is pumped to the top of the spray drying chamber, where a single nozzle assembly is mounted. Drying air (220 - 245 °C) enters from the top around the atomization zone and is directed downwards towards the chamber base. Drying air is then exhausted from the top of the chamber through two ducts. An integrated fluid bed is located at the base of the drying chamber. A powder layer (0,75 m) is formed in the fluid bed and is held under vigorous fluidization. The atomized spray of concentrate contacts the fluidized powder creating ideal conditions for agglomeration. The powder is discharged from the drying chamber via a rotary valve into a vibrating fluid bed.

The MSD differs from conventional one and two-stage spray dryers in many ways. It is important that it can be operated both as a producer of agglomerated products and as a producer of standard powders. It is possible to control the properties of the final product such as particle size and insolubility index; temperature of the inlet and outlet air, the size of the droplets of concentrated milk after spraying as well as residence time in the drying chamber are of importance. In Table 1 examples of the properties of a skim-milk powder obtained by MSD processing are shown (Van Mil & Hols, 1988).

Of particular importance is first of all the large average particle size and especially, the low percentage of particles smaller than 100 µm. Secondly instant properties as insolubility index, wettability and dispersibility prove even better than those required for instant powder. In the literature specifications for non-fat instant powder require an insolubility index of 1.0 and a dispersibility of 85 % (Pisecky, 1986).

A different new drying technique which should be mentioned is the Filtermat dryer. In fact it is also a three-stage dryer with a moving polyester belt as second drying stage; the concentrate is atomized in a drying chamber (the first stage); the powder reaches the moving belt, which is located on the bottom of the drying chamber; the belt has an open filter-like structure and there is a continuous flow of drying air through the belt (the second stage); finally, the belt transports the powder to a secondary drying chamber (the third stage). The process is particularly suited for products which are difficult to dry by using

regular one or two-stage dryers, for instance sticky products with a high fat content.

Table 1. Properties of powder obtained by MSD Processing

Property	Skim-milk powder	Instant whole milk powder
Moisture %	3.5	2.5
Particle density (g/ml)	1.37	1.24
Bulk density (g/ml)	0.44	0.35
Bulk density (tapped)(g/ml)	0.49	0.41
Particle size		
average (um)	277	438
% <100 um	5.3	5.5
ISi (ml)	0.05	0.05
Free fat (%)	-	1.87
Wettability (s)	21	6
Dispersibility (%)	99	97
Flowability (s)	14	20

Reduction of the food temperature

It is generally recognized that frozen and chilled foods have obtained an important position on the market nowadays (Venkat, 1988). Considerable attention has been paid to factors that influence the microbiological quality of these foods (Nickerson & Sinskey, 1977). The critical steps for frozen foods were found to be the stages of pre-cooling and after thawing. Technological progress has been made especially in these areas. It has been reported that several disadvantages of conventional thawing as growth of bacteria, drip loss, surface oxidation and color changes can be avoided by use of microwaves (Rosenberg & Bögl, 1987a). Nowadays it is considered to be a good manufacturing practice to give a short and efficient blanching treatment to the product to be frozen. Furthermore, modern equipment enables the processor to achieve quick and efficient freezing rates, thus preventing outgrowth of micro-organisms during this critical stage. Much effort is given to the development of computer models that are able to calculate the freezing and thawing behaviour of foods (Hendrickx et al., 1988).

Keeping foods at refrigerator temperatures above freezing has become common practice. There is an increasing number of companies offering ready-to-serve meals, pasteurized and kept under chilled conditions (2-5 °C). The shelf-life of these products may vary between 4 and 6 weeks depending on the type of meal, the quality of the ingredients, pretreatment of the meal before refrigeration and hygienic conditions during processing. An extremely critical factor is keeping the proper

temperature during storage (Notermans & Beckers, 1988). Elimination of logistic bottlenecks is of crucial importance in order to make the most of the shelf-life period for the consumers' benefit. Modern pasteurization techniques as microwave are becoming part of complete processing lines for the production of refrigerated meals; at the present in the Netherlands two companies are known to use microwave as pasteurization technique and to put a complete series of chilled pasta-meals on the market place.

### Centrifugation and filtration

In food processing another route exists to achieve reduction of microbiological counts without affecting other quality parameters. This technique splits the main food stream into two streams, one carries the micro-organisms, the other one carries the sensitive food components. Both streams are sterilized separately and recombined after treatment. In this connection two examples should be mentioned, one based on centrifugation and the other on microfiltration.

The first one, a classical example of a split-stream process, is the process of reducing spores of butyric acid bacteria in cheese milk by means of a bacteria removal separator or Bactofuge (van den Berg et al., 1988). The Bactofuge was based on the observation that effective separation between bacteria concentrate and product could be obtained by means of a centrifugal milk clarifier if the bacteria concentrate was continuously removed from the bowl. Spores of butyric acid bacteria in cheese milk may lead to butyric acid fermentation in cheese, which is associated with the occurrence of the defect of "late blowing". Particularly the larger types of semi-hard and hard cheese, such as Gouda, which are salted after souring by means of a brining process, are susceptible to these micro-organisms. The Bactotherm process offers a method of physical nature to improve the bacterial quality of cheese milk. The process is described in Figure 2. Pasteurized cheese milk is fed to the Bactofuge.

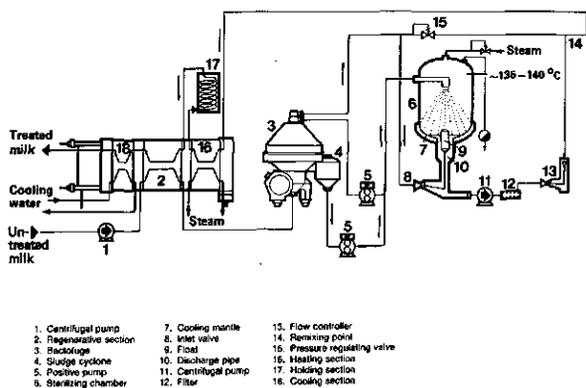


Figure 2. Bactotherm process line.

Bacterial bacto-fugate and bacteria-rich sludge are pumped into the sterilizer and heated to at least 130 °C. Next the sterilized bacto-fugate and bacto-fugated milk are mixed, pasteurized and cooled to the renneting temperature. The average decrease in the number of spores of butyric acid bacteria was found to be between 97.4 and 98.7 % depending on the temperature of bacto-fugated cheese milk. Bacto-fugation of cheese milk has been put into practice in the Netherlands since the beginning of the seventies.

As a second example microfiltration should be mentioned. Its potential use to retain bacteria from liquid food products has been studied in the dairy industry, the juice industry and breweries (Strathmann, 1988). Microfilterable particles lie within the range of 0.1 - 1.0 microns. It has long been known that a membrane filter with a pore size of approximately 0.2 micron can filter bacteria from a water solution. Theoretically, this technique could be used to reduce the amount of bacteria and spores in skimmilk (Malmberg & Holm, 1988). The choice of the pore size of the filter is extremely important in order to obtain selective removal of bacteria; if a smaller pore size is chosen the filter will rapidly be blocked by protein. The process is as follows: milk is centrifuged and the skim-milk is filtered; the retentate which involves 10 % of the skim-milk then contains nearly all the bacteria. Next retentate and cream can be sterilized and mixed with the low-bacteria filtered skim-milk, the permeate. The resulting milk was found to be superior in quality as compared to regular heat-sterilized milk. The microfiltration technique used in the example is cross-flow filtration. This means that the product, in this case the skim-milk, circulates at a high speed along the filter surface at the same time as a part of the circulating milk, the retentate, is bled off to avoid too much concentration of fat, protein and bacteria. It was observed that the fouling rate of the microfilter can be reduced by application of the cross-filtration technique. Nowadays microfiltration is applied commercially in Sweden for part of the production of consumer milk. It remains to be seen how quickly microfiltration as a sterilization technique will penetrate into other segments of the food industry. However, it can be expected that split-stream processes will prove increasingly to be useful in order to meet requirements for high quality fresh or fresh-like foods.

#### Radiation and application of electric energy

In this area many new techniques draw the attention of potential users. These techniques include treatments by infrared or ultraviolet radiation, gamma-rays, microwaves, electrical high voltage impulses and Ohmic heating.

#### Infrared radiation

The potential uses of infrared radiation have been described by Van Zuilichem et al. (1988): these included inactivation of trypsin inhibitors and lipoxigenase in soy flour, microbiological decontamination of spices, seed-dehulling of cocoa-beans and decontamination of a variety of cocoa products. Commercial applications of infrared are reported to be found in the cocoa-industry today.

## Gamma-radiation

The situation with regard to gamma-radiation was recently summarized by Diehl (1988). It was mentioned that the existing knowledge on food irradiation was collected 10 to 20 years ago. The bottleneck for general application of this technology is the present situation of legal uncertainty. Large differences exist in national regulations, not at least for as far as the issue of labeling is concerned. Commercial uses of food irradiation presently include treatment of spices, dehydrated vegetables, food ingredients as gum arabic, potatoes and onions for sprout inhibition and frozen seafood and chickenmeat.

## Microwaves

Among the new processing techniques preservation by means of microwaves is industrially used in several applications. Microwaves are non-ionizing electromagnetic waves which generate vibrations in water molecules contained in the food, resulting in a heating effect.

In a recent publication Rosenberg and Bögl (1987b) discussed the question whether a substance or micro-organisms subjected to microwave treatment may be expected to show changes that are not due to heat development only in addition to the purely thermal effects of microwave energy. Based on an extensive survey of the literature they concluded that microwave energy is very well suited for pasteurization and sterilization of food. The short time needed for heating, and thus the relative low thermal exposure of the food, is advantageous. Beside the speed of heating there is also the absence of large temperature gradients which are almost unavoidable in conventional processing. Examples of commercial applications are in the first place tempering of frozen food from its frozen storage temperature to a temperature that is higher but still below the freezing point of water. The food remains frozen, so that no drip and weight-loss, bacterial growth or other deleterious effects can occur. A second application is the use of microwaves for the pasteurization of chilled-foods, the advantage of the relatively low thermal exposure (2 till 4 minutes at 80 °C) is obvious: damage to structure of the food and deleterious effects on its taste are avoided whereas an acceptable shelf-life period can be achieved as a result of sufficient microbiological reduction during processing. Other examples of industrial application include the cooking of meat and meat-products as well as the dehydration of for instance pasta (Steele, 1987; Decareau, 1986). The development of a special, continuous microwave vacuum dryer for drying fruit concentrates has been reported (Steele, 1987).

Recently, it was concluded that if the trend towards application of precooked

products in the restaurant business continues and even spreads to consumer products, industrial microwave processing will gain significant importance. In the meantime it has been recognized that much research still has to be done. Areas of interest are (1) the penetration depth in the product as a function of the product temperature, moisture content of the product, dielectric activity, and wave frequency, (2) the absorption of energy in the first few centimetres of the product resulting in overheating on the product surface, (3) the need for knowledge of three-dimensional temperature profiles within solid foods and (4), the need for sensors which will enable us to study the temperature in the product during processing. It is expected that the

differences in costs between microwave and conventional processing techniques in terms of capital, labor, space, energy saving and reduced product losses will diminish; this will provide an incentive for development of new applications including those based on combinations of conventional and microwave treatments (IFT, 1989).

### Ohmic heating

Finally, preservation by Ohmic heating should be mentioned. This process is based on the utilization of heat which is produced within a certain food when an electric current is passed through it. The technique requires products with an acceptable conductivity; usually this refers to pumpable products with a water content of at least 40 % and sufficient salts. The heating effect is similar to that obtained by microwaves to the extent that electrical energy is transformed into thermal energy volumetrically throughout the products. In contrast to microwave heating it seems that in employing Ohmic heating the depth of penetration is not limited. Similar to microwave heating it is difficult to achieve homogeneous heating of particles larger than 25-30 mm. A process schedule is shown in Figure 3.

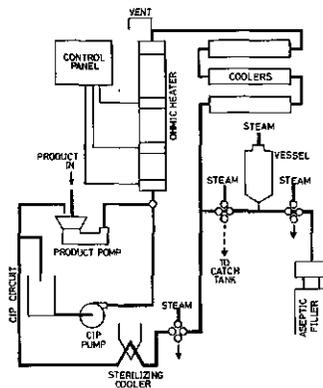


Figure 9. Ohmic heating system.

Ohmic heating has been tested on pilot-scale as a continuous sterilization technique which could be combined with aseptic packaging. Major advantage of the technique is that due to very rapid rates of heating sterilization temperatures can be increased to 140 °C without significant damage to either the liquid phase or the particulates. Successful tests on sliced vegetables, pieces of meat, whole fruits, pasta and soup have been reported (Messelink, 1988; Skudder, 1988).

## Discussion

Nowadays many preservation methods have become available for the food processing industry. Designers and builders of equipment offer new techniques as well as modifications of existing techniques, which will enable the food processor to produce foods according to the consumers' wishes with respect to safety, wholesomeness, taste, appearance and fresh or fresh-like character. Development in food processing both in the past and in the future greatly depends on progress made in basic sciences as physics, chemistry and microbiology as well as in the area of process engineering. It is obvious that an interdisciplinary approach is the basic requirement for progress in food processing. In fact it is a challenge to food producers and equipment suppliers to combine efforts in order to achieve mutual benefits.

## References

- Van den Berg, G., C.B.G. Daamen & J. Stadhouders, 1988. Het bactofugeren van kaasmelk. *Zuivelzicht* 1: 22-24.
- Decareau, R.V., 1986. Microwave food processing equipment throughout the world. *Food Technology*. 40(6): 99-105.
- Diehl, J.F., 1988. Food irradiation and combination processes: the state of the art. In: *Progress in food preservation processes I*: 237-247. Ceria, Brussels.
- Hanemaaijer, J.H., 1985. Microfiltration in whey processing. *Desalination* 53: 143-155.
- Hallström, B., 1988. Heat and food products, p. 25. Elsevier, New York.
- Hendrickx, M., K. van Genechten & P. Tobback, 1988. Transmission line matrix (TLM)-models for predicting freezing and thawing times of foods. In: *Progress in food preservation processes I*: 81-99. Ceria, Brussels.
- Hermans, W.F., 1988. In flow fraction specific thermal processing of liquid foods containing particulates. In: *Progress in food preservation processes I*: 207-212. Ceria, Brussels.
- Heutink, R., 1986. Tomato juices and tomato juice concentrates: a study of factors contributing to their gross viscosity. Dissertation Agricultural University, Wageningen, Netherlands.
- I.F.T., 1986. Effects of food processing on nutritive values. *Food Technology* 40(12): 109-116.
- I.F.T., 1989. Microwave food processing. *Food Technology* 43(1): 117-126.
- Malmberg, R. & S. Holm, 1988. Producing low bacteria milk by microfiltration. *European Food and Dairy Journal* 54(1): 30-32.
- Messelink, G.J., 1988. Ohmic heating: een nieuwe techniek voor het steriliseren van voedingsmiddelen. *Voedingsmiddelentechnologie*. 23: 47-48.
- Van Mil, P.J.J.M. & G. Hols, 1988. Eigenschappen van enkele zuivelproducten gedroogd in een meerfasendroger. *Voedingsmiddelentechnologie* 21: 21-24.
- Nickerson, J.T. & A.J. Sinskey, 1977. *Microbiology of foods and food processing*. Elsevier, New York.
- Notermans, S.H.W. & H.J. Beckers, 1988. Gepasteuriseerde maaltijden en botulisme: risico-evaluatie. *Voedingsmiddelentechnologie* 23: 42-45.
- Ohlsson, T., 1988. The cook value as tool in optimizing thermal processes. In: *Progress in food preservation processes I*, 51-58. Ceria, Brussels.

- Van Pelt, W.H.J.M. & H.A. Jansen, 1988. Freeze concentration economics and applications. In: S. Bruin (ed), Preconcentration and drying of food materials, 77-86. Elsevier, Amsterdam.
- Pisecky, J., 1986. Standards, specifications and test methods for dry milk products. In: D.McCarthy (ed), Concentration and drying of foods. Elsevier, London.
- Rosenberg, U. & W. Bögl, 1987a. Microwave thawing, drying and baking in the food industry. *Food Technology* 41(6): 85-91.
- Rosenberg, U. & W. Bögl, 1987b. Microwave pasteurization, sterilization, blanching and pest control in the food industry. *Food Technology* 41(6): 92-99.
- Skudder, P.J., 1988. Development of the ohmic heating process for continuous sterilization of particulate food products. In: Progress in food preservation processes I, 271-279. Ceria, Brussels.
- Steele, R.J., 1987. Microwaves in the food industry. *CSIRO Food Research Quarterly* 47: 73-78.
- Strathmann, H., 1988. Stand der Membrantechnik und ihre wirtschaftliche Bedeutung. *Swiss Chem.* 10: 8-16.
- Venkat, K., 1988. From spartan simplicity to epicurean delight: technological challenges to the food industry. In: *Food Science: basic research for technological progress*. Pudoc, Wageningen.
- Versteeg, C., 1985. Process and quality control aspects of reverse osmosis concentration of milk. *Australian Journal of Dairy Technology*. 40: 102-107.
- De Wit, J.N., 1977. De invloed van verhitten en homogeniseren op de eigenschappen van emulsies in zuivelprodukten. *Voedingsmiddelentech nologie*. 47/48: 12-16.
- De Wit, J.N. & G. Klarenbeek, 1989. Technological and functional aspects of milk proteins. In: C.A. Barth & E. Schlimme (ed.), *Milk proteins*, 211-222, Steinkopff, Darmstadt.
- Van Zuilichem, D.J. B. Schulte & W. Stolp, 1988. Directe verwarming van levensmiddelen door infrarode warmtestraling. *Voedingsmiddelentech nologie* 23: 35-40.

# Microbiological papers

## MICROBIOLOGICAL METHODS TO EVALUATE THE INNER QUALITY OF ORANGE JUICES

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### Summary

Heated orange juices have mutagenic and cytotoxic effects on Salmonella typhimurium TA 100 under specific conditions. These effects were dose related and related to the amount of heat load and can be ascribed to Maillard intermediary products (MIP), since the heat treatments were mild and did not cause detectable colour and flavour changes. The method can be used as a rapid and inexpensive measure of heat load for prediction of shelf-life of orange juices. This would help the fruit-juice technologist in establishing the conditions of heat treatments and storage and in evaluating the quality of (concentrated) juices.

Descriptors: orange juice; heat load; Maillard reaction; inner quality; shelf-life; mutagenicity; cytotoxicity.

### Introduction

Most orange juices on the market are reconstituted from concentrates. As can be seen in Fig. 1 the juices receive at least two heat treatments, one from concentration and a second one from pasteurization before and/or during filling. Juices packed directly are heat treated for pasteurization to inactivate enzymes (pectinesterase) and to prevent microbiological spoilage. Although high technology in citrus processing results in high quality products, the products received by the consumer do not always have this high quality. Browning and development of off-flavours during storage and distribution are responsible for the quality loss of the product (Handwerk & Coleman, 1988; Klim & Nagy, 1988).

### Browning of orange juice

This phenomenon is due to non-enzymic reactions between reducing sugars and amino acids, called Maillard reaction after the French chemist Louis Maillard who published his observation in 1912 that solutions containing amino acids and reducing sugars turn brown on heating (Mauron, 1981; Danehy, 1986). In the meantime it has been shown that Maillard reaction also lead to off-flavours in orange juice (Varsel, 1980; Marshall et al., 1986; Graumlich et al., 1986).

Orange juice contains sugars, amino acids and ascorbic acid which can undergo Maillard reactions during heat treatment and storage. These reactions are initiated by the formation of Maillard intermediary products (MIP), which are not themselves perceived sensorially but which are the precursors reacting further to form brown pigments and off-flavours. According to Lee & Nagy (1988b) important precursors in citrus juices are also ascorbic acid and acid catalysed break-down products of reducing sugars. The shelf-life of (reconstituted) juices therefore depends to a large extent on the concentration of MIP at the moment of

packing. The concentration of MIP is therefore an indicator for the inner quality of orange juices.

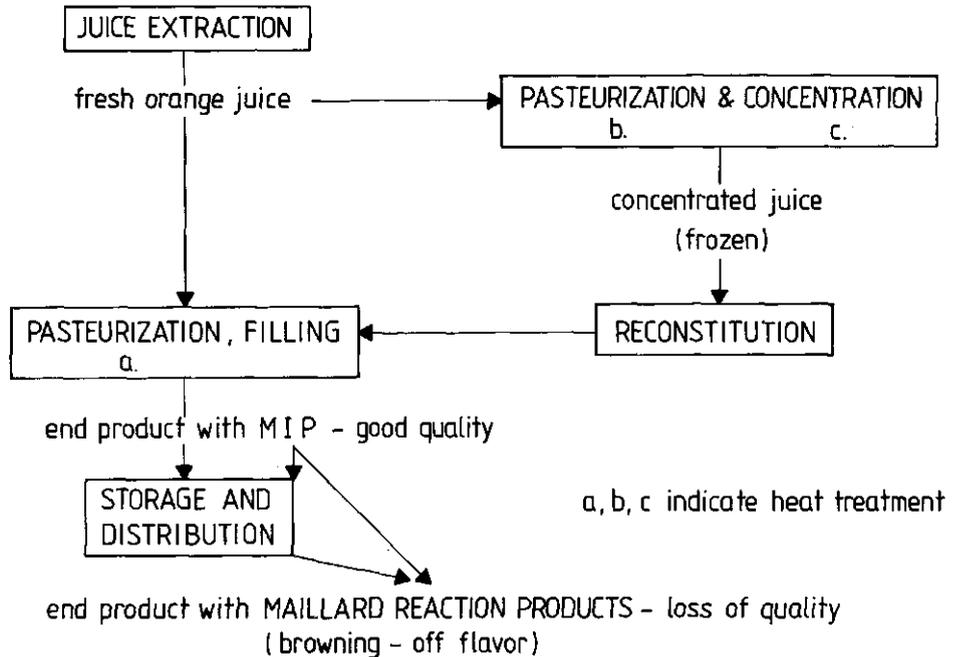


Fig. 1. Flow sheet of processing of orange juice.

#### Early detection of Maillard reaction products at subsensory levels

So a rapid and simple method of measuring MIP would be very useful to manufacturers of orange juice. Unfortunately lack of knowledge of the chemical nature of MIP has so far prevented elaboration of a chemical analytical method. Methods used such as colour change measurement (Meydav et al., 1977; Mannheim & Passy, 1979; Robertson & Reeves, 1981; Kanner et al., 1982), determination of furfural (Nagy & Randall, 1973; Kanner et al., 1982) and hydroxymethyl furfural (Wucherpfennig & Burkardt, 1983; Lee & Nagy, 1988a) contents, and loss of ascorbic acid measurement (Curl, 1949; Saguy et al., 1978; Maeda & Mussa, 1986; Trammell et al., 1986) describe an advanced stage of Maillard reactions, at which sensory deterioration is already noticeable. We have therefore developed microbiological methods to measure concentration of MIP as indicator of the heat load which orange juices have received. It is hoped that these methods will help processors to assess the inner quality of concentrated juices used as a raw material and to examine conditions of heat treatment in their own plant. In this way the shelf-life of juices may be prolonged by choosing conditions for further heat treatments that take account of heat load from previous treatments.

### Mutagenicity according to Ames

The Ames test measures mutagenicity by using several mutant strains of the bacterium Salmonella typhimurium as tester. This mutant cannot make the essential amino acid histidine (HIS). However exposure to mutagens corrects the defect, enabling some of the bacteria to grow forming colonies on minimal agar plates containing only trace of HIS (Maron & Ames, 1983). The bacteria acquiring this correction (reversion) are called revertants; their numbers per dose of mutagen is a measure of the mutagenic activity.

The literature reports mutagenic activity of Maillard browned foodstuffs in the Ames test (Pariza et al., 1979; 1983). Moreover model systems of solutions containing equimolar concentration of sugars and amino acids heated intensively at 100 °C - 121 °C for 1-10 h have been reported to show mutagenic activity towards Salmonella typhimurium strain TA 100 (Shinohara et al., 1980; Powrie et al., 1981). Based on this information we conducted studies using samples from laboratory prepared orange juice heated at 93 °C for 30 s to 30 min and neutralized to pH 7.4 after heat treatment (Ekasari et al., 1986a). These heat treatments are in the range of industrial conditions and do not result in direct colour change. Commercial juices were also studied without further heat treatment but also neutralized before testing. When using the original protocol of Ames standard plate test, no mutagenic response was observed in any heated orange juice. However such a response did appear when in addition to neutralization the protocol was modified to include 4 h preincubation at pH 7.4 and 37 °C. A detailed description of sample preparation and assay is given in Ekasari et al. (1986a and 1986b). The response can be expressed as mutation ratio (explained on Table 1).

Table 1. Dose-response effects for the mutagenicity of heated orange juices.<sup>a</sup>

Times of heating orange juices at 93 °C:	Mutation ratio <sup>b</sup>				
	amount of sample (ml)				
	0.1	0.2	0.3	0.4	0.5
0 (unheated)	1.4 <sup>c</sup>	1.3 <sup>c</sup>	1.3 <sup>c</sup>	1.2 <sup>c</sup>	1.3 <sup>c</sup>
30 s	2.1	2.8	3.5	3.1	3.3
1 min	3.5	4.6	5.6	4.6	3.7
2 min	2.8	6.2	5.4	4.2	3.3
5 min	2.8	4.6	3.8	3.4	2.7
30 min	3.5	4.3	2.8	-	-

<sup>a</sup> Samples were laboratory-prepared orange juice heated at 93 °C (30 s--30 min) and submitted to the modified Salmonella mutagenicity assay (4 h preincubation at pH 7.4 and 37 °C with Salmonella typhimurium TA 100).

<sup>b</sup> Mutation ratio (number of induced revertant colonies per plate divided by number of spontaneous revertant colonies per plate)  $\geq$  2.0 indicates positive mutagenic response. The number of spontaneous revertants were in the range of 93-174 colonies per plate.

<sup>c</sup> Unheated juice was not mutagenic.

Table 1 shows the mutation ratio depended on heat treatment and dose, a clear indication of the presence of a mutagenic compound or compounds. For both factors, an increase in mutagenicity is followed by a decrease due to a cytotoxic effect. This is also seen in the shape of the dose-response curves obtained when plotting number of revertants against dose of sample (Ekasari et al., 1988). Comparison of a result from a test sample with Table 1 or with the curves in Ekasari et al. (1988) can be used to judge the heat load and/or the inner quality of juices.

It should be noted that there is a limited auxotrophic (non-revertant) growth due to the small amount of HIS in the orange juice and the medium. This auxotrophic growth does not appear as colonies but as a so-called "background lawn", which can only be seen under the microscope. Samples carrying a high heat load i.e. heated for longer than 2 min at 93 °C show a strong cytotoxic effect as indicated by the reduced background lawn of the corresponding agar plate. This observation led us to kinetic measurement of the cytotoxicity.

#### Cytotoxicity measured by conductance changes

Similar to the mutagenic effect, a cytotoxic effect was observed only under our specific test conditions i.e. pH adjustment of samples to 7.4 and 4 h preincubation at pH 7.4 and 37 °C (Ekasari et al., 1986b). We have used the kinetic measurement of the Malthus microbiological growth analyser M128. This instrument monitors changes in conductance of the medium associated with bacterial metabolism; the time required to reach acceleration of conductance changes is defined as detection time (DT, h; Eden & Eden, 1985). We assume that detection time is influenced by the cytotoxic effects. The orange juice samples were prepared as for the mutagenicity assay. The assay procedure was simpler than the modified mutagenicity test and is described in Ekasari et al. (1988). We observed that detection time correlated with the heat load; the unheated juice had the shortest detection time and juice with the highest heat load had the longest detection time.

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

#### Use of the modified Salmonella mutagenicity assay to classify orange juices

We have been able to classify commercial and industrial juices by their mutagenicity data; results were compared with the dose--response curves presented in Ekasari et al. (1988) and interpreted as heat load.

Frozen concentrate ranked between the single strength juice directly packed and the reconstituted juice (Table 2). We may suppose that the directly packed juice had the mildest heat treatment which was only pasteurized once. The frozen concentrate received more heat treatment during concentration and the reconstituted juices were subjected to an even more intensive heat treatment: concentration and pasteurization for aseptic filling. The mutagenicity test therefore does give information on the inner quality of juices. Of course, juices with a lower plus score are not necessarily better than the ones with a higher plus score. These differences may not be relevant in a distribution system where refrigeration is maintained. However, if we would buy concentrated juices for reconstitution, we would certainly be guided by our test.

Table 2. Classification of commercial orange juices by their mutagenic responses<sup>a</sup> interpreted as heat load.

Sample	Package	Presumed heat load <sup>b</sup>	Technological history
Single-strength	carton	+	pasteurized at 82 °C for 4 sec; aseptic-fill
Single-strength	glass	+	unknown
Frozen concentrate	carton can	++	unknown
Reconstituted	Pure Pak	+++	aseptic-fill
Reconstituted	Tetra Brik	+++	aseptic-fill
Reconstituted	PKL-Combibloc	+++	aseptic-fill
Reconstituted	Pure Pak	+++++	aseptic-fill

<sup>a</sup> Modified Salmonella mutagenicity assay (Ekasari et al., 1986a).

<sup>b</sup> Ranged from lowest heat load (+) to highest heat load (+++++).

Table 3 presents results from industrial juices. Juices 1 and 2 were reconstituted juices prepared from the same concentrate and processed on the same date. The mutagenicity test showed that juice 2 carried a higher heat load than juice 1. Indeed juice 2 has been subjected to a higher temperature and was kept at relatively high temperature after canning to dry the can and avoid rusting. This results show that we can also use the test to evaluate process condition in the factory.

Table 3. Heat load of industrial orange juices subjected to tunnel pasteurization.

Sample <sup>a</sup>	Heat treatment	Package	Heat load <sup>b</sup>
Juice 1	74 °C for 14 min	glass	+++
Juice 2	85 °C for 10 min	metal can	+++++

<sup>a</sup> Made from the same concentrate on the same date.

<sup>b</sup> See Table 2 for classification.

### Final remarks

It must be stressed that heat treated orange juices at their natural acidity show neither mutagenic nor cytotoxic effects. These properties are only obtained under specific conditions of the tests, which do not apply to the condition of human consumption of orange juices.

### Acknowledgment

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## References

- Curl, A.L., 1949. Ascorbic acid losses and darkening on storage at 49 °C of synthetic mixtures analogous to orange juice. *Food Research* 14:9-14.
- Danehy, J.P., 1986. Maillard reactions: Non-enzymatic browning in food systems with special reference to the development of flavor. In: C.O. Chichester, E.M. Mrak, B.S. Schweigert (Eds.): *Advances in Food Research* 30. Academic Press, New York. p. 77-138.
- Eden, R.F., Eden, G., 1985. *Impedance Microbiology*. A.N. Sharpe (Ed.). Research Studies Press, Letchworth, England.
- Ekasari, I., Jongen, W.M.F., 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 Chemistry* 21:125-131.
- Ekasari, I., Jongen, W.M.F., Pilnik, W., 1986b. Detection of Maillard intermediary product in orange juice by bacterial mutagenicity assay. Proceedings international symposium federation fruit juice producers 19:409-418. The Hague.
- Ekasari, I., Jongen, W.M.F., Vermunt, A.E.M., Pilnik, W., 1988. Measurement of heat load in orange juices: Use of microbiological methods. *Food Technology* 42(5):124-128.
- Graumlich, T.R., Marcy, J.E., Adams, J.P., 1986. Aseptically packaged orange juice and concentrate: A review of the influence of processing and packaging conditions on quality. *Journal of Agricultural and Food Chemistry* 34:402-405.
- Handwerk, R.L., Coleman, R.L., 1988. Approaches to the citrus browning problem. *Journal of Agricultural and Food Chemistry* 36:232-236.
- Kanner, J., Fishbein, J., Shalom, P., Harel, S., Ben-Gera, I., 1982. Storage stability of orange juice concentrate packaged aseptically. *Journal of Food Science* 47:429-436.
- Klim, M., Nagy, S., 1988. An improved method to determine nonenzymic browning in citrus juices. *Journal of Agricultural and Food Chemistry* 36:1271-1274.
- Lee, H.S., Nagy, S., 1988a. Quality changes and nonenzymatic browning intermediates in grapefruit juice during storage. *Journal of Food Science* 53:168-180.
- Lee, H.S., Nagy, S., 1988b. Relationship of sugar degradation to detrimental changes in citrus juice quality. *Food Technology* 42(11):91-97.
- Maeda, E.E., Mussa, D.M.D.N., 1986. The stability of vitamin C (L-Ascorbic acid) in bottled and canned orange juice. *Food Chemistry* 22:51-58.
- Mannheim, C.H., Havkin, M., 1981. Shelflife of aseptically bottled orange juice. *Journal of Food Processing and Preservation* 5:1-6.
- Mannheim, C.H., Passy, N., 1979. The effect of deaeration methods on quality attributes of bottled orange juice and grapefruit juice. *Confructa* 24:175-187.
- Marshall, M., Nagy, S., Rouseff, R., 1986. Factors impacting on the quality of stored citrus fruit beverages. In: G. Charalambous (Ed.): *The shelflife of Foods and Beverages*. Elsevier Science Publishers, Amsterdam. p. 237-254.
- Maron, D.M., Ames, B.N., 1983. Revised methods of the Salmonella mutagenicity test. *Mutation Research* 113:173-215.
- Mauron, J., 1981. The Maillard reaction in food; A critical review from the nutritional standpoint. In: C. Eriksson (Ed.): *Progress in Food and Nutrition Science* 5. Pergamon Press, New York. p. 5-35.
- Meydav, S., Saguy, I., Kopelman, I.J., 1977. Browning determination in citrus products. *Journal of Agricultural and Food Chemistry* 25:602-604.

- Nagy, S., Randall, V., 1973. Use of furfural content as an index of storage temperature abuse in commercially processed orange juice. *Journal of Agricultural and Food Chemistry* 21:272-275.
- Pariza, M.W., Ashoor, S.H., Chu, F.S., 1979. Mutagens in heat processed meat, bakery and cereal products. *Food Cosmetic Toxicology* 17:429-430.
- Pariza, M.W., Lorets, L.J., Storkson, J.M., Holland, N.C., 1983. Mutagens and modulator of mutagenesis in fried ground beef. *Cancer Research* 43(suppl):2445-2465.
- Powrie, W.D., Wu, Ch.H., Rosin, M.P., Stich, H.F., 1981. Clastogenic and mutagenic activities of Maillard reaction model systems. *Journal of Food Science* 46:1433-1438.
- Robertson, G.L., Reeves, M.J., 1981. Relationship between colour and brown pigment concentration in orange juices subjected to storage temperature abuse. *Journal of Food Technology* 18:535-541.
- Saguy, I., Kopelman, I.J., Mizrahi, S., 1978. Simulation of ascorbic acid stability during heat processing and concentration of grapefruit juice. *Journal of Food Process Engineering* 2:213-225.
- Shinohara, K., Wu, R.T., Jahan, N., Tahaka, M., Moringa, N., Murakami, H., Omura, H., 1980. Mutagenicity of the browning mixtures by amino-carbonyl reactions on Salmonella typhimurium TA 100. *Agricultural Biological Chemistry* 44:671-672.
- Trammell, D.E., Dalsis, D.E., Malone, C.T., 1986. Effect of oxygen on taste, ascorbic acid loss and browning for HTST-pasteurized, single-strength orange juice. *Journal of Food Science* 51:1021-1023.
- Varsel, C., 1980. Citrus juice processing as related to quality and nutrition. In: S. Nagy & J.A. Attaway (Eds.): *Citrus Nutrition and Quality*. American Chemical Society, Washington D.C. p. 225-271.
- Wucherpfennig, K., Burkhardt, D., 1983. Importance of final temperature with regard to the cooling of hot filled fruit juices. *Flüssiges Obst* 50:424-426.

## BASIC RESEARCH ON TRADITIONAL FERMENTED FOODS FOR TECHNOLOGICAL PROGRESS

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### Summary

A brief review is given of studies on some traditional fermented foods at the Department of Food Science, Agricultural University, Wageningen, Netherlands, as continuation of previous investigations in the United States and Indonesia. It includes studies on soyabean tempe, tempe bongkrek, tapé and ragi.

On soyabean tempe, it includes the search for the typical mould species required for the fermentation, the development of an inoculum suitable for the traditional manufacturer as well as for industrial use and on the inhibitive effect of Rhizopus oligosporus and some other species to production of aflatoxin by Aspergillus flavus and A. parasiticus. Aspects on tempe bongkrek include growth and toxin production of Pseudomonas cocovenans, inhibitive effect of R. oligosporus on toxin production by P. cocovenans, prevention of toxin production by NaCl, and chemical analysis of bongkrek acid and toxoflavin.

An inoculum was developed for fermentation of tapé consisting of a mixture of Amylomyces rouxii and Endomycopsis chodati, which were isolated from ragi.

Descriptors: food, fermentation, tempe, bongkrek, tapé, ragi, Rhizopus, Aspergillus, Amylomyces, Endomycopsis, aflatoxin, toxoflavin.

### Introduction

This paper is a brief summary of studies on some traditional fermented foods at the Agricultural University, Wageningen, Netherlands as a continuation of previous investigations in the United States and Indonesia. The motive of these studies was to discover the underlying principles of the fermentation processes. Basic knowledge of these processes is essential to work out ways of improving existing traditional manufacturing methods for the production of foods with better qualities. With few exceptions, the studies were started with traditional food products of which useful scientific data as background information were practically non-existent or at least confusing. Only the products themselves and traditional manufacturing methods could be traced from traditional manufacturers. These people had learned to make the products from their parents and grandparents without knowledge of the underlying biotechnological principles. Consequently, most of the studies started by collecting data from observations of the products themselves and from interviews with manufacturers about the production methods and with sellers and consumers about the desired qualities. These data served as background information for planning further investigations.

The studies on traditional fermented foods started with the investigation of soyabean tempe at the USDA Northern Regional Research

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Center, Peoria, Illinois (NRRC). It was continued at the Institute of Technology, Bandung, Indonesia (ITB), where it was extended with preliminary investigations on other traditional Indonesian fermented foods. Part of these studies were continued at the Department of Food Science, Agricultural University, Wageningen, Netherlands (AUW). These studies provided first information for use of basic research in technological progress.

Due to editorial limitation, this paper is restricted to a brief summary of the studies on soyabean tempe, tempe bongkrek, tapé and ragi. However it is not restricted to those parts of the studies which at AUW, but also mentions previous history.

#### Tempe kedelai (soyabean tempe)

Tempe kedelai or tempe for short is a compact soyabean cake completely covered and penetrated with white mycelium of Rhizopus sp. When the study on tempe started in 1960, it was not clear from the available limited literature of those days, which mould species were responsible for the fermentation of soyabeans into tempe. Reports from various sources differed. Since micro-organisms are the most essential component of a fermentation process, the first concern was to find out which mould species were required for tempe fermentation. For this purpose, many mould cultures from the NRRC culture collection, supposedly originating from tempe or used to make tempe, and newly isolated cultures from fresh commercial tempe sent from Indonesia, were selected and tested for ability to produce good tempe at 25 °C within 40 to 48 h. This preliminary study allowed the conclusion that at least four species of Rhizopus fermented soyabeans into tempe. These were R. oligosporus, R. stolonifer, R. oryzae and R. arrhizus (Ko & Hesseltine, 1961). This part of the study was accompanied by miscellaneous experiments, including comparative tests on the suitability of whole soyabeans, dehulled soyabeans, soyabean grit and extracted soyabean flakes for making tempe, determination of some properties of the isolated mould cultures like maximum, minimum and temperature range for optimum mycelium growth and spore formation, ability to decompose starch and to liquefy gelatin and testing packing materials to replace the traditionally used banana leaves (Hesseltine et al., 1963). These preliminary studies at NRRC were continued by Hesseltine and his staff during the following years and resulted in many publications on various aspects of tempe fermentations.

#### Typical Indonesian tempe mould species

In Indonesia, the study was continued at ITB. A rather extensive survey was set up to determine which mould species were generally used by traditional Indonesian manufacturers to make good tempe. More than a hundred cultures were isolated from more than 80 tempe samples, collected from markets in various parts of Indonesia. Areas where tempe samples were obtained ranged from sea level up to 1000 m altitude with mean temperatures ranging from 15 °C to above 30 °C. Collection sites were selected intentionally to determine whether mould cultures from cooler regions for making tempe would be different from those in warm places. Most of the cultures isolated from tempe of good quality and producing good tempe in pure culture were Rhizopus oligosporus. Obviously, R. oligosporus is the principle species used in Indonesia for the preparation of tempe (Hesseltine et al., 1963).

## Tempe inoculum

At that stage of development, the logical follow-up was to consider preparing a tempe inoculum from a pure culture of R. oligosporus. Traditional tempe starters, whether made from tempe cake or by growing the required mould on certain leaves, are basically correct and satisfactory for small-scale production. For modern production on industrial scale, however, technical modification will be required. So attempts were made to devise a simple method suitable for application by traditional manufacturers and for use on industrial scale.

Most successful was the preparation of an inoculum using traditionally cooked or steamed rice as growth medium. Initially, the rice was inoculated with a pure culture of R. oligosporus. After incubation at 37 °C for 8-9 days, the moulded and dried rice was pulverized. One gram of the powder, which contained  $10^8$ - $10^9$  mould spores, was enough for inoculation of about 2 kg of cooked soyabeans. Once such an inoculum was obtained from a laboratory or other sources, a new supply can be easily made by inoculating a small amount of it to cooked rice and processing it according to the described procedure. This can be done by the traditional manufacturer himself without requiring expensive and sophisticated equipment. The process can be repeated, as required. Although started with a pure culture of R. oligosporus, the resulting inoculum is not pure, because the mould is grown on rice cooked or steamed without pressure. This allowed the survival of bacterial spores. A dry powder made in this way contained  $10^4$ - $10^5$  bacterial spores per gram. However, its quality as tempe inoculum is not necessarily inferior. This was demonstrated in an experimental plant that used the inoculum for more than a year and produced about 75 kg tempe of good quality daily (Rusmin & Ko, 1974).

This experience is in accordance with recent reports. Although not yet closely studied scientifically, tempe made with traditional starters, containing different micro-organisms, is claimed to have better qualities than tempe made with an inoculum containing only a single strain of Rhizopus (Jutono, 1985).

For production on a large scale, it may be preferred to use a pure inoculum or a starter made from a mixture of pure cultures for better control of the process. Pure starters can easily be obtained by minor modification of the method. Instead of cooking or steaming the rice without pressure, it should be sterilized.

The method is therefore suitable for small-scale or for industrial-scale production of tempe.

## Inhibitive effect of R. oligosporus to aflatoxin production

When studies on food fermentation were initiated in Agricultural University in 1968, the general feeling in Western countries about the presence of moulds in foods was not positive. At that time, much attention was paid to the discovery of aflatoxin in moulded peanuts. The death of more than 100 000 turkeys after feeding them on mouldy groundnuts some years earlier made a deep impression. It aroused suspicion about any presence of moulds in foods. This suspicion was automatically also directed to tempe. Although harmful effects of tempe had never been reported, there was not yet any scientific data to substantiate it. The absence of aflatoxin from tempe was briefly mentioned in certain publications at that time, but it was not substantiated by detailed information.

To prove that R. oligosporus does not produce aflatoxin, the following

experiments were designed.

One series of flasks containing synthetic Modified Meyer Medium was inoculated with spores of R. flavus. Another series was inoculated with spores of R. oligosporus and a third with a mixture of spores of the two mould species. The inoculated flasks were incubated as stationary cultures at 30 °C. At certain intervals, one set of 3 flasks, each from a different series, were removed at the same time from the incubator. The cultures containing pure A. flavus and those containing pure R. oligosporus showed normal growth and sporulation. But growth and sporulation of the mixed cultures were much less than the corresponding pure cultures. As could be expected, from the flasks containing only A. flavus, aflatoxin could easily be detected, and aflatoxin could not be detected in cultures containing only R. oligosporus. The mixed cultures were a surprise. It contained far less aflatoxin than the corresponding cultures of pure A. flavus (Figure 1). Similar results were obtained when soyabeans were used as growth substrate instead of a synthetic medium. Since soyabean is the raw material for making tempe, the results indicate that when fermenting tempe is contaminated takes place with an aflatoxin producing strain of A. flavus, the production of aflatoxin is inhibited (Ko, 1974).

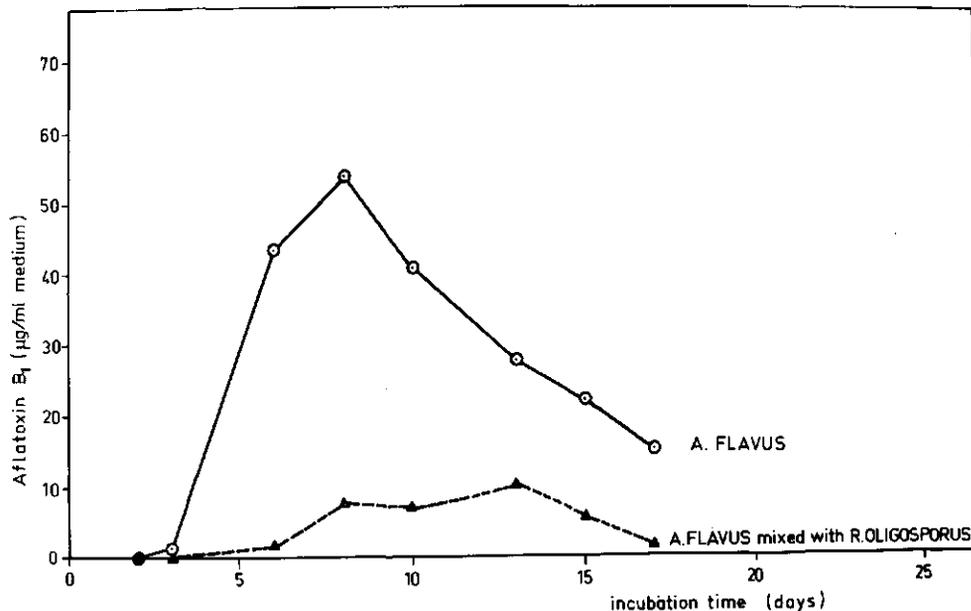


Fig. 1. Comparison of aflatoxin B<sub>1</sub> accumulation in a pure culture of Aspergillus flavus (○) with that in a mixed culture of A. flavus and Rhizopus oligosporus (▲).

Extended investigations showed that other species of Rhizopus, including R. oryzae and R. chinensis, showed the same inhibiting properties. Similarly, species of Neurospora used for the fermentation of peanut presscake into oncom, and certain strains of Penicillium roqueforti have the same inhibitory properties to aflatoxin-producing

strains of A. flavus (Ko, 1981).

These results confirmed that R. oligosporus and some other mould species used for the fermentation of certain food products do not produce aflatoxin and inhibit aflatoxin production by A. flavus and A. parasiticus.

Further studies were started to discover the basic mechanism of the inhibitory effect of mould species to aflatoxin production by other species. But no significant results have been obtained.

Other studies related to tempe

Gradually, other aspects connecting with tempe attracted the attention of other workers at the Agricultural University. Roozen and de Groot (1985) investigated the changes of activity and nature of trypsin inhibitors in soyabeans during tempe fermentation. More about the continuation of studies on tempe is presented in Nout's contribution to this book.

#### Tempe bongkrek

Tempe bongkrek is another variety of tempe. Soyabean tempe, which was never reported to be poisonous, is rather different with tempe bongkrek. Its basic raw material is partly defatted coconut. If this is contaminated with Pseudomonas cocovenenans, it can be poisonous. There are still some rather serious outbreaks of food poisoning by consumption of tempe bongkrek. During 1988, three outbreaks have already reported involving more than 400 victims, of which 42 were fatal. Although more than a half century ago several studies were devoted to this problem, many microbiological aspects still deserve serious attention. Those conducted at the Agricultural University were the following.

#### Growth and toxin production of P. cocovenenans

Investigation of the growth and toxin production of P. cocovenenans disclosed the unfortunate circumstance that the conditions under which tempe bongkrek is fermented, coincide with those of the optimum growth and toxin production of P. cocovenenans (Ko, 1985).

#### Inhibitive effect of R. oligosporus on toxin production

Similar with soyabean tempe, the required mould for the fermentation of tempe bongkrek is R. oligosporus. If P. cocovenenans is present as a contaminant in a coconut substrate to be fermented into bongkrek, it is consequently mixed in the growth substrate together with R. oligosporus. This became the underlying idea for the study of the interaction between R. oligosporus and P. cocovenenans when they grow together in a coconut growth medium. One of the observations was that P. cocovenenans did not produce toxins at 30 °C within 48 h, if its number at the start of the fermentation was less than 10 times the number of R. oligosporus spores inoculated as starter. These observations suggest that, during the manufacture of tempe bongkrek, the chance of toxin production by P. cocovenenans can be reduced by heavy inoculation with R. oligosporus spores (Ko & Kelholt, 1981).

## Prevention of toxin production by NaCl

Contamination with P. cocovenenans can be avoided easily by simple hygiene. However many manufacturers of tempe bongkrek belong to the less educated part of the people and even if they are aware of hygienic methods, many are too poor to afford the purchase of an autoclave or other equipments. This makes it difficult or even impossible to prevent occasional contamination with P. cocovenenans before or during fermentation. Provided that the raw material does not yet contain toxins, a method to prevent toxin production during the fermentation period should therefore be directed to the suppression of toxin production, even if P. cocovenenans is present. The investigation showed that addition of a mass fraction of NaCl of 1.5-2.0% to the raw material was an adequate method. Initially, salt was investigated to test its ability to kill P. cocovenenans. Unexpectedly salt suppressed toxin production at concentrations far below its lethal concentration. In addition, the required amount of salt did not disturb the fermentation process and was just enough to improve the taste of the product. The choice to investigate the effect of salt was not by chance. It was based on several considerations. Salt is available almost everywhere and is not expensive. It is widely used as food preservative to inhibit growth of certain micro-organisms. For taste, it is usually added to dishes made of tempe bongkrek. Therefore, the presence of salt in the product is quite acceptable. Ultimately, the choice proved appropriate for the purpose (Ko et al., 1977).

## Chemical analysis

During the entire study, the methods of detection, isolation, purification and measurement of content of the toxins were one of the main concerns. Initially, the methods described separately in literature for each of the toxins, were thoroughly tested. Gradually, a method was developed by combination, modification and adjustment of separately described methods. This includes the extraction of both toxins together from the fermented product with chloroform. The following separation of the toxins from each other was based on the difference of solubilities in petroleum ether and in water. Bongkrek acid is soluble in petroleum ether, but not in water. On the other hand, toxoflavin is not soluble in petroleum ether, but dissolves well in water. After separation, each of the toxins were purified and measured spectrometrically. The absorption spectrum of bongkrek acid in methanol has maxima at wavelengths of 237 nm and 267 nm, and a minimum at 249 nm. Calculation of the concentration in methanolic solution was based on absorbance at  $\lambda = 267$  nm, the highest of the two peaks. An aqueous solution of toxoflavin has an absorption spectrum with a rather flat peak at 400 nm and a much higher and sharper peak at 258 nm. Calculation of concentration of toxoflavin in aqueous solution was based on absorbance at this high peak (Ko & Keiholt, 1981).

The method proved satisfactory and was used during most of the studies. However it required laborious separation and purification of each of the toxins. This encouraged us to look for improvements. While continuing the current studies, ways were examined of measuring the toxins by HPLC. In cooperation with Voragen and his staff of the Food Chemistry Department, a HPLC method was developed to measure the toxins without separating them and thus avoiding laborious purification (Voragen et al., 1982).

## Tapé and ragi

Tapé is a popular Indonesian delicacy which is prepared by fermenting glutinous rice or cassava tuber with ragi. Ragi is a flattened round cake 2-3 cm in diameter made from rice flour. Because of its content of fungi and yeasts, it is used as inoculum for the fermentation of tapé.

Similar to the study on soyabean tempe, this study was started by collecting ragi samples from different places in Indonesia. Many fungi and yeasts were isolated from the samples. Attempts to find out which of the isolated micro-organisms or combinations of them were essential for the fermentation of tapé were initially not successful. Later the investigation was continued at the Agricultural University. After laborious selection and combination of old and new isolates of moulds and yeast cultures, a combination of Amylomyces rouxii and Endomycoopsis chodati was ultimately found to be satisfactory for fermentation of glutinous rice into tapé of good quality. Also cassava could be fermented into good tapé with this combination.

As for tempe, a method for the preparation of a starter made from pure cultures of A. rouxii and E. chodati was developed. Tests on its keeping qualities showed that after storage at room temperature for more than 5 months, the inoculum was still as active as a newly prepared one (Ko, 1972). The combination of A. rouxii and E. chodati, found in this study, became the starting point of an extensive study by Steinkraus and his team at the New York State Agricultural Experiment Station, Cornell University, Geneva, New York: it investigated the biochemical changes that occur during tapé fermentation (Cronk et al., 1977, 1979).

Furthermore, at the Malaysian Agricultural Research and Development Institute, Kuala Lumpur, Malaysia, the study was extended by the development of a production method for application at modern industrial scale (Merican & Yeoh, 1980).

## References

- Cronk, T.C.; Steinkraus, K.H.; Hackler, L.R.; Mattick, L.R.; 1977. Indonesian tapé ketan fermentation. *Applied Environmental Microbiology* 33:1067-1073.
- Cronk, T.C.; Mattick, L.R.; Steinkraus, K.H.; Hackler, L.R.; 1979. Production of higher alcohols during Indonesian tapé ketan fermentation. *Applied Environmental Microbiology* 37:892-896.
- Hesseltine, C.W.; Smith, M.; Bradle, B; Ko Swan Djien; 1963. Investigations of tempeh, an Indonesian food. *Developm. Industr. Microbiology* 4:275-287.
- Jutono; 1985. The microbiology of usar, a traditional tempe inoculum. *Proceedings Asian Symposium Non-salted soybean fermentation, Tsukuba, Japan.* p. 50-59.
- Ko Swan Djien; 1972. Tapé fermentation. *Applied Microbiology* 23:976-978.
- Ko Swan Djien; 1974. Self-protection of fermented foods against aflatoxin. *Proceedings IV International Congress Food Science Technology III:244-253.*
- Ko Swan Djien; 1981. Fermentation of foods by moulds. In: R.A. Samson, E.S. Hoekstra & C.A.N. van Oorschot (Eds.): *Introduction to food-borne fungi.* Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. p. 236-241.
- Ko Swan Djien; 1985. Growth and toxin production of Pseudomonas cocovenenans, the so-called "Bongkrek bacteria". *ASEAN Food J.* 1:78-84.
- Ko Swan Djien; Hesseltine, C.W.; 1961. Indonesian fermented foods. *Soybean Digest* 22:14-15.

- Ko Swan Djien; Kelholt, A.J.; 1981. The inhibitive effect of Rhizopus oligosporus on toxin production by Pseudomonas cocovenenans in tempe bongkrek. *Annales Bogorienses* 7(3):97-106.
- Ko Swan Djien; Kelholt, A.J.; Kampelmacher, E.H.; 1977. Inhibition of toxin production in tempe bongkrek. *Proceedings 5th International Conference on Global Impacts of Applied Microbiology, Bangkok, Thailand.* p. 375-388.
- Merican, Z.; Yeoh Quee Lan; 1980. Penyediaan tapai secara moden. Rep. 2nd Workshop on Solid Substrate Fermentation, ASEAN sub-Committee on Protein, 27-29 November 1980, Kuala Lumpur, Malaysia. p. 10-12.
- Roozen, J.P.; de Groot, J.; 1985. Electrophoresis and assay of trypsin inhibitors in different stages of tempe production. *J. Food Biochem.* 9:37-48.
- Rusmin, S.; Ko Swan Djien; 1974. Rice-grown Rhizopus oligosporus inoculum for tempeh fermentation. *Applied Environmental Microbiology* 28:347-350.
- Voragen, A.G.J.; de Kok, H.A.M.; Kelholt, A.J.; Schols, H.A.; Ko Swan Djien; 1982. Determination of bongkrek acid and toxoflavin by High Pressure Liquid Chromatography. *Food Chemistry* 9:167-174.

## QUALITY UPGRADING OF TRADITIONAL FERMENTED FOODS

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### Summary

Food fermentations were studied at the Department of Food Science and Technology, University of Nairobi, Kenya, and at Wageningen.

For African traditional beers, study included the manufacturing process, microbiology, nutritional value and preservation of busaa, a Kenyan maize beer.

Tempe serves as a model in our research on fungal solid substrate fermentations. The implications of lactic fermentation during the preparatory soaking of soya beans were reviewed, with particular reference to the growth of undesirable bacteria, i.e. Bacillus cereus and Staphylococcus aureus. In addition, the influence of environmental conditions on the kinetics of fungal growth were studied.

In mycology, the reduction of growth and aflatoxin accumulation in Aspergillus flavus and A. parasiticus in mixed cultures with Rhizopus and Neurospora spp. was reviewed. This inhibition was caused by a complex of factors including competition for nutrients, inhibition of aflatoxin production, and biodegradation of aflatoxin.

Lactic fermentation protected infant foods against the multiplication of contaminating micro-organisms. Particularly under conditions of poor hygiene in tropical climates, fermentative acidification offers good prospects for food protection.

Descriptors: fermented food, maize, beer, soya bean, tempe, Rhizopus, Neurospora, Aspergillus, aflatoxin, infant, weaning, sorghum, millet, legumes.

### Introduction

Fermented foods are a major group of ingredients in the human diet for several reasons, including increased shelf-life, attractive flavour, colour and texture, improvement of palatability and nutritional value, and reduction of bulk and required cooking time.

Traditionally, fermented foods have been manufactured on a small scale by household methods of food processing. Whereas the production of cheese, soya-sauce, lager beer, and fermented sausages has grown in scale in industrial societies, many fermented products are still prepared in the household or community in developing countries. In such situations, fermentation is essential as a means of preservation of food.

This review summarizes the author's explorations into aspects of health, nutrition, product development and process analysis, with special regard to tropical fermented foods.

## African traditional beers

The manufacture of traditional beer is common in Africa. The use of a variety of locally available raw materials, e.g. maize, sorghum, millets, cassava, banana, sugar-cane, and sometimes wheat and barley, results in many products (Steinkraus, 1983; Campbell-Platt, 1987). Beers such as Nigerian pito and burukutu, Kenyan busaa and chekwe, Tanzanian pombe, and Ethiopian borde and talla have social and ceremonial purposes in traditional village life. Generally they resemble thin porridges with 4-13% dry matter. Often alcoholic and lactic fermentations occur, resulting in a typical sour taste (pH 3.5-4.5) and low alcohol content (1-4% v/v). At the time of consumption, the products are still fermenting and the shelf-life is only 1-2 days due to continuing acidification in the absence of preservation. Though there are regional variations, production is carried out in 3 stages: malting; preparation of a soured mass of partly gelatinized starch; and the liquid (beer) fermentation.

For the preparation of malt, cereal grains are soaked and allowed to germinate for 3-4 days at ambient temperatures and are then sun-dried. Beside amylolytic and proteolytic enzymes, such malts contain high total microbial counts of  $10^6$ - $10^9$   $g^{-1}$ . Consequently, they will be partly responsible for the inoculation of the liquid fermentation. Malting studies (Novellie, 1962; Nout & Davies, 1982) on sorghum, finger millet and barley have shown that sorghum and finger millet produce malts containing predominantly  $\alpha$ -amylase and only a little  $\beta$ -amylase. So the brewing process with these malts is slower and yields less fermentable extract than with barley malt.

The starchy mass is prepared as a slurry or a stiff dough which undergoes a natural mixed lactic fermentation for 2-3 days. Subsequently, the soured mass (pH 3.5-4.5) is heated by boiling, baking or roasting, causing some gelatinization of starch and a desirable fried flavour. This heat treatment is not adequate to inactivate its flora of lactic acid bacteria, which will continue their activity during liquid fermentation.

During liquid fermentation, the gelatinized starch and the malt are mixed with water and left to ferment for 1-3 days at temperatures ranging from ambient up to about 50 °C. The brewing (generation of extract) and fermentation take place simultaneously. The brew is then filtered through grass sieves or cloth to remove coarse particles before consumption. The microbiology of South African kaffir beer has been studied (van der Walt, 1956) as well as that of Kenyan busaa (Nout, 1980a) and the activity of Saccharomyces and Candida yeasts and Lactobacillus spp. was demonstrated.

The high content of dry matter and the synthesis of vitamins during germination of the malt and fermentation of the beer give traditional African beers a nutritive value superior to European lager-type beers (Chevassus-Agnes et al., 1976; Nout, 1987). Urbanization taking place in Africa has detached many Africans from their traditional customs and rules (Beckman, 1988). This is one of the factors responsible for a strong increase in the consumption of low-cost alcoholic products, particularly in urban slums. In some countries, e.g. Kenya, this has precipitated the prohibition of all traditional alcoholic products. Unfortunately such measures usually lead to an increased consumption of illicit traditional spirits which are more harmful to public health (Nout, 1979) than the prohibited beers.

Traditional African beers have a significant nutritional potential, are affordable and offer a superior alternative to traditional distilled

spirits. The status of these beers should be improved by developing dependable processes for their manufacture. Kenyan traditional busaa could be preserved by bottling and pasteurization (Nout, 1980b). Research has resulted in medium-scale production of chibuku maize beer in Tanzania and Zambia, and in large-scale industrial brewing of Bantu sorghum beer in South Africa (Rothschild, 1972).

### Tempe

Following up on the work of Ko (elsewhere in this volume), several aspects of the tempe fermentation were investigated.

Similar to the traditional African beers, fresh tempe is a "living" product containing a multitude of micro-organisms and active enzymes. For experimental purposes, measures were defined (Nout et al., 1985) to evaluate the acceptability of fresh tempe. Tempe should not be regarded as a product exclusively fermented by moulds, since it also contains many bacteria and yeasts, increasing its acceptability.

Comparisons of commercial processes and the acceptability and shelf-life of the resulting products suggested that fermentative acidification during the first stage (i.e. soaking of soya beans in water) contributed to the quality of fresh tempe. Inspired by earlier work on the microbiology of the sour-dough fermentation (Creemers-Molenaar et al., 1985; Nout & Creemers-Molenaar, 1986), we developed a simple method to stimulate the souring during the soaking stage by lactic fermentation, accelerated by recycling the inoculum (Nout et al., 1987c).

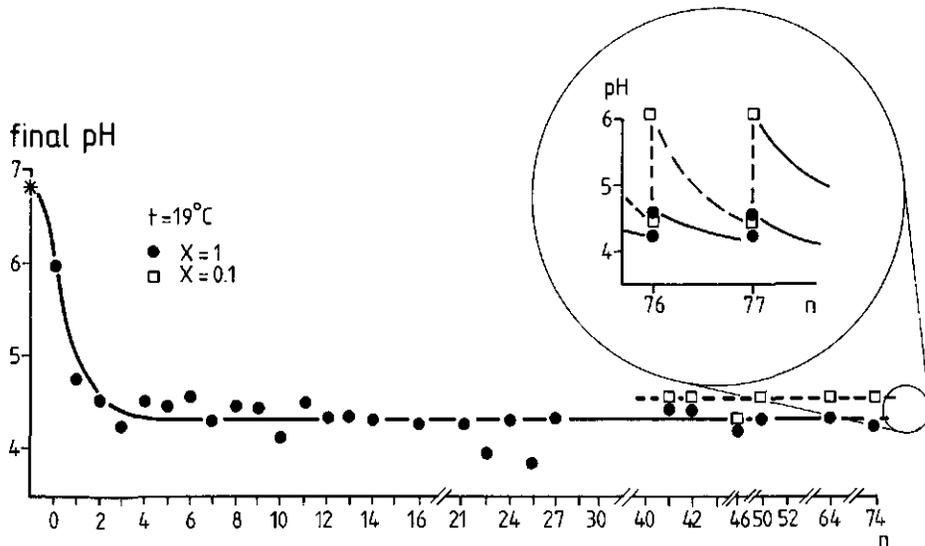


Fig. 1. Influence of  $n$  (number of recyclings) on the final pH of soak water. Inset: effect of  $X$  (recycling rate) on pH fluctuation of soak water. \*, pH at start of first soak.

Figure 1 illustrates the pH of the soakwater after  $n$  recyclings at an incubation temperature of 19 °C. Each soaking had a duration of 24 h; a recycling rate ( $x$ ) = 1 proved to be sufficient to prevent large fluctuation in pH at the start of each soak. After only a few soakings, the pH at the end of each soaking had fallen to a fairly constant value of 4.3. This rapid and predictable acidification could be achieved over the temperature range 19-45 °C. Such soaks were dominated mainly by Lactobacillus plantarum and Saccharomyces dairensis. In particular, L.plantarum was demonstrated to contribute to the acceptability and shelf-life of fresh tempe.

At that time, Samson et al. (1987) published a survey on the microbial composition of tempe and tofu (tahu). Since Bacillus cereus and Staphylococcus aureus exceeded  $10^5$  g<sup>-1</sup> in about 10% of Dutch commercial tempe, the growth of these bacteria during manufacture and storage of tempe was studied. For B.cereus (Nout et al., 1987a), development during the fermentation stage could be prevented if the raw material had been acidified to pH  $\leq$  5.5 by fermentation during the soaking stage. Inadequate acidification of soya beans could not be countered by the inclusion of an inoculum of lactobacilli at the start of the fungal fermentation. Although the lactobacilli multiplied well during the fermentation, their acid production was too late to prevent the growth of B.cereus.

The growth of Staphylococcus aureus during the soaking of soya beans was inhibited if accelerated acidification resulted in a final pH of about 4.0. During the fermentation of tempe, the growth of S.aureus was not influenced by the presence of Rhizopus oligosporus or Lactobacillus plantarum, or by the low oxygen concentration in fermenting tempe. However the production of staphylococcal enterotoxins was reduced in the presence of L.plantarum or low volume fraction of oxygen, 0.02. Remarkably, the thermal resistance of the enterotoxins thus formed in tempe was considerably less than in buffered brain heart infusion broth (Nout et al., 1988).

Those studies underline the importance of adequate souring during the soaking step. In properly made tempe heated before consumption, for instance, by frying, the risk of food-borne intoxication from either B.cereus or S.aureus is negligible. The value of the fermentative acidification during soaking of soya beans was also demonstrated by Tunçel et al. (in press) who investigated several processes for the manufacture of tempe starter. The fermentative souring of raw materials before inoculation with pure fungal cultures contributed to the microbiological safety of resulting starters and to the acceptability of the resulting tempe.

Quite a different aspect of tempe fermentation is presented by the limitations of heat and mass transfer taking place during solid-substrate fermentation (SSF) with fungi, resulting in a considerable increase in the temperature, accumulation of CO<sub>2</sub> and depletion of O<sub>2</sub> inside fermenting mass. To understand the influence of the size of the lump and incubation conditions on the extent and kinetics of fungal biomass production during SSF, we are trying to construct a multiparameter model. A first requirement for such studies is a reliable method to measure fungal biomass. To this effect we evaluated the use of ergosterol as a chemical index for biomass of Rhizopus oligosporus (Nout et al., 1987b). Although we gained some interesting results on the physiology of the tempe mould, the production of ergosterol was demonstrated to be influenced by oxygen concentration and mycelium age, to such an extent that especially in bulk SSF ergosterol is not a suitable index for biomass production. Instead we now use gravimetric methods in synthetic

solid substrates. In addition, the possible quantitative use of immuno-assays in natural solid substrates is being evaluated. With simplified solid media, the influence of temperature, and O<sub>2</sub> and CO<sub>2</sub> concentrations on the biomass production by Rhizopus oligosporus were measured and compared with published data on growth. The effect of temperature could be described by the Ratkowsky equation (Ratkowsky et al., 1983). The effect of O<sub>2</sub> follows Monod kinetics, whereas the CO<sub>2</sub> effect has not been explained yet. Other parameters e.g.  $\mu_{max}$ , pH, inoculum rate) need to be studied to arrive at a model which can simulate solid-substrate fermentations.

#### Aflatoxin accumulation in mixed fungal cultures

Ko reported earlier on the influence of certain fungi on the growth and aflatoxin B<sub>1</sub> accumulation by Aspergillus flavus and A.parasiticus (this volume). The importance of preventing contaminations with aflatoxins of (fermented) foods as well as animal feed ingredients prompted us to continue the investigation of the role of Rhizopus and Neurospora spp. in mixed cultures with Aspergillus. In solid and liquid cultures of natural and synthetic origin, aflatoxin B<sub>1</sub> accumulation in simultaneously inoculated mixed cultures of A.flavus and A.parasiticus with Rhizopus or Neurospora spp. was a third and 0.2% of that with cultures of Aspergillus.

Preliminary studies with selected Rhizopus and Neurospora strains investigated the mechanism of the reduction of accumulated aflatoxin. At least three phenomena were shown to be involved: competition for carbon sources; formation of compounds inhibitory to the aflatoxin synthesis; ability of the tested Rhizopus and Neurospora spp. to metabolize aflatoxin B<sub>1</sub> (Nout, in press, 1989a).

#### Infant foods

Over 14 million infants and children younger than 5 years die annually in the tropical regions of the world (Unicef, 1986). Major causes are infections with pathogenic bacteria, parasites and viruses causing watery diarrhoea; and inadequate intake of major nutrients and energy resulting in protein-energy malnutrition.

In co-operation with the Department of Human Nutrition of the Agricultural University, research is aiming at the development of infant foods to meet the requirements (1) that they are prepared from locally available and accepted ingredients; (2) that their composition guarantees adequate supply of protein and energy; (3) that growth or survival of contaminating pathogens is minimal at 30 °C for 24 h after preparation.

Adequacy of protein supply is achieved with composite mixes of cereals and legumes, e.g. sorghum with groundnut or pigeon pea. An energy concentration of 4.2 MJ/l of prepared infant food requires a dry matter content of about 20%. In order to ensure the desired liquid consistency of the porridge, 1% sorghum malt is used in the process to liquefy gelatinized starch. Preliminary results (Nout et al., 1987d) demonstrated that lactic fermentation of porridge ingredients resulted in final products with antimicrobial properties. In storage and challenge tests with Salmonella typhimurium in which the prepared porridges were contaminated on purpose, there was a rapid destruction of viable Salmonella cells. By lactic fermentation a wide variety of porridge ingredients including cereals, legumes and starchy root crops were protected against the multiplication of several bacteria including

Salmonella, Shigella, Citrobacter, Enterobacter, Escherichia and Yersinia spp. (Nout et al., in press, 1989b).

In principle, lactic fermentation of formulated infant foods offers interesting prospects for the improvement of their shelf-life and safety. Since the sour taste of lactic fermented foods is well accepted in some African countries (Nout, 1981), the production of fermented infant food is compatible with cultural food preferences there. To support production and consumer trials, several aspects require further study, including the influence of the fermentation on the nutritional value of porridges, and analyses of cost, hardware, manpower and logistics for fermentation processes at a small scale under primitive conditions.

#### References

- Beckman, V.; 1988. Alcohol, another trap for Africa. Libris, Örebro, Sweden.
- Campbell-Platt, G.; 1987. Fermented foods of the world - a dictionary and guide. University Press, Cambridge, UK.
- Chevassus-Agnes, S.; Favier, J.C.; Joseph, A.; 1976. Technologie traditionnelle et valeur nutritive des "bières" de sorgho du Cameroun. Cahiers de Nutrition et de Diététique 11:89-104.
- Creemers-Molenaar, T.; Nout, M.J.R.; Bonants-van Laarhoven, T.M.G.; 1985. Microbiological aspects of wheat sour-dough. Antonie van Leeuwenhoek 51:607-608.
- Nout, M.J.R.; 1979. The manufacture and consumption of Chang'aa (Nubian Gin). Lebensmittel Wissenschaft und Technologie 12:212-216.
- Nout, M.J.R.; 1980a. Microbiological aspects of the traditional manufacture of Busaa, a Kenyan opaque maize beer. Chemie Technologie Mikrobiologie der Lebensmittel 6:137-142.
- Nout, M.J.R.; 1980b. Process development and preservation of Busaa, a Kenyan traditional opaque maize beer. Chemie Technologie Mikrobiologie der Lebensmittel 6:175-182.
- Nout, M.J.R.; 1981. The role of lactic acid fermentations in the context of food manufacture in Kenya. Sixth International Conference on "Global Impacts of Applied Microbiology". Academic Press, London, pp.169-175.
- Nout, M.J.R.; 1987. Chemical composition and nutrient balance of Busaa, a Kenyan opaque maize beer. Chemie Mikrobiologie Technologie der Lebensmittel 11:51-55.
- Nout, M.J.R.; Beernink, G.; Bonants-van Laarhoven, T.M.G.; 1987a. Growth of *Bacillus cereus* in soybean tempeh. International Journal of Food Microbiology 4:293-301.
- Nout, M.J.R.; Bonants-van Laarhoven, T.M.G.; de Dreu, R.; Gerats, I.A.G.M.; 1985. Influence of some process variables and storage conditions on the quality and shelf-life of soybean tempeh. Antonie van Leeuwenhoek 51:532-534.
- Nout, M.J.R.; Bonants-van Laarhoven, T.M.G.; de Jongh, P.; de Koster, P.; 1987b. Ergosterol content of *Rhizopus oligosporus* NRRL 5905 grown in liquid and solid substrates. Applied Microbiology and Biotechnology 26:456-461.
- Nout, M.J.R.; Creemers-Molenaar, T.; 1986. Microbiological properties of some wheatmeal sourdough starters. Chemie Mikrobiologie Technologie der Lebensmittel 10:162-167.
- Nout, M.J.R.; Davies, B.J.; 1982. Malting characteristics of finger millet, sorghum and barley. Journal of the Institute of Brewing 88:157-163.

- Nout, M.J.R.; de Dreu, M.A.; Zuurbier, A.M.; Bonants-van Laarhoven, T.M.G.; 1987c. Ecology of controlled soybean acidification for tempeh manufacture. *Food Microbiology* 4:165-172.
- Nout, M.J.R.; Hautvast, J.G.A.J.; van der Haar, F.; Marks W.E.W.; Rombouts, F.M.; 1987d. Formulation and microbiological stability of cereal-based composite weaning foods. In: D. Alnwick, S. Moses & O.G. Schmidt (Eds): "Improving young child feeding in Eastern and Southern Africa. Household-level food technology". Proc. Workshop Nairobi, Kenya 12-16 Oct. 1987. pp. 245-260. Int. Developm. Res. Centre Ottawa Canada 1988 (IDRC-265e).
- Nout, M.J.R.; Notermans, S.; Rombouts, F.M.; 1988. Effect of environmental conditions during soya-bean fermentation on the growth of *Staphylococcus aureus* and production and thermal stability of enterotoxins A and B. *Int. J. Food Microbiology* 7:299-309.
- Nout, M.J.R.; 1989a. Effect of *Rhizopus* and *Neurospora* spp. on growth of *Aspergillus flavus* and *Aspergillus parasiticus* and accumulation of aflatoxin B<sub>1</sub> in groundnut. *Mycol. Res.*, in press.
- Nout, M.J.R.; Rombouts, F.M. & Havelaar, A.; 1989b. Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic micro-organisms. *Int. J. Food microbiol.*, in press.
- Novellie, L.; 1962. Kaffircorn malting and brewing studies. XII - Effect of malting conditions on malting losses and total amylase activity. *Journal of the Science of Food and Agriculture* 13:121-123.
- Ratkowski, D.A.; Lowry, R.K.; McMeekin, T.A.; Stokes, A.N.; Chandler, R.E.; 1983. Model for bacterial culture growth throughout the entire biokinetic temperature range. *Journal of Bacteriology* 154:1222-1226.
- Rothschild, D.; 1972. Push-button beer at Congella. *Food industries of South Africa* 24(3):19-21.
- Samson, R.A.; van Kooij, J.A.; de Boer, E.; 1987. Microbiological quality of commercial tempeh in the Netherlands. *Journal of Food Protection* 50:92-94.
- Steinkraus, K.H.; 1983. Handbook of indigenous fermented foods. Marcel Dekker, Inc., New York, Basel.
- Tunçel, G.; Nout, M.J.R.; Rombouts, F.M.. Effect of acidification on the microbiological composition and performance of tempe starter. *Food Microbiology*, in press.
- Unicef; 1986.
- Walt, J.P. van der; 1956. Kaffircorn malting and brewing studies. II - Studies on the microbiology of kaffir beer. *Journal of the Science of Food and Agriculture* 7:105-113.

## REFERENCE MATERIALS FOR WATER AND FOOD MICROBIOLOGY

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### Summary

The National Institute of Public Health and Environmental Protection and the Community Bureau of Reference currently develop reference materials for quality control of microbiological methods. The materials consist of gelatin capsules filled with spray dried milk, which has artificially been contaminated with a specific micro-organism. For water microbiology reference materials are being developed with the test strains: WR1 Escherichia coli, WR3 Enterobacter cloacae, WR51 Micrococcus varians, and WR63 Enterococcus faecium. The contamination level of these materials is about 500 cfu/capsule. Reference materials with the test strains WR1 and WR3 have (successfully) been used in three collaborative studies with laboratories of the EEC. For food microbiology reference materials are being developed with the test strains: Salmonella typhimurium, Listeria monocytogenes (both with a contamination level of about 5 cfu/capsule) and Staphylococcus aureus (with a contamination level of about 10<sup>4</sup> cfu/capsule). Reference materials with test strain Salmonella typhimurium have successfully been used in a collaborative study with 31 laboratories of the EEC.

Descriptors: reference materials, water microbiology, food microbiology, Escherichia coli, Enterobacter cloacae, Salmonella typhimurium.

### Introduction

Reference materials have been developed in many disciplines of science, like chemistry and physics. The development of microbiological reference materials however is hampered by the instability of living organisms, the dependence of the outcome of an analytical procedure on the physiological state of the organisms and the non-existence of definitive methods to verify reference materials. Reference materials consisting of artificially contaminated spray-dried milk, as developed at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands (RIVM) may be a step forward in the process of quality assurance in analytical microbiology. The materials are being developed under contract with the Community Bureau of Reference (BCR). Applications can be made in the analysis of water, food, and pharmaceutical products: (1) for quality control of measurements for the routine practice in a laboratory; (2) to compare the efficiency of different (isolation or enumeration) methods; (3) as a standardized sample in collaborative studies.

The developed reference materials are tested for their suitability as a standardized sample in collaborative studies with laboratories (about 40) of the EEC. Up to now studies for water microbiology were organized with reference materials containing test strains WR1 Escherichia coli and WR3 Enterobacter cloacae, for food microbiology with reference materials containing test strain Salmonella typhimurium. When a reference material has proven its suitability, it will be proposed for certification by the BCR. At this moment no reference materials are certified.

### Preparation of reference material

The selected test strain is first cultured in liquid or on solid medium. The incubation time and temperature are dependent on the test strain but are usually 37 °C for 48--72 h. After the incubation time the culture is concentrated by centrifugation or by harvesting the culture from the solid medium with sterile milk and glass beads. The concentrated culture is added to sterile concentrated milk with about 25% dry matter. The milk suspension is mixed well and subsequently spray dried, at an inlet temperature of about 200 °C and an outlet temperature of about 70--75 °C. This produces a highly contaminated milk powder which can be kept for over a number of years. This powder is mixed with uncontaminated milk powder to the desired contamination level. The resulting mixture is used for filling gelatin capsules (about 0.2 g milkpowder/capsule), the final step in the preparation of the reference materials. The capsules are checked for contamination level, homogeneity and stability before shipment to participating laboratories by (air) mail.

### Results and discussion

#### Reference materials for water microbiology

Two reference materials containing the test strains WR1 Escherichia coli and WR3 Enterobacter cloacae were developed. The mean contamination level of both reference materials was 400--500 colony forming units per capsule (cfu/capsule). The test strains are typical coliform bacteria. Test strain WR1 is thermotolerant (maximum growth temperature,  $T_{max} = 45.5$  °C on laurylsulphate agar), test strain WR3 however is not able to grow at 44 °C. The  $T_{max}$  of this latter strain is 42.5 °C on the same medium (Havelaar et al., 1987). Test strain WR3 can therefore detect false positive results, resulting from poor adjustment of the incubator. Both reference materials were used in three collaborative studies, (in November 1986, in June 1987 and in November 1988), with about 40 participating laboratories of the European Community.

The design was the same in all three trials. Each participating laboratory received four capsules of each test strain in two different shipments. Each capsule was dissolved in one liter peptone saline solution. The solution represented a simulated water sample. In total eight simulated water samples were analysed by means of membrane filtration techniques by three culturing methods: (1) coliform bacteria by a reference method (called Reference; media and membrane filters were supplied by RIVM); (2) coliform bacteria by national standard methods (called TOTCOL); (3) thermotolerant coliform bacteria by national standard methods (called THCOL).

The results of the first and the second trials were analysed statistically (Mooijman et al., 1988) and are summarized below (the analyses of the third trial have not yet been finished). The results of the Reference method were expected to be the same in each laboratory. However in both trials and for both test strains significant differences were found between laboratories. These differences could partly be explained by problems with the reconstitution of the capsules. Improvements for some of these problems were incorporated in the second trial, resulting in less extremely high counts compared with the first trial. Still, further improvements of the reconstitution procedure were necessary to solve all problems and are evaluated in the third trial.

Significant differences between laboratories were also found with the TOTCOL and THCOL methods in both trials. Some of these differences could be explained by the use of different media. Seventeen participating laboratories found growth of test strain WR3 at a nominal incubation temperature of 44 °C (THCOL), during the first trial. During the second trial 10 participating laboratories found growth of test strain WR3 at this temperature. Possible explanations for the growth of WR3 were: the use of a non-vented incubator; or the use of a vented incubator combined with high stacks of Petri dishes.

Within some laboratories significant differences existed between the different culturing methods in both trials. These differences could partly be explained by the use of different media.

Overall it was concluded that the reference materials were suitable for the collaborative studies. Beside the reference materials for Escherichia coli and Enterobacter cloacae, reference materials with test strains WR63 Enterococcus faecium and WR51 Micrococcus varians have been developed. The contamination level of these two latter reference materials is also about 500 cfu/capsule. Test strain WR63 is a typical strain of the group D faecal streptococci. Test strain WR51 does not belong to this group but may give false positive results on streptococci media in case the azide concentration is too low. Both reference materials (with test strain WR51 and WR63) have been used in a collaborative study in February 1989.

#### Reference materials for food microbiology

A reference material for Salmonella typhimurium was developed with a mean contamination level of about 5 salmonellae per capsule. The distribution of the salmonellae over the capsules does not differ significantly from a Poisson distribution (In 't Veld et al., 1988). Due to the low contamination level there is a chance (of about 1% when the mean contamination level is about 5) that a capsule will not contain Salmonella. The stability of the materials is at least 4 months when stored at 4 °C (Beckers et al., 1985). This reference material has been used in a collaborative study with 31 laboratories in the European Community (In 't Veld et al., 1988). Each laboratory investigated 20 capsules. The mean contamination level of the samples was 4.35 salmonellae /capsule. The capsules were added to buffered peptone water (BPw). After incubation the BPw-culture was used to inoculate 3 selective enrichment broths: (1) Muller Kaufmann's tetrathionate brilliant green bile broth (TBB); (2) Rappaport Vassiliadis' malachite green magnesium chloride broth (RV); (3) a broth routinely used at the participating laboratories (OWN).

Significant differences were found between the 3 selective enrichment broths; TBB gave 78.7 %, RV 87.6 % and OWN 93.0 % positive isolations. Twentysix laboratories (84 %) isolated Salmonella in at least 18 of the 20 capsules with the OWN method. It was concluded that the Salmonella reference materials can be used to test laboratory performance. Beside the isolation of Salmonella from reference materials a laboratory has to be able to isolate Salmonella from food samples. The composition of a food and the micro-organisms in it influence the isolation of Salmonella. To test these influences a Salmonella capsule is added to a 1:10 suspension of the food in the BPw. A study of Beckers et al. (1986) showed a decrease to 50--60% positive isolations when foods were investigated together with the Salmonella reference material. Hence the isolation of low numbers of salmonellae from a number of foods (for example minced meat) is frequently not possible.

Beside reference materials for Salmonella, materials for Listeria monocytogenes and for Staphylococcus aureus have been developed. The Listeria reference materials have the same contamination level as the Salmonella reference materials and can be used to test the isolation procedure for Listeria. The Listeria reference materials will be tested in a trial to be organized in June 1989. The reference materials for Staphylococcus aureus have a contamination level of about  $10^4$  colony forming units per capsule and can be used to test different counting procedures. For this purpose a capsule is dissolved in 10 ml peptone saline in 30 minutes at a temperature of 37.5 °C. After this, 0.1 ml (containing about 100 colony forming units) is plated on the media under investigation. After incubation of the media the number of colonies is counted and the yield can be compared between the different media. To test the influence of a food on the enumeration of the reference strain, 20 g of food is suspended in 180 ml of peptone saline. A capsule is added to 10 ml of the suspension and treated as described above.

#### References

- Beckers, H.J., F.M. van Leusden, M.J.M. Meyssen & E.H. Kampelmacher, 1985. Reference material for the evaluation of a standard method for the detection of salmonellas in foods and feeding stuffs. *Journal of Applied Bacteriology* 59: 507-512.
- Beckers, H.J., F.M. van Leusden, D. Roberts, O. Pietsch, T.H. Price, M. van Schothorst, R.R. Beumer, R. Peters & E.H. Kampelmacher, 1986. Evaluation of reference samples for the detection of Salmonella. *International Journal of Food Microbiology* 3: 287-298.
- Havelaar, A.H., W.M. Hogeboom, V.M. Sekhuis & E.H.W. van Erne, 1987. Proficiency testing of water microbiology laboratories in the Netherlands. *Journal of Applied Bacteriology* 62: 555-564.
- In 't Veld, P.H., J.H. Hoekstra & H.J. Beckers, 1988. Detection of Salmonella in reference samples. BCR-Food Trial 1. Bilthoven, National Institute of Public Health and Environmental Protection, The Netherlands (report 148205001).
- Mooijman, K.A., S.H. Heisterkamp & A.H. Havelaar, 1988. The first international trial with reference materials for water microbiology. Bilthoven, National Institute of Public Health and Environmental Protection, The Netherlands (report 148602001).

## CHEMICAL PRESERVATION OF MAYONNAISE-BASED SALADS.

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### Summary

Mayonnaise-based salads are composed of solid ingredients of plant and animal origin mixed with a dressing. Factors contributing to their shelf-life are low pH, presence of acidulants and preservatives, and low storage temperature. Major spoilage organisms are yeasts, lactic acid bacteria and a few moulds. These salads do not normally permit growth of pathogenic micro-organisms. Legally permitted preservatives, such as benzoic acid and sorbic acid are effective in these products with low pH. However a major draw-back is their solubility in oil, which makes them less effective with increasing oil content below a critical pH of 4.9 (benzoic acid) or 5.1 (sorbic acid). Vacuum or gas packaging is effective only if dissolved oxygen is removed, and it is effective only against yeasts and moulds. In view of consumers preference for less sour salads with no preservatives added, the application of strict GMP rules to production and storage of salads is all the more important. Descriptors: mayonnaise-based salads, yeasts, lactic acid bacteria, pathogenic micro-organisms, preservation, acetic acid, benzoic acid, sorbic acid.

### Introduction

The production of commercially prepared mayonnaise-based salads has increased strongly in recent years. In addition to the traditional salads, mostly based on potatoes, many new salads with a more varied composition have been introduced. These salads consist of a liquid phase of a mayonnaise-like salad dressing and solid ingredients, including vegetables, fruit, meat and fish. The dressings are oil-in-water emulsions with soya oil, egg yolk, sugar, salt, acetic acid and stabilizers as main components (Brocklehurst & Lund, 1984).

From a microbiological point of view, the dressings are rather stable products, due to their low pH and high concentration of acetic acid (Holzapffel & Mossel, 1968; Smittle, 1977; Brocklehurst, 1983). After adding the solid ingredients, water, sugar, salt, acetic acid and oil diffuse, so that the salads are less stable than their sauces (Brocklehurst & Lund, 1984; Tunalay et al., 1985; Davies & Brocklehurst, 1986).

To guarantee a sufficiently long shelf-life, salads are stored at temperatures below 7 °C. Also, chemical preservatives, such as benzoic acid and sorbic acid may be added. In addition to microbial spoilage, chemical and physical instability may occur. Oxidation of fats may be due to exposure to light and the presence of oxygen and ingredients with catalytic activity. Physical instability of the emulsion may be caused, for instance, by preparation methods and types of thickeners used (Zschaler, 1976; Harrison & Cunningham, 1985). In this study, only those factors are discussed that influence the microbial stability of salads.

Consumer trends are towards salads without chemical preservatives and with a less sour taste. The achievement of salads with desirable taste and acceptable shelf-life without preservatives is a true challenge (Debevere, 1987).

#### Microbiology of mayonnaise-based salads

Because of the chemical composition of the salads and the low storage temperature, the micro-organisms causing spoilage are lactic acid bacteria, yeasts, moulds and bacilli. Species of lactic acid bacteria which are regularly isolated from salads are Lactobacillus plantarum, Lb.brevis, Lb.fructivorans, Lb.buchneri, Pediococcus spp, Leuconostoc paramesenteroides, Leuconostoc mesenteroides. The most important spoilage yeasts are Saccharomyces exiguus and Sacch.dairensis. Pichia membranaefaciens, Debaryomyces klockeri, Geotrichum candidum, some Candida spp. and Zygosaccharomyces bailii are also found in salads (Smittle, 1977; Baumgart, 1977; Smittle & Flowers, 1982; Brocklehurst et al., 1983; Brocklehurst & Lund, 1984; van Ansenwoude, 1986). Moulds that grow on the surface of the salads are usually Penicillium or Aspergillus spp. (van Ansenwoude, 1986). Besides those micro-organisms, bacilli, streptococci, micrococci and pseudomonads are isolated from spoiled salads (Terry & Overcast, 1976; Smittle, 1977).

Mainly through the acetic acid content in the aqueous phase, the low pH and the low temperature of storage, mayonnaise-based salads do not normally permit growth of pathogenic micro-organisms. Some pathogens, when inoculated into the salad, can survive some time, but they cannot multiply (Holtzapffel & Mossel, 1968; Fowler & Clark, 1975; Smittle, 1977). In a recent study with seven commercial salads of widely different composition Prade (1988) came to the same conclusion (Table 1). Problems with pathogenic micro-organisms may arise when the manufacturers make concessions to the demands of the consumer for less sour salads. In salads with very little acetic acid and a pH above 5.0, pathogens might have the opportunity to multiply, especially if cold storage is interrupted.

Table 1. Survival of some pathogens in commercial salads of different composition (pH 4.6 to 5.4), stored at 7 °C.

Pathogen	D (d)*	
	average	variation among salads
<u>Salmonella typhimurium</u>	28	6 - 100
<u>Yersinia enterocolitica</u>	6	4 - 8
<u>Aeromonas hydrophila</u>	3	2 - 5
<u>Listeria monocytogenes</u>	37	10 - 100
<u>Staphylococcus aureus</u>	10	6 - 20
<u>Bacillus cereus</u> (spores)	63	13 - >100
<u>Clostridium perfringens</u> (spores)	54	13 - >100

\* D defined as time (in days) to reduce the number of survivors by a factor of 10.

### Microbiological criteria

Most of the ingredients used in mayonnaise-based salads are heated by pasteurization, blanching or cooking. Contamination of the product will occur during processing, for instance cutting, slicing, mixing of the ingredients and filling. Some unheated ingredients can cause a contamination too. For this group of products, hygiene in the factory is important. Unhygienic conditions in the factory can lead to a product-adapted microflora, which will easily be spread in the products. Because of the need for a rather long shelf-life of these products (4-6 weeks in the refrigerator), low initial contamination is essential.

For mayonnaise-based salads, some microbiological specifications have been established under Dutch law:

- no pathogens nor their toxins may be present; coagulase-positive staphylococci are considered to be absent if population of viable cells of this type in the product is less than 500 per gram;
- the viable number of moulds and yeasts together should be less than  $10^4$  per gram;
- the viable number of Enterobacteriaceae should be less than  $10^3$  per gram.

Some salad-producing factories have their own specifications for the microbiological quality of the salads: total aerobic plate count ( $<10^5$  per gram) and number of lactobacilli ( $<10^5$  per gram). These numbers are important for shelf-life. If the number of lactobacilli is too high, the sensoric quality of the salad may decrease.

### Factors that influence microbial stability

The microbial stability of salads is largely determined by initial contamination (nature and number of micro-organisms); pH and types of acids present, preservatives, gas composition and storage temperature. Some of these factors are discussed below.

#### pH

For stability of salads, pH plays an important role. The lower the pH, the more stable the product. This is caused by pH itself, the type of acid used, the amount of acid necessary to achieve the low pH, and the degree of dissociation of the acid. At low pH, more acid is undissociated. It is generally accepted that undissociated acid molecules can easily enter the microbial cell and there give rise to antimicrobial activity.

#### Type of acid

The antimicrobial activity of an acidulant depends on the dissociation constant ( $K$ ), as well as on the specific properties of the acid. The  $pK$  of some organic acids are summarized in Table 2. The concentration of undissociated molecules at any pH can be calculated with the equation

$$pH = pK + \log [\text{dis. acid}]/[\text{undis. acid}] \quad (1)$$

In many foods, acetic acid is used as an acidulant. In addition, other acids may be used. Some experiments make it appear that a combination of these acids results in a synergistic antimicrobial effect (Baird-Parker, 1980). Research by Smeenk (1986), however, demonstrated that no synergism is achieved by combination of several organic acids (acetic, lactic,

citric, malic and tartaric acids). She tested the antimicrobial activity of these acids on several strains of *Lactobacillus* and yeasts, in a culture broth and concluded that yeasts are only inhibited by acetic acid, and that lactobacilli are inhibited by both acetic and lactic acids. This conclusion was confirmed by experiments of Eringfeld (1988), who inoculated sauces with *Lactobacillus plantarum* and *Saccharomyces cerevisiae*. Some of her results are summarized in Figure 1.

Table 2. The pK<sub>a</sub> of some organic acids.

Acid	pK <sub>a</sub>
<u>Acidulants</u>	
tartaric	2.98
citric	3.09
malic	3.40
lactic	3.86
acetic	4.76
<u>Preservatives</u>	
benzoic	4.18
sorbic	4.76
propionic	4.85

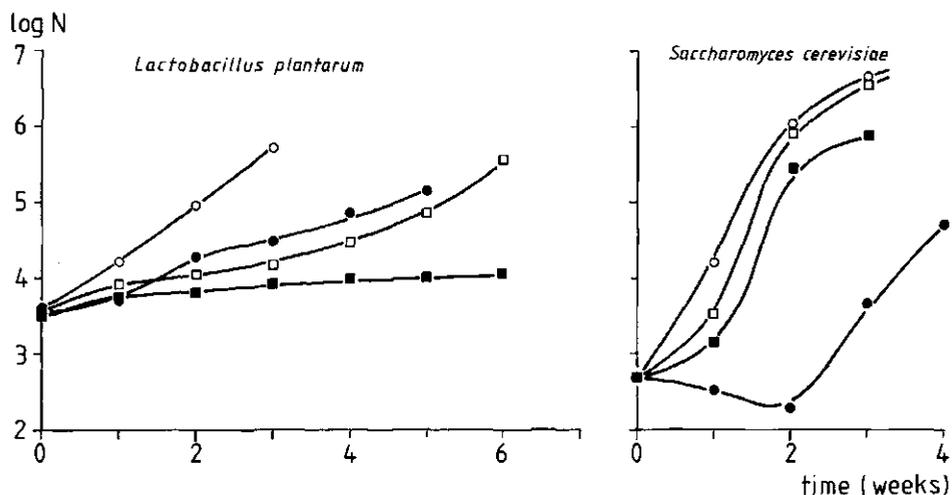


Fig. 1. The effect of acetic and lactic acids on the growth of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in sauce. pH, 4.5; temperature, 7 °C. Acetic acid in aqueous phase 0.13 mol/l (5 g/kg acetic acid in sauce), o; acetic acid 0.26 mol/l, ●; acetic acid 0.13 mol/l and lactic acid 0.13 mol/l, □ ; acetic acid 0.13 mol/l and lactic acid 0.26 mol/l, ■.

#### Chemical preservatives

Products can be preserved by adding water-soluble substances (such as sugar and salt, to lower the water activity or acids to lower the pH).

Chemical preservatives in a narrower sense are those additives used in low concentration ( $< 0.5$  g/kg) to inhibit microbial growth. According to Dutch law only two chemical preservatives are permitted in salads: sorbic acid and benzoic acid and their sodium and calcium salts, to a total concentration of 1 g/kg of the acids.

Sorbic acid mainly inhibits growth of yeasts and moulds, although some moulds are resistant and some can metabolize it. Bacteria are also inhibited by sorbic acid, but to a lesser extent than yeasts and moulds.

Benzoic acid is more inhibitory to bacteria than to yeasts and moulds. As with other acids, the degree of dissociation of the preservatives is important (Table 1).

A great disadvantage of these preservatives is their solubility in oil. While microbial growth has to be inhibited in the aqueous phase of the product, these preservatives are better soluble in oil than in water. The partition of these acids in oil and water is expressed in the distribution coefficient ( $k$ ), which is the ratio of the concentration (g/g) of the (undissociated) acid in oil and the concentration (g/g) of the undissociated fraction of the acid in the water phase. The distribution coefficient in an oil-water system is about 3.0 for sorbic acid and 6-13 for benzoic acid. In concentrations legally permitted, the distribution coefficient for benzoic acid is about 6 but, at higher values, it may be higher (Lubieniecki-von Schelhorn, 1967; Breeuwer, 1988). As a consequence, the amount of undissociated acid in the aqueous phase (the actively inhibiting form) not only depends on pH, but also on the oil content. This is illustrated in Figure 2.

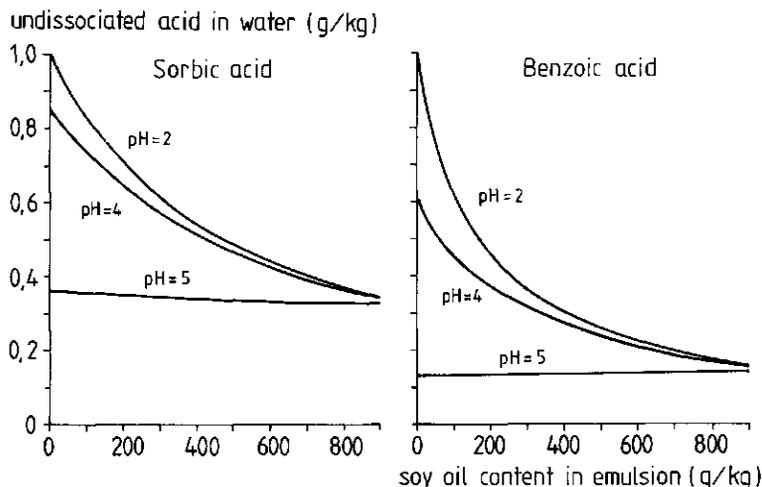


Fig. 2. Concentration of undissociated benzoic acid and sorbic acid in the water phase, as determined by oil content and pH (Breeuwer, 1988).

This figure shows that at pH relevant to salads the concentration of undissociated sorbic acid and benzoic acid in the water phase decreases as the oil content in the emulsion increases. It also shows that there is a critical pH at which the concentration of undissociated acid is independent of the oil content. This pH is 5.1 for sorbic acid and 4.9 for benzoic acid. In fact, this critical pH is determined by the distribution coefficient  $k$  of the acid and its  $pK$ , according to the

equation:

$$\text{pH}_{\text{crit}} = \text{pK}_a + \log (k - 1), \quad (2)$$

which implies that the critical pH is equal to the  $\text{pK}_a$ , if the distribution coefficient  $k$  of the acid equals 2.

The concentration of undissociated sorbic acid or benzoic acid in the water phase of an emulsion can be calculated from the equation, derived by Breeuwer (1988):

$$[\text{undis. acid}]_w = \frac{[\text{acid}]}{k \times \text{oil fr.} + \text{water fr.} + 10^{\text{pH} - \text{pK}} \times \text{water fr.}} \quad (3)$$

in which

$[\text{undis. acid}]_w$  = concentration (g/g) of undissociated acid in the water phase

$[\text{acid}]$  = concentration of benzoic or sorbic acid added to the emulsion (g/g)

$k$  = distribution coefficient of acid as defined above  
oil fraction (g/g) + water fraction (g/g) = 1.

Experiments by Breeuwer (1988) confirmed that the extent of growth inhibition can be related to the calculated concentration of undissociated preservative in the aqueous phase. Some of his results are shown in Figure 3.

Equations 2 and 3 apply to emulsions. In salads, the situation is further complicated by absorption phenomena of the preservatives to solid components (Davies & Brocklehurst, 1986).

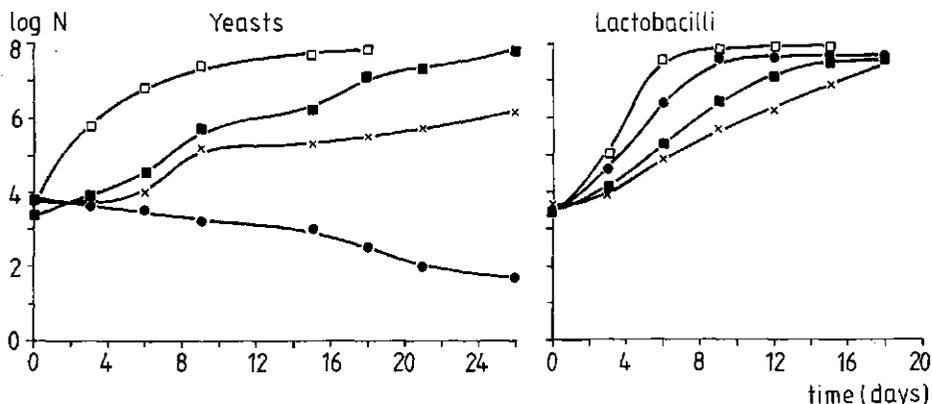


Fig. 3. Growth of yeasts and lactobacilli in an emulsion with 5 g/kg acetic acid in the aqueous phase and 1 g/kg preservative. pH, 4.0; temperature, 15 °C; 500 g/kg oil, 0.4 mg undissociated sorbic acid per gram water, ●; 500 g/kg oil, 0.3 mg undissociated benzoic acid per gram water, ■; 100 g/kg oil, 0.4 mg undissociated benzoic acid per gram water, x; 500 g/kg oil, no preservative, □.

## Modified gas composition

Modified and controlled gas atmosphere is often used in storage of bulk products like fruit and potatoes. Up till now, packaging of food with modified gas composition is almost exclusively used for meat and meat products. However with improving technical means and the development of appropriate packaging material, other products can also be packaged in this way. Packaging of salads under modified gas atmosphere would be appropriate to suppress oxidation of fats and growth of micro-organisms.

Miltenburg (1986) tested salads, flushed with  $N_2$  and with a mixture of  $CO_2$  (10% volume fractions) and  $N_2$  (90% volume fraction). Lactobacilli cannot be inhibited by these gases. Yeasts were inhibited only by the mixture, and not by  $N_2$  alone. These results were obtained with salads prepared under vacuum. In a normal salad, prepared in the presence of oxygen, the growth of yeasts was not inhibited by flushing the headspace with  $CO_2/N_2$ . It seems that so much oxygen is left in the salads that the yeasts can grow normally. The method can probably be improved by evacuating the air from the package before adding the gas. This method was used by Eringfeld (1988), who put sauce under vacuum and then added a mixture of  $CO_2$  (10% or 20% volume fractions) and  $N_2$ . Again only yeasts and not lactobacilli were inhibited.

## Conclusions

- Major spoilage organisms of mayonnaise-based salads are yeasts, lactic acid bacteria and a few moulds.

- Such salads do not normally permit growth of pathogenic micro-organisms, due to low pH, presence of organic acids and low storage temperatures.

- Legally permitted preservatives, such as benzoic acid and sorbic acid are effective in these products with low pH. However, a major draw-back is their solubility in oil which causes them to be less effective with increasing oil content below a critical pH of 4.9 (benzoic acid) or 5.1 (sorbic acid).

- Vacuum or gas packaging is effective only if dissolved oxygen is removed, and its effectiveness is limited to yeasts and moulds only.

- Shelf-life of mayonnaise-based salads without preservatives depends on initial contamination, pH, concentration of undissociated acetic acid in the water phase, and storage temperature. In view of consumers preference for less sour salads with no preservatives added, the application of strict GMP rules to production and storage of salads is becoming all the more important.

## References

- Ansenwoude, G.H.M.G. van; 1986. Bederf van salades. M.Sc.Thesis, Agricultural University, Department of Food Science, Wageningen, Netherlands.
- Baird-Parker, A.C.; 1980. Organic acids. In: Microbial ecology of foods. Vol I. Factors affecting life and death of microorganisms. The International Commission on Microbiological Specifications for Foods. Academic Press, New York and London. p. 126-135.
- Baumgart, J.; 1977. Zur mikrobiologischen Stabilität von Feinkost-Salaten. *Alimenta* 16:197-200.
- Breeuwer, P.; 1988. De verdeling van conserveermiddelen over de olie- en waterfase in salades. M.Sc.Thesis, Agricultural University, Department of Food Science, Wageningen, Netherlands.

- Brocklehurst, T.F.; 1984. A delicatessen problem. *Food Manufacture* (april) 65-67.
- Brocklehurst, T.F.; Lund, B.M.; 1984. Microbiological changes in mayonnaise-based salads during storage. *Food Microbiol.* 1:5-12.
- Brocklehurst, T.F.; White, C.A.; Dennis, C.; 1983. The microflora of stored coleslaw and factors affecting the growth of spoilage yeasts in coleslaw. *J. Appl. Bacteriol.* 55:57-63.
- Davies, A.M.C.; Brocklehurst, T.F.; 1986. Near infrared reflectance analysis of oil concentration in an emulsion: a study of oil and water migrations in a mayonnaise-based salad. *J. Sci. Food Agric.* 37:310-316.
- Debevere, J.M.; 1987. The use of buffered acidulant systems to improve the microbiological stability of acid foods. *Food Microbiol.* 4:105-114.
- Eringfeld, M.P.L.A.; 1988. Verbetering van de microbiële stabiliteit van salades. M.Sc.Thesis, Agricultural University, Department of Food Science, Wageningen, Netherlands.
- Fowler, J.L.; Clark, W.S.; 1975. Microbiology of delicatessen salads. *J. Milk Food Techn.* 38:146-149.
- Harrison, L.J.; Cunningham, F.E.; 1985. Factors influencing the quality of mayonnaise : a review. *J. Food Quality* 8:1-20.
- Holtzapffel, D.; Mossel, D.A.A.; 1968. The survival of pathogenic bacteria in, and the microbial spoilage of, salads, containing meat, fish and vegetables. *J. Food Technol.* 3:223-239.
- Kirsop, B.H.; Brocklehurst, T.F.; 1982. The spoilage of mayonnaise-based delicatessen salads. *Process Biochem.* (sept/oct) 9-11, 14.
- Lubieniecki-von Schelhorn, M.; 1967. Untersuchungen über die Verteilung von Konservierungsstoffen zwischen Fett und Wasser, I. Mitteilung. *Zeitsch.Lebensm.Unters. Forschung* 131:329-345.
- Miltenburg, S.; 1986. Microbieel bederf van salades op basis van mayonaise. M.Sc.Thesis, Agricultural University, Department of Food Science, Wageningen, Netherlands.
- Prade, E.H.; 1988. Het gedrag van pathogenen in salades. M.Sc.Thesis, Agricultural University, Department of Food Science, Wageningen, Netherlands.
- Smeenk, I.; 1986. Microbiële stabilisatie van salades. M.Sc.Thesis, Agricultural University, Department of Food Science, Wageningen, Netherlands.
- Smittle, R.B.; 1977. Microbiology of mayonnaise and salad dressing: a review. *J. Food Protect.* 40:415-422.
- Smittle, R.B.; Flowers, R.S.; 1982. Acid tolerant microorganisms involved in the spoilage of salad dressings. *J. Food Protect.* 45:977-983.
- Stöltzing, U.; 1987. Einfluss von Essigsäure und Puffersubstanzen auf die Haltbarkeit und die sensorischen Eigenschaften unkonserverter Salatmayonnaise. *Lebensmitteltechnik* 3:96-99.
- Terry, R.C.; Overcast, W.W.; 1976. A research note : A microbiological profile of commercially prepared salads. *J. Food Sci.* 41:211-213.
- Tunaley, A.; Brownsey, G.; Brocklehurst, T.F.; 1985. Changes in mayonnaise-based salads during storage. *Lebensm. Wissensch. u. Technol.* 18:220-224.
- Zschaler R.; 1976. Einfluss von physikalischen und chemischen Faktoren auf die Haltbarkeit von Feinkost-Erzeugnissen. *Alimenta* 15:185-188.

# Chemical papers

## HEAD-SPACE VOLATILES AND DETERIORATION REACTIONS DURING STORAGE OF DEHYDRATED MASHED POTATOES

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### Summary

During storage of potato flakes and granules, the formation of head-space volatiles was monitored. C1-C6 straight chain hydrocarbons and hexanal (oxidation products of lipids) and methylpropanal and methylbutanal (non-enzymic browning products) were evaluated. It appeared to be meaningful to monitor these head-space volatiles in order to study the influence of gas atmosphere (air, nitrogen, carbon dioxide), sulphite and antioxidants on the relative importance of the two major deterioration reactions determining shelf-life of dehydrated mashed potatoes: oxidation of lipids and non-enzymic browning.

Descriptors: oxidation of lipids; non-enzymic browning.

### Introduction

Fresh cooked potatoes have a mild but characteristic aroma. Steam distillation during the drying process drives off volatile flavour components and consequently dehydrated potatoes are at best practically devoid of potato aroma (Sapers, 1975). Chemical changes in dehydrated potatoes during processing or storage, which cause the formation of volatile substances, are likely to produce an off-flavour, thus limiting shelf-life of dehydrated (mashed) potatoes.

Two deterioration reactions play a major role during processing and storage of dry potato flakes and granules: non-enzymic browning (Strecker degradation) and oxidation of lipids. Both reactions produce volatile off-flavours and their significance for shelf-life of dehydrated potatoes has been recognized (Burton, 1945; 1949; Hendel et al., 1955).

Various components can be detected by analysis of the vapour above dehydrated potato products (head-space). Buttery & Teranishi (1963) measured hexanal as autoxidation product of linoleic acid and "the browning aldehydes" 2-methylpropanal (MP) and 2-/3-methylbutanal (MB) in the head-space of potato granules. Sapers et al. (1970; 1972) determined the same components in the head-space of puffed dehydrated potatoes and potato flakes. Straight-chain hydrocarbons, C1-C6, which are secondary oxidation products of unsaturated fatty acids, are formed parallel to aldehydes like hexanal. These volatiles were determined in head-space of potato flakes by Arnaud & Wuhrmann (1974) and Löliger & Jent (1983). These hydrocarbons (C1-C6) did not cause an off-flavour but could be used to determine the state of lipid oxidation (Löliger & Jent, 1983).

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In this study, the formation of volatiles in the head-space of potato flakes and granules has been studied in relation to the influence of additives (sulphite, antioxidants), moisture content and storage gas on deterioration during storage (shelf-life) (Keijbets et al., 1988).

### Materials and methods

#### Potato flakes and granules processing, packaging and storage

Potato flakes were processed at the IBVL pilot plant by a standard procedure (Adler, 1971). Potato tubers (cv. Bintje; dry matter about 220 g/kg reducing sugars 2.5--6.6 g/kg), were stored at 8 °C with sprout inhibitor (C)IPC. They were steam-peeled, sliced 15 mm thick, washed, precooked for 20 min. 70 °C, cooled for 20 min in water (4 °C), steam-cooked for 30--35 min, mashed in a ricer and drum-dried. The drying conditions were steam pressure 0.3--0.4 MPa, drum speed 2--3 rotations per min, drying time 12--20 s, distance between drum and last feeding roll 0.5 mm. After mashing and before drying, emulsifier, antioxidants and sulphite were added and mixed into the mash. A monoglyceride mixture of palmitic and stearic acid, 1:1 by mass (Eastman, Kodak, USA Food Industries, Netherlands), 5 g/kg on potato dry basis was used as an emulsifier. Mixtures of BHA/BHT (in groundnut oil), BHA (in propylene glycol) (Naarden Chemie, Netherlands) and ascorbyl palmitate (AP) (Hoffmann-La Roche, Switzerland) were used as antioxidants. AP content added to dry matter was 350 mg/kg dry matter. After drum-drying, the flakes were broken and sieved (4 mm), packed within 24 h in lacquered cans of capacity 2 litres under air, nitrogen or carbon dioxide (< 1 % oxygen) and stored at 20 °C, until analysis. Potato granules were manufactured in an industrial plant according to the add-back process (Adler, 1971). The granules were packed and stored under the same conditions as the flakes.

#### Analyses

Moisture, sugar, sulphite and BHA/BHT content were determined by methods previously described by Keijbets et al. (1988). A static head-space technique was used to determine the concentration of volatiles. Head-space in the cans with potato flakes/granules was sampled by piercing the top of the cans with a small stainless steel sampling device with a sealing membrane. Gas samples were taken with a gastight needle. The volatiles were measured on a Hewlett-Packard HP5750 gas chromatograph with a flame-ionization detector for total hydrocarbons (C1-C6), methylpropanal (MP) and methylbutanals (MB). A 7.31 m x 0.32 cm stainless steel column packed with 20 % Carbowax 20M on Chromosorb WAW (60-80 mesh) was used. The GC conditions were oven temperature 75 °C and volume rate of carrier gas nitrogen 20 ml/min. Chromatograms were integrated with an HP3370A integrator. Hexanal was measured by GLC of vapour of potato flakes/granules suspended in boiling water (Buttery & Teranishi, 1963; Sapers et al., 1970). In a 300-ml serum flask plus cap, 15 g of potato product were weighed, 2 ml internal reference solution of ethylbutyrate, 5 mg/l was added followed by 150 ml of boiling tap-water. The flask was then closed and shaken for 1 min.

Headspace gas 2.5 ml was injected into a Perkin-Elmer 3B GC, fitted with a 3.05 x m 0.51 cm glass column packed with 10 % Fluorad FC-431 on Chromosorb WHP (100-200 mesh) and flame-ionization detector. Oven temperature was 110 °C, volume rate of carrier gas nitrogen 30 ml/min. Chromatograms were integrated with an HP3390A integrator. The hexanal concentration was calculated using cochromatography of a solution containing hexanal and ethylbutyrate (5 mg/l) as a reference substance.

### Results and discussion

A series of samples of potato flakes with different contents of moisture (40-120 g/kg), sulphite and antioxidant were produced on the pilot plant. The moisture content was varied by changing the drum speed. Potato granules were produced from the same lot of Bintje potatoes as the flakes, with which they were compared.

The influence of storage gas (air, nitrogen, carbon dioxide), sulphite and antioxidant on formation of volatiles in the head-space of potato flakes and granules are presented in a series of graphs (Figures 1-6).

#### Storage atmosphere

During storage of potato flakes with low moisture content (40-50 g/kg) in air, C1-C6 hydrocarbons were the predominant volatiles (Figure 1).

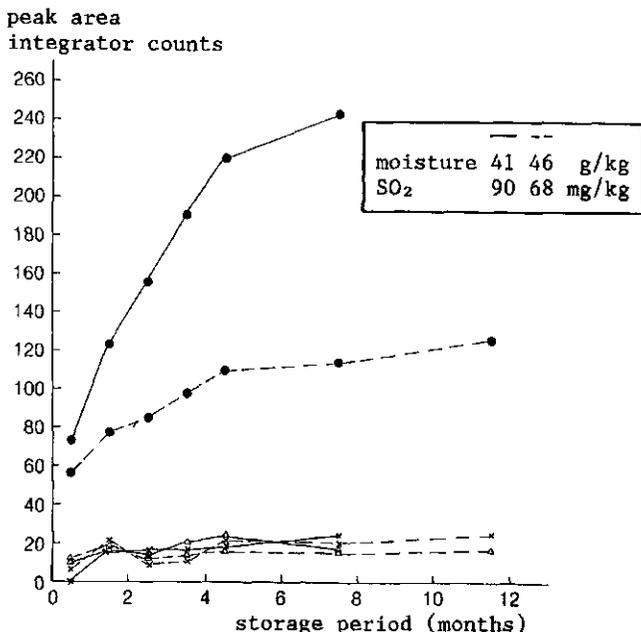


Fig. 1 Development of head-space volatiles [ $\bullet$  C1-C6 hydrocarbons,  $\Delta$  methylpropanal,  $\times$  methylbutanal(s)] during storage of low-moisture potato flakes with (--) or without (—) BHA/BHT in air at 20 °C

There was a rapid increase of C1-C6 hydrocarbons during storage at 20 °C, but hardly any change in MP and MB. The moisture content of these potato flakes was near the monolayer moisture content calculated by the BET procedure of Salwin (1959). Sapers et al. (1974) calculated a value of 55,1 g/kg, while Strolle & Cording (1965) determined this to be between 51 and 58 g/kg. At this monolayer moisture content, flakes would be optimally protected against oxygen, thus against oxidation of lipids.

At increasing moisture content (about 70 g/kg) less C1-C6 hydrocarbons were formed, but more MP and MB (Figure 2). At 100 g/kg, the concentration of C1-C6 (and hexanal) were very low, therefore MP and MB became the major volatiles (Figure 3).

peak area  
integrator counts

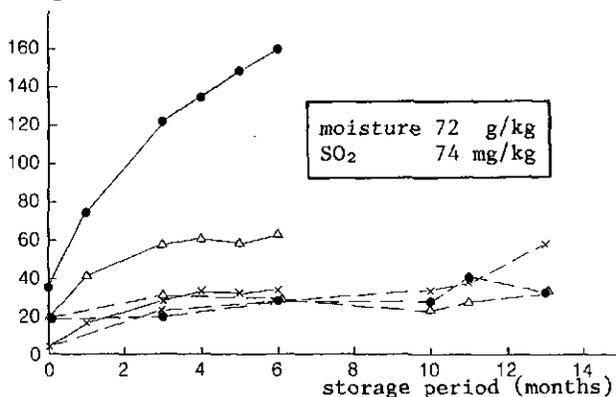


Fig. 2 Development of head-space volatiles [● C1-C6 hydrocarbons, Δ methylpropanal, x methylbutanal(s)] during storage of intermediate moisture potato flakes in air (-) or nitrogen (--) at 20 °C

peak area  
integrator counts

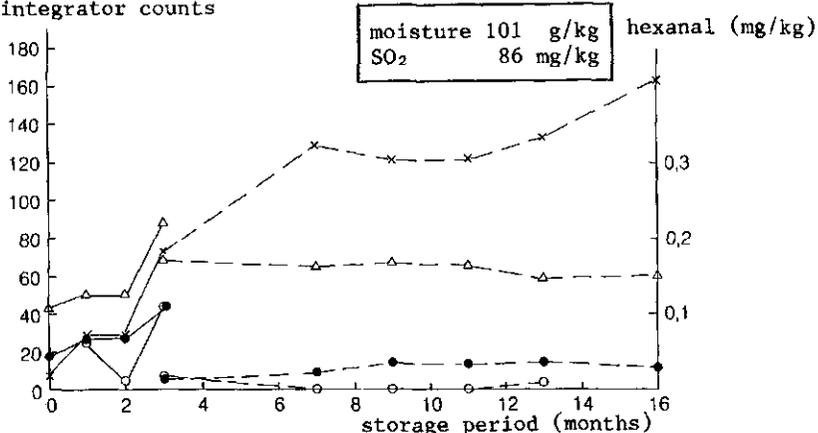


Fig. 3 Development of head-space volatiles [○ hexanal, ● C1-C6 hydrocarbons, Δ methylpropanal, x methylbutanal(s)] during storage of high-moisture potato flakes in air (-) or nitrogen (--) at 20 °C

This is even more clear in Figure 4, where potato flakes from the same production date with different moisture and sulphite contents are compared. These results show that lipid oxidation becomes a minor factor to non-enzymic browning in deterioration of potato flakes at increasing moisture content.

Whistler & Daniel (1985) make a clear distinction between Maillard reaction and Strecker degradation. The former is a complex reaction between amino groups of an amino acid or protein and reducing sugar leading to brown pigments, the latter involves interaction of  $\alpha$ -dicarbonyl compounds (which may originate in the Maillard reaction !) and  $\alpha$ -amino acids but does not result in pigment formation. MP, 2-MB and 3-MB are Strecker degradation products of the  $\alpha$ -amino acids L-valine, L-isoleucine and L-leucine. The Strecker degradation produces carbon dioxide. Sulphite, that inhibits non-enzymic browning, has little effect on this reaction.

Storage of potato flakes in nitrogen is an effective way to inhibit lipid oxidation. Development of C1-C6 and hexanal was largely retarded in nitrogen (Figures 2, 3 and 4). The Strecker degradation products became the predominant volatiles, mainly at high moisture contents (90--120 g/kg) (contrast Figures 2 and 3, Figures 4a,b and 4c,d). Contrary to storage in air, MB contents were higher than those of MP. The formation of MB appeared to be less oxygen-dependent than that of MP.

There was some indication that storage in carbon dioxide prohibited the development of MP and particularly MB at high moisture content (92 g/kg) in comparison with nitrogen (Figure 5). Increase in the moisture content from 77 to 92 g/kg strongly stimulated the formation of MB in the nitrogen head-space, but not in carbon dioxide. This may be a typical example of product inhibition by carbon dioxide in the Strecker degradation.

#### Antioxidants

Addition of BHA/BHT to potato flakes had a similar effect during storage in air to replacement of air by nitrogen. The development of C1-C6 HC was retarded (Figure 1). AP also had an antioxidative effect, though less than BHA/BHT (not shown). Löliger & Jent (1983) also demonstrated that C1-C6 concentration decreased when BHA/BHT was used. A natural rosemary extract had the same effect as BHA/BHT.

#### Sulphite

Sulphite, already at very low content (8 - 14 mg/kg), inhibited the formation of MP but hardly that of MB (Figure 4). The effect of sulphite is most obvious at high moisture content (Figures 4a,b against Figures 4c,d). Because of the relevance of Strecker degradation products for storage in nitrogen, low contents of sulphite (< 100 mg/kg) were effective in improving shelf-life of flakes in nitrogen at least at not too high contents of moisture (50--70 g/kg) (Keijbets et al., 1988). Beyond 70 g/kg, particularly at 90--120 g/kg, sulphite was less effective because it did not inhibit MB, the dominant volatile for high contents of moisture in nitrogen storage (Figures 4c,d).

The different behaviour of MP and MB (effect of CO<sub>2</sub>, sulphite) characterizes MB as a typical Strecker degradation product, but MP as a Maillard reaction product.

	A	B	C	D	
moisture	82	78	115	121	g/kg
SO <sub>2</sub>	0	14	0	8	mg/kg
BHA	5	5	3	4	mg/kg
AP	all 350				mg/kg

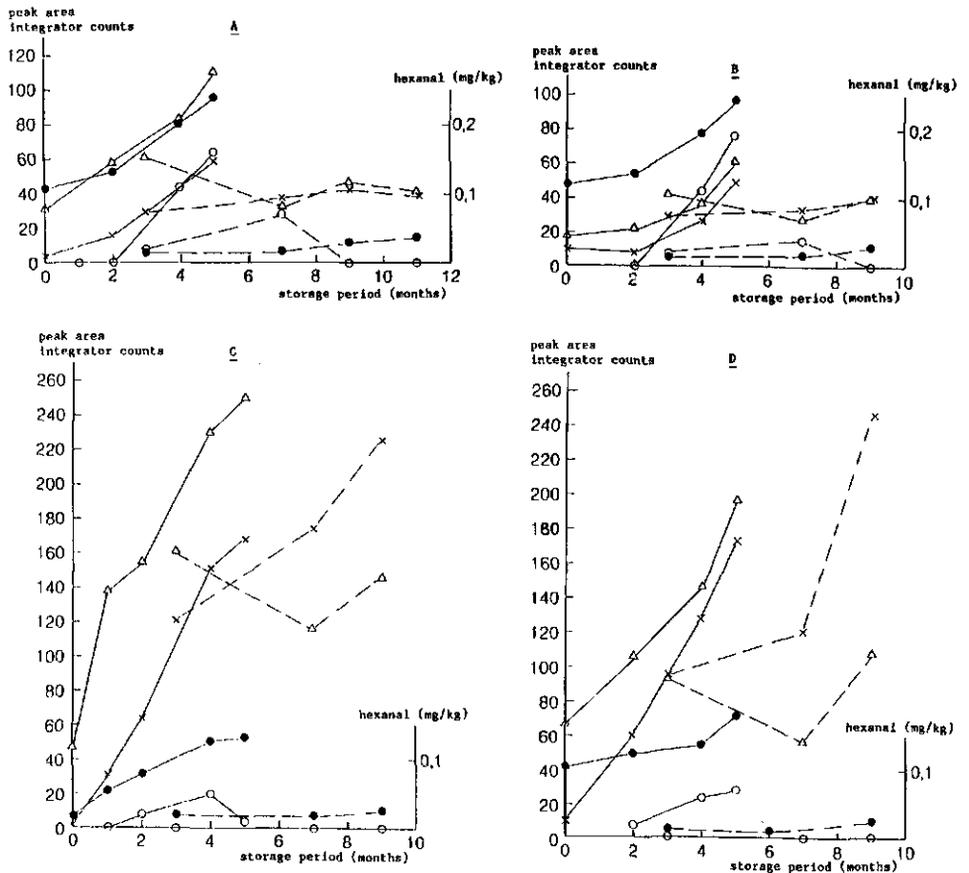


Fig. 4 Development of head-space volatiles [o hexanal, • Cl-C6 hydrocarbons, Δ methylpropanal, x methylbutanal(s)] during storage of intermediate and high-moisture potato flakes with or without sulphite in air (—) or nitrogen (--) at 20 °C

	A	B
moisture	77	92 g/kg
SO <sub>2</sub>	91	105 mg/kg
BHA	11	19 mg/kg

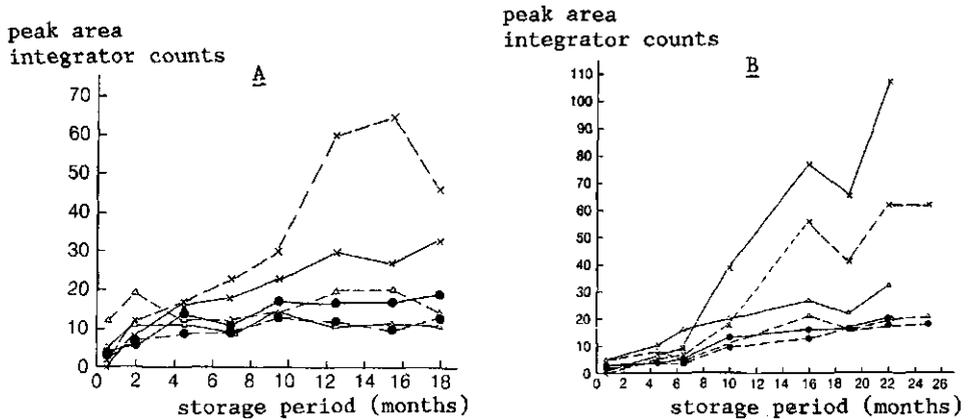


Fig. 5 Development of head-space volatiles [o hexanal, • C1-C6 hydrocarbons, Δ methylpropanal, x methylbutanal(s)] during storage of intermediate and high-moisture potato flakes in nitrogen (—) or carbon dioxide (--) at 20 °C

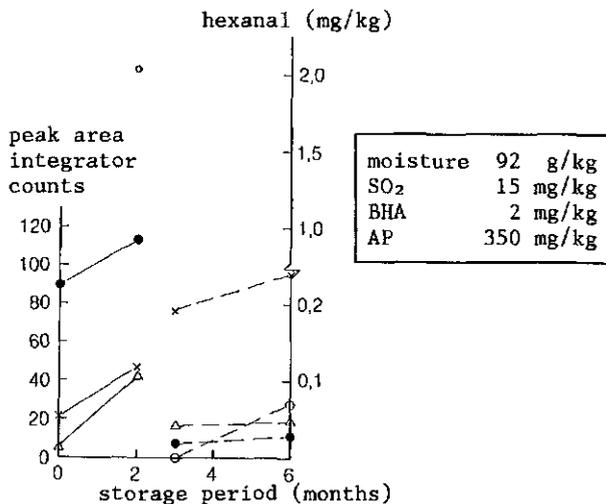


Fig. 6 Development of head-space volatiles [o hexanal, • C1-C6 hydrocarbons, Δ methylpropanal, x methylbutanal(s)] during storage of potato granules in air (—) or nitrogen (--) at 20 °C

## Potato granules

Deterioration by lipid oxidation was much more severe in potato granules than in flakes produced from the same potato raw material, as shown by the rapid development of C1-C6 and hexanal (Figure 6 against Figures 4b,d). This was true for those samples stored either in air or nitrogen. The development of MP in potato granules was less than in flakes. The rapid oxidation of lipids in these granules coincides with a description characterized as slightly oxidized (Escher et al., 1981).

## Conclusions

Measurement of head-space volatiles during storage of potato flakes and granules is a useful tool to clarify in the course of deteriorations limiting shelf-life. Composition of the gas atmosphere, sulphite and antioxidants influence the relative importance of these reactions: lipid oxidation and non-enzymic browning/Strecker degradation.

- During storage in air at low moisture content (40--50 g/kg) C1-C6 hydrocarbons are the major volatiles (lipid oxidation). At increasing moisture content, methylpropanal and methylbutanals become dominant in the head-space (Strecker degradation).
- Antioxidants with air or nitrogen storage inhibit the development of C1-C6 hydrocarbons and hexanal (lipid oxidation), so that the Strecker degradation aldehydes become important volatiles, in particular at high moisture contents (90--120 g/kg).
- Sulphite is an effective inhibitor of methylpropanal formation in head-space, mainly at intermediate moisture contents (50--70 g/kg). As a result, sulphite at low contents would increase the shelf-life of potato products when stored in nitrogen. This is mainly due to the significance of Strecker degradation during storage in nitrogen. At high moisture contents (90--120 g/kg) methylbutanal dominates the head-space with nitrogen storage.
- Carbon dioxide inhibits the development of methylbutanal and to a lesser extent of methylpropanal for potato flakes with a high moisture content (90 g/kg).
- Deterioration by oxidation of lipids was much more severe in potato granules than in flakes.

## Acknowledgment

Mrs G. Ebbenhorst-Seller and J. Ruisch analysed the potato flakes and granules.

## References

- Adler, G., 1971. Kartoffeln und Kartoffelerzeugnisse. Verlag Paul Parey, Berlin-Hamburg, 202 pp.
- Arnaud, M. & J.J. Wuhrmann, 1974. Dehydrated food oxidation as measured by thermal release of hydrocarbons. Proc. IV Int. Congr. Fd Sci. Technol, Madrid.
- Burton, W.G., 1945. Mashed potato powder. III. High temperature browning of mashed potato powder. J. Soc. chem. Ind. 64: 215-218.

- Burton, W.G., 1949. Mashed potato powder. IV. Deterioration due to oxydative changes. *J. Soc. Chem. Ind.* 68: 149-151.
- Buttery, R.G. & R. Teranishi, 1963. Measurements of fat autooxidation and browning aldehydes in food vapors by direct vapor injection gas-liquid chromatography. *J. agric. Food Chem.* 11: 504-507.
- Escher, F., P. Rotach, A. Denzler & J. Solms, 1981. Herstellung von granulätähnlichen Produkten aus walzengetrockneten Kartoffelflocken. *Lebensm. - Wiss. u.-Technol.* 14: 260-267.
- Hendel, C.E., H.K. Burr & M.M. Boggs, 1955. Non-enzymatic browning and sulfite flavor in dehydrated white potato. Effects of sulfite level and method of sulfite application. *Food Technol.* 9: 627-629.
- Keijbets, M.J.H., J.W. Ludwig, & G. Ebbenhorst-Seller, 1988. Houdbaarheid van gedroogde aardappelpuree: invloed van grondstof, toevoegmiddelen en bewaaratmosfeer, chemische en sensorische aspecten, IBVL-Rapport 680.
- Löliger, J. & A. Jent, 1983. Analytical methods for quality control of dried potato flakes. *Am. Potato J.* 60: 511-524.
- Salwin, H., 1959. Defining minimum moisture contents for dehydrated foods. *Food Technol.* 13: 594-595.
- Sapers, G.M., 1975. Flavor and stability of dehydrated potato products. *J. agric. Food Chem.* 23: 1027-1032.
- Sapers, G.M., O. Panasiuk, F.B. Talley, S.F. Osman & R.L. Shaw, 1972. Flavor quality and stability of potato flakes. Volatile components associated with storage changes. *J. Food Sci.* 37: 579-583.
- Sapers, G.M., O. Panasiuk, F.B. Talley & R.L. Shaw, 1974. Flavor quality and stability of potato flakes. Effect of drying conditions, moisture content and packaging. *J. Food. Sci.* 39: 555-558.
- Sapers, G.M., J.F. Sullivan & F.B. Talley, 1970. Flavor quality in explosion puffed dehydrated potato. 1. A gas chromatographic method for the determination of aldehydes associated with flavor quality. *J. Food Sci.* 35: 728-730.
- Strolle, E.O. & J. Cording jr., 1965. Moisture equilibria of dehydrated mashed potato flakes. *Food Technol.* 19: 583-585.
- Whistler, R.L. & J.R. Daniel, 1985. Carbohydrates. In: *Food Chemistry*, O.R. Fennema (Ed.), 2nd Edn. Marcel Dekker, Inc., New York-Basel.

## FPLC-HPLC: a useful combination for screening hemicellulase activities in technical enzyme preparations

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### Summary

Technical enzyme preparations were screened by a combination of FPLC (Fast Protein Liquid Chromatography) and HPLC. They were first fractionated by FPLC using an anion-exchanger (Mono Q). The obtained fractions were screened for enzyme activities by measuring the amount of reducing sugars released by these fractions after incubation with various substrates. Fractions which showed xylanase activity were used to determine the degradation pattern by HPLC: endo and exo-activity were detected by analysing the sample on combined Biogel TSK 40 XL, 30 XL and 20 XL columns; xylose and xylose-oligomers were analysed on a Aminex HPX 87P column. Descriptors: FPLC, HPLC, screening,  $\beta$ -xylanase,  $\beta$ -xylosidase.

### Introduction

#### Classification

Xylan-degrading enzymes can be classified into three groups of enzymes -endo-xylanases which degrade xylan by hydrolysis into low oligomeric xylose fractions; this group can be subdivided into endo-xylanases able to hydrolyse also the xylose-arabinose bond and endo-xylanases unable to hydrolyse the xylose-arabinose bond, -exo-xylanases which degrades xylan from the non-reducing end into xylose- $\beta$ -xylosidase which degrades low oligomeric xylose-fractions into xylose (Reilly, 1981; Dekker, 1976).

#### Xylan structure

Xylans are mainly composed of a xylan backbone with single unit side chains of arabinose and methyl-glucuronic acid. For a complete enzymic breakdown of xylan one probably needs a combination of not only different xylan-degrading enzymes but also  $\alpha$ -arabinofuranosidase and  $\alpha$ -glucuronidase (Biely, 1985). Endo-(1,4)- $\beta$ -xylanases (E.C.3.2.1.8) cleave the  $\beta$ -(1,4)-bond between two xylose-units. The  $\beta$ -xylosidases (E.C.3.2.1.37) split of xylose by cleaving the  $\beta$ -(1,4)-bond between two xylose-units from the non-reducing end. The sugars which occur as a side-chain of the xylan backbone can be released by two important enzymes, namely  $\alpha$ -glucuronidase (E.C.3.2.1) and  $\alpha$ -L-arabinofuranosidase (E.C.3.2.1.55). The  $\alpha$ -glucuronidase cleaves the  $\alpha$ -(1,2)-bond between a xylose and a methyl-glucuronic acid. The  $\alpha$ -L-arabinofuranosidase cleaves the  $\alpha$ -(1,3)-bond between a xylose and an arabinofuranose (Biely, 1985).

Depending on the source of the xylan (softwood or hardwood xylan) xylose can be highly acetylated (Timell, 1964 and 1965). This acetylation can play an important role in the total degradation of xylan in raw materials. Esterase is the enzyme needed to remove these acetyl groups from xylose.

By only measuring the amount of reducing sugars released by xylan-de-

grading enzymes it is not possible to make conclusions about the nature of the xylan degrading enzymes (see classification) or the number of enzymes present in a technical enzyme preparation.

Combination of FPLC and HPLC gives a powerful method for screening technical enzyme preparations.

### Materials and Methods

Enzyme preparations used were obtained from Gist-Brocades (Seclin, France), Röhm (Darmstadt, FRG) and Novo (Basel, Switzerland).

Fractionation of the enzyme preparations was carried out on a Mono Q column (anion exchanger) with a FPLC (Fast Protein Liquid Chromatograph, Pharmacia). Degradation products of xylan incubated with different enzyme fractions, were analysed with three Biogel-TSK columns in series (Biogel TSK 40+30+20 XL; Biorad) and an Aminex HPX 87P column (Biorad) on a SP 8200 Spectra Physics HPLC.

Enzyme activities were measured according to Beldman et al (1985). Enzymes and substrates were incubated in sodium acetate buffer pH 5.0 at 30°C and the activities calculated from the increase in reducing end groups as measured by the Nelson-Somogyi method (Somogyi, 1952) or from the release of p-nitrophenol from the p-nitrophenylglycoside as measured spectrophotometrically at 400 nm. One unit of activity is defined as the amount of enzyme that catalyses the release of 1 µmol of xylose or p-nitrophenol from the substrate per minute at 30°C.

### Results and discussion

#### Screening technical enzyme preparations

Table 1 summarizes the technical enzyme preparations which were fractionated by Fast Protein Liquid Chromatography (FPLC).

Table 1. Technical enzyme preparations screened by FPLC; Roman numbers refer to figure 1 and 2.

Code	Name of enzyme preparation
I	Xylanase ( <u>Trichoderma resei</u> )
II	Maxazym CL2000
III	Xylanase ( <u>Aspergillus niger</u> )
IV	KPB 00I 3L
V	Rapidase C600
VI	Xylanase ( <u>Disporotrichum dimorphosporum</u> )
VII	Xylanase 5000
VIII	Veron HE

The collected fractions were analysed for xylanase and β-xylosidase activity (substrates used were oat spelt xylan and PNP-β-xylopyranoside respectively). Figure 1 shows the elution-patterns of the different enzyme preparations on FPLC, including the xylanase and β-xylosidase activity.

Xylan was incubated for 24 h. with fraction 2 of all the fractionated preparations (figure 1). Degradation products were analysed on an Aminex HPX 87P column by HPLC (figure 2).

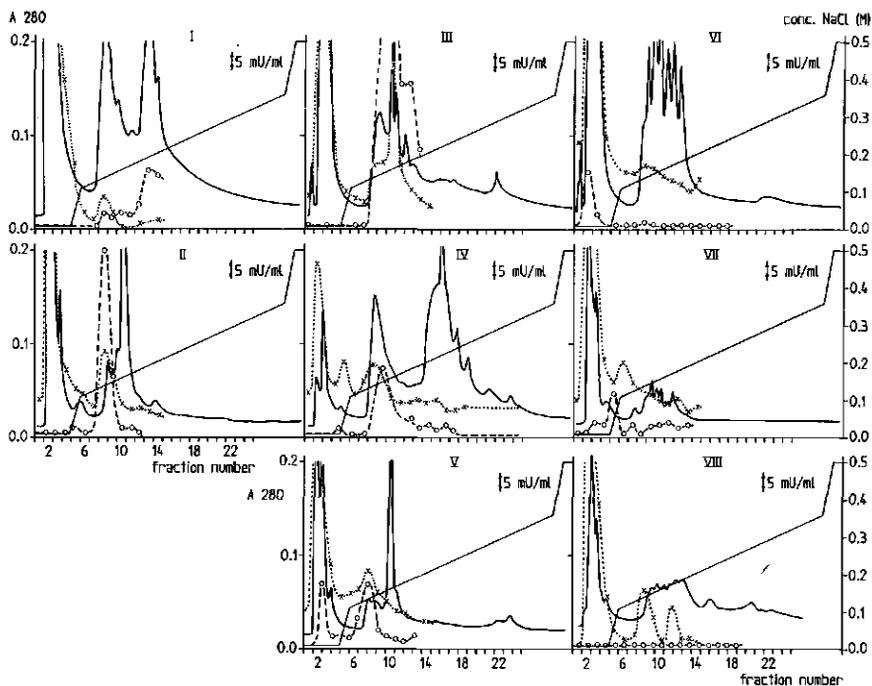


Fig. 1. FPLC-Elution pattern of the technical enzyme preparations (1 ml fractions). Buffer used was 0.02 M piperazine pH 5.0 and 0.02 M piperazine pH 5.0 containing 0.5 M NaCl (flow 1.0 ml/min).

Monitoring:

— A280  
 - - \* - Xylanase-activity  
 — o —  $\beta$ -Xylosidase-activity

Fractions having the same elution volume on the Mono Q column show remarkable similarities in the degradation pattern of xylan on HPLC, suggesting that these fractions probably contain the same types of enzyme or combination of enzymes.

As expected, fractions with  $\beta$ -xylanase and  $\beta$ -xylosidase activity result in monomeric xylose and xylobiose as end-product (preparation I and III), whereas other fractions containing only  $\beta$ -xylanase result in xylo-triose and xylobiose as end-products (preparation II, IV, V, VI and VII). The amount of oligomers formed is related to the amount of  $\beta$ -xylanase and/or  $\beta$ -xylosidase present. When large amounts of  $\beta$ -xylosidase are present, large amounts of xylose will be formed. In case of small amounts of  $\beta$ -xylosidase present in the fraction, only small amounts of xylose will be formed.

The fractions of the *Aspergillus niger* preparation (III), which showed xylanase and/or  $\beta$ -xylosidase activity, were incubated with oat spelt xylan. The incubations were analysed after 3 hours for molecular weight distribution and for low oligomeric neutral sugars.

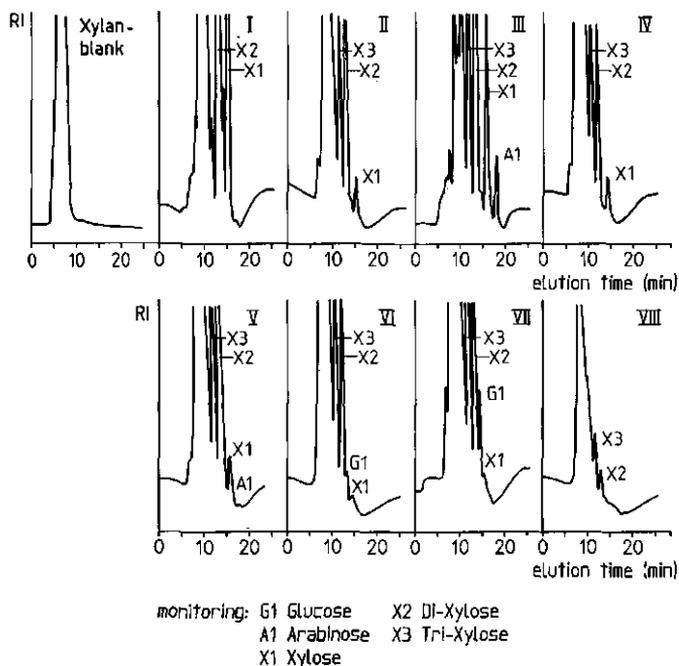


Fig. 2. HPLC elution pattern of xylan incubated with fractions 2 of the technical enzyme preparations (figure 2) for 24 h. Column: Aminex HPX 87P. Elution with millipore water, flow 0.5 ml/min at 85°C.

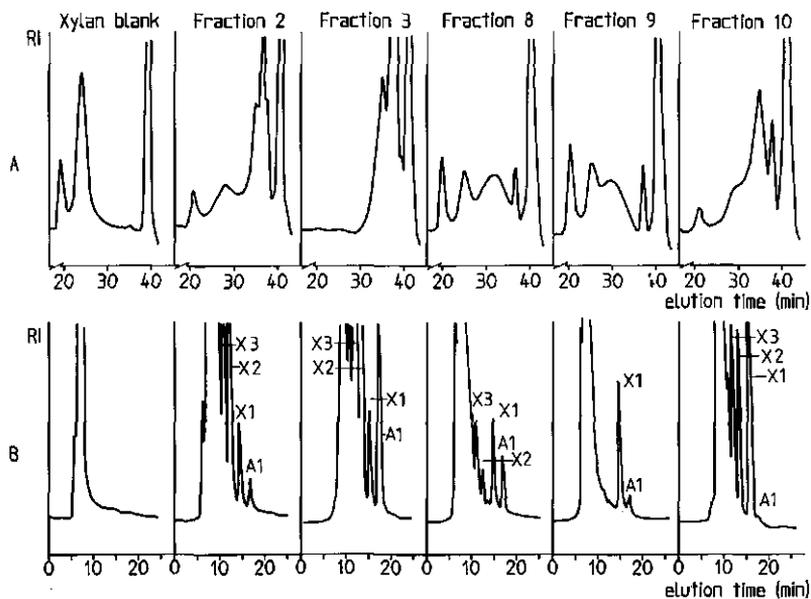


Fig. 3. HPLC elution pattern of xylan incubated with fractions 2, 3, 8, 9 and 10 of the *Aspergillus niger* (III, figure 1) for 3 h. Columns used:  
 A. Biogel TSK 40+30+20 XL: Elution with 0.4 M HAC pH 3.0 (flow 0.8 ml/min; T 30°C; Range detector 1.0).  
 B. Aminex HPX 87P: Elution with millipore water, flow 0.5 ml/min at 85°C.

The elution patterns shown in figure 3.a. indicate differences in endo and exo-activity of the fractions 2, 3, 8, 9 and 10. Fraction 8 and 9 show a single peak in figure 3.a. which according to figure 3.b. coincides with xylose and/or arabinose. The figure also shows that different xylan-active fractions can have the same degradation products in a different ratio. The presence of large amounts of arabinose, figure 3.b. fraction 3, implies  $\alpha$ -L-arabinofuranosidase.

### Conclusions

FPLC-HPLC is a very useful combination for screening technical enzyme preparations. It not only results in an overview of the number of certain enzymes (for example xylanases), but also in the type of enzymes (i.e. endo-xylanases and exo-xylanases). Measuring the release of reducing sugars from a heterogeneous polysaccharide substrate does not allow conclusions with regard to the active enzyme types. For example, when arabinoxylan is used as a substrate for activity measurements it is possible that the reducing sugars released are arabinose and not xylose. This indicates arabinofuranosidase activity and not xylanase or xylosidase activity.

When using a standard fractionation procedure on FPLC, we were able to identify enzymes in a technical enzyme preparation by their retention times using purified and well characterised enzymes as references. These purified enzymes must then however originate from the same type of micro-organism.

Screening in this way gives an impression of the types of enzymes present in technical enzyme preparations. This is very helpful when working with crude fermentation liquors of micro-organisms grown on different substrates. Running samples on FPLC or HPLC takes 30-45 minutes each, depending on the column and procedure used. Including some short sample pretreatments it is a time saving screenings-method.

### References

- Beldman, G., Searle-Van Leeuwen, M.J.F., Rombouts, F.M. and A.G.J. Voragen, 1985, The cellulases of *Trichoderma viride*, European Journal of Biochemistry, 146, 301-308.
- Biely, P., 1985, Microbial xylanolytic systems, Trends in Biotechnology, 3, 286-290.
- Dekker, R.F.H. and Richards, G.N., 1976, Hemicellulases: their occurrence, purification, properties and mode of action, Advances in Carbohydrate Chemistry and Biochemistry, 32, 278.
- Reilly, P.J., 1981, The conversion of agricultural by-products to sugars, Engineering Research Institute, Iowa, 199 p.
- Somogyi, M., 1952, Notes on sugar determination, Journal of Biological Chemistry, 195, 19-23.
- Timell, T.E., 1964, Wood hemicelluloses: Part I, Advances in Carbohydrate Chemistry and Biochemistry, 19, 247.
- Timell, T.E., 1965, Wood hemicelluloses: Part II, Advances in Carbohydrate Chemistry and Biochemistry, 20, 433.

# TESTING OF FOOD CONTACT MATERIALS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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## Summary

A method to test food contact materials by gas chromatography -massspectrometry to meet the Food and Commodity Act is described. The identification of an antioxidant in nipples is given.

Descriptors: food contact materials; antioxidants; nipples; gas chromatography-mass spectrometry, thio-bis-(2-methyl-4-hydroxy-5-tertbutyl)benzene.

## Introduction

Legislation of food contact materials in the Netherlands forms part of the Food and Commodity Act (Warenwet). Surveillance of the commodities is exercised by sixteen regional Food Inspection Services. Supervision that calls for extensive background knowledge of the products in question, or advanced analytical techniques or a special way of assessment, is exercised by small specialized units (Centres of Expertise, CE) in the food inspection organization. The (CE) on food contact materials forms part of the Food Inspection Service in Utrecht. In this centre, packaging materials and food utensils, notably those manufactured from organic components, are examined for compliance with the relevant legislation. Reliable analytical methods to investigate the food contact materials contribute to the safety of the products available by the technological progress.

### Basis for the activities

The basis of the work of the CE on food contact materials is the Packaging and Food Utensils Regulation which stipulates what classes of materials are allowed for food contact use, what raw materials, processing aids and additives are allowed for preparation of different materials and, last but not least, to what extent migration of components of such materials, together or individually, is acceptable. There are ten chapters in the regulation, each dealing with one particular class of material. Some classes have been subdivided. The plastics class, for example, comprises 24 types of plastics. Each class or subclass has its own positive list.

### Criteria in testing

From the Packaging and Food Utensils Regulation, the following criteria may be taken to establish compliance of the material with the regulation:

- nature of the material
- composition of the material
- content of specified components
- total amount of components migrating (global migration)
- amount of specified constituent migrating (specific migration)
- leaching of colouring matters

- sensorial quality of the material
- mechanical quality of the material
- contaminants.

### Techniques

The techniques used in examination of the materials include: Infrared spectroscopy, gas chromatography and liquid chromatography, thin layer chromatography (HPTLC). In this paper, only the gas chromatography will be described. The other techniques are described by Van Battum & Van Lierop (1988).

#### Gas chromatography and mass spectrometry

Head space gas chromatography (HSGC) is used to determine the content of low-molecular-weight volatile components in the material for instance monomers and residual solvents. The apparatus is a PE Sigma 2000 gas chromatograph with an HS-100 sampling device. The gas chromatograph may be operated by flame-ionization, alkali flame-ionization detection or electron-capture detection. Capillary gas chromatography with flame-ionization detection (GC) or with mass-spectrometric detection (GC-MS) is employed to detect the 'gas chromatographable' components in an extract of the food contact material in question, as well as for the determination of specific migration. The apparatus for GC is an HP 5880A gas chromatograph, connected with an HP 3500 laboratory data system. The one for GC-MS consists of a Finnigan 4000 gas chromatograph-mass spectrometer combination, and a Super Incos data system with National Bureau of Standards (NBS) library. The mass spectrometer was operated in the electron impact mode.

Fused silica capillary column (Hewlett Packard Ultra 1) coated with cross-linked methylsilicone gum. Length 50 m; internal diameter 0.32 mm; coating thickness 0.52  $\mu\text{m}$ ; column directly connected to the MS source, temperature of injector 200  $^{\circ}\text{C}$ ; temperature of separator 240  $^{\circ}\text{C}$ ; temperature program 3 min at 60  $^{\circ}\text{C}$ , 10  $^{\circ}\text{C}/\text{min}$  to 300  $^{\circ}\text{C}$ . Volume rate of helium carrier about 1 ml/min; MS pressure 5E-7 torr; injection 1  $\mu\text{l}$  splitless.

#### Reference materials

For comparison, reference materials are necessary. As a rule, small samples of additives or processing chemicals for reference purposes are obtainable only from the producer or from the manufacturer of the material. As commercial names of additives have very little in common with the chemical names used in positive lists, collecting the reference materials is quite time-consuming. On receipt, the reference materials are coded and analytical parameters are determined, i.e. infrared and mass-spectra are taken, retention index for GC and HPLC and chromatographic index values for HPTLC are determined, and the colour of spots in HPTLC is recorded. All data are stored in data bases and so a continually growing set of data is available for the identification of components of such materials.

#### Sampling of food contact materials

Within the framework of surveillance, sampling of materials from all over the country is a complicated matter. In addition to domestic manufacturers and converters, many foreign companies operate on the Dutch

market. Moreover there are many importers and numerous agencies in the field, some of which sell not only food contact materials, but also other packaging materials not subject to the Regulation. Sampling would be most efficient when arranged with the firms.

#### Testing procedure

Full testing of a food contact material for compliance with the Regulation (i.e. examination the nature of the material, composition, volatiles content, migration of constituents and sensorial quality) is a labour-intensive matter. In practice, therefore, a step-by-step procedure is exercised by the CE.

The procedure is as follows

1. Introduce 150 g of material into a small glass vial of capacity 15ml.
2. Add 1 ml acetone containing 30 mg/l of each of the internal standards C12, C20 and C24 alkane. (add 200 mg C12, C20 and C24, respectively per kg of polymer).
3. Cap the vial with a Teflon-coated septum.
4. Place the vial for half an hour in a ultrasonic bath.
5. Inject 1 ul into the GC or GC-MS system.
6. Determine relative retention time of peaks with respect to C12, C20 or C24 for identification of the component.
7. Determine relative response of components with respect to C12, C20 or C24 to estimate the amount of each component extracted.

#### Results

##### Composition of the material

The composition of the materials is investigated by extraction of the material with a suitable solvent, followed by analysis of the extract. For GC or GC-MS, a sample of the material is extracted with diethyl ether or acetone to which small amounts of C12, C20 and C24 alkanes have been added as internal standards. These standards serve as references in the identification of components by retention index. In figure 1 is a gas chromatogram of a mixture of hydrocarbons C9-C20 and C22-C25. The Kovacs indices of interesting components from the packaging materials can be obtained. Figure 2 shows the reconstructed ion chromatogram of a mixture of reference compounds. Figure 3 illustrates the possibilities of the data system. With single-ion detection, the detectionlevel is considerably lowered and more compounds can be analysed. They serve also as yardsticks for the semiquantitative determination of the amount of components extracted.

Figure 4 gives the reconstructed ion chromatogram of the acetone extract of a rubber nipple. Peak 1185 could be identified by using our MS library of about 300 references. It coincided with the antioxidant thio-bis(2-methyl-4-hydroxy-5-tertiarbutyl)benzene. The data system of the GC-MS allows subtraction of the reference spectrum from the sample spectrum (figure 5). Such a figure is often more useful for the identification than the data of purity fit and refit also produced by the data system of GC-MS.

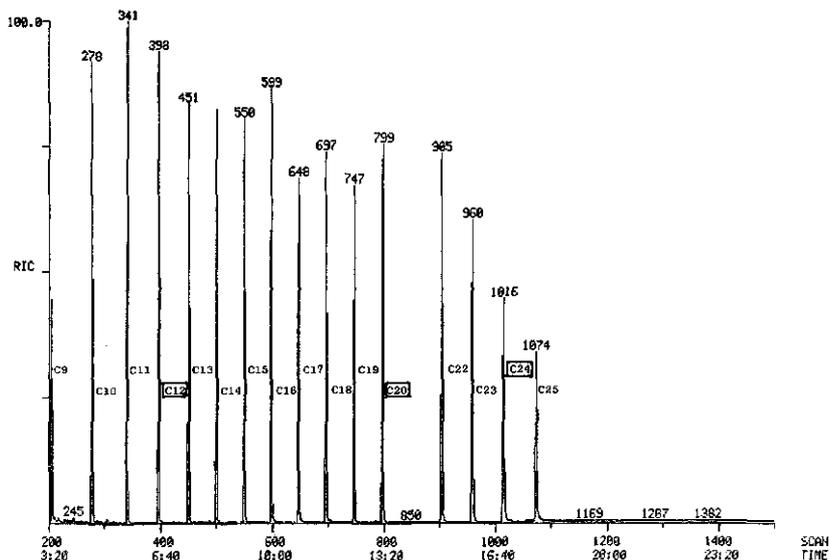


Figure 1. Reconstructed ion chromatogram/relative ion current of a mixture of hydrocarbons C9-C20 and C22-C25.

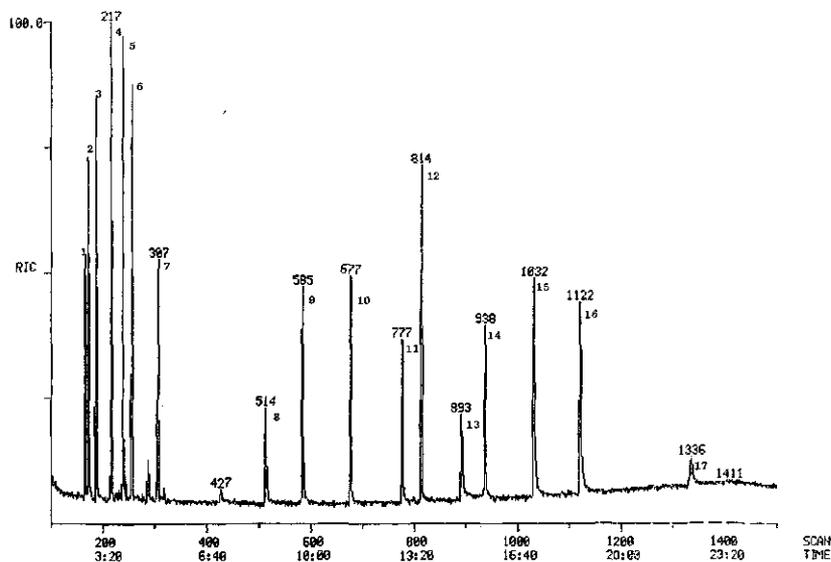


Figure 2. Relative ion current of mixture of reference compounds: (1) ethyl benzene; (2) metaxylene; (3) styrene; (4) isopropylbenzene; (5) propylbenzene; (6) alpha-methylstyrene; (7) styrene oxide; (8) dimethylphtalate; (9) diethylphtalate; (10) dipropylphtalate; (11) dibutylphtalate; (12) eicosane (C20 alkane); (13) dibutylsebacate; (14) acetyl triethylcitrate; (15) di-(2-ethylhexyl)adipate; (16) di-(2-ethylhexyl)phtalate; (17) di-n-octylazelate.

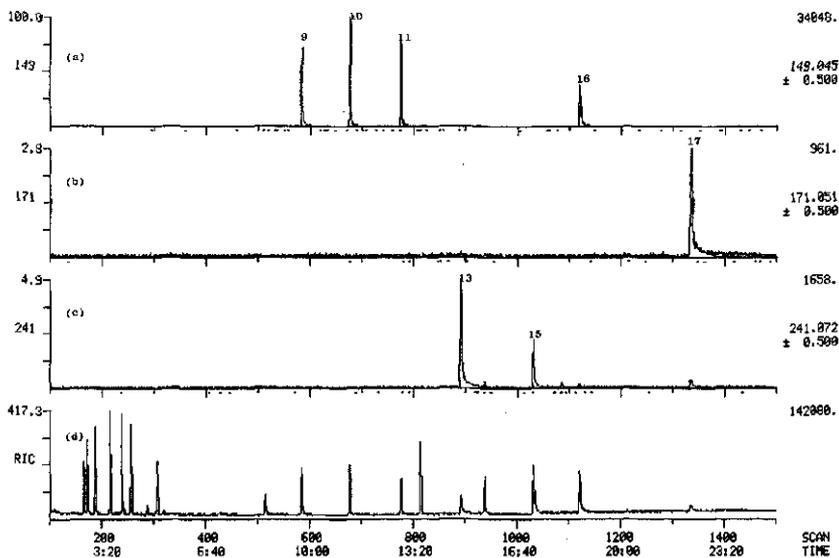


Figure 3. Single-ion detection chromatogram for (a) phthalates with  $m/z$  149; (b) di-*n*-octylazelaate with  $m/z$  171; (c) dibutyl sebacate and di-(2-ethylhexyl)adipate with  $m/z$  241; (d) relative ion current of mixture of reference compounds (see fig. 2).

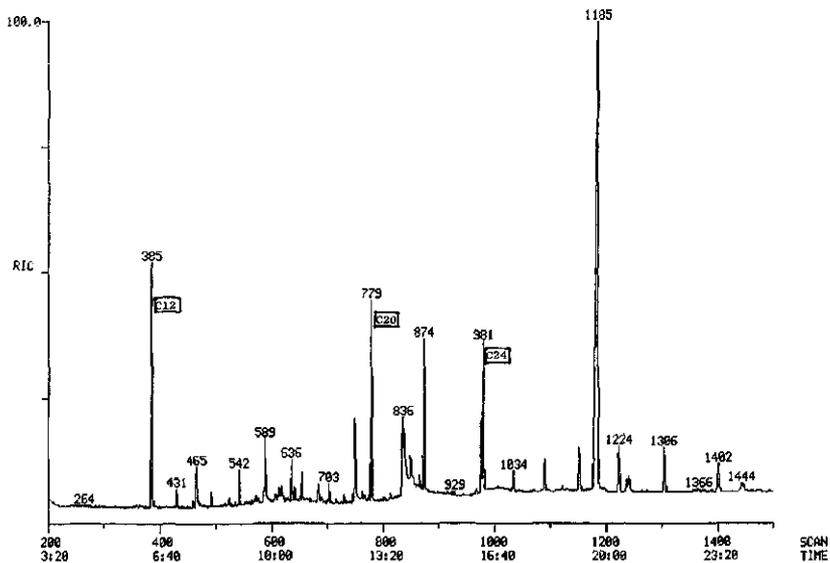


Figure 4. Relative ion current of acetone extract of a rubber nipple.

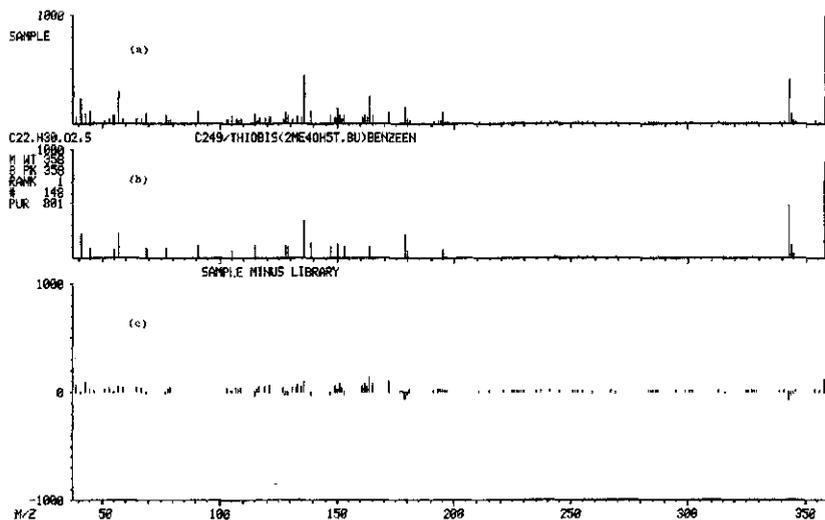


Figure 5. Identification of peak 1185 (see fig. 4). (a) mass spectrum of sample peak 1185; (b) library mass spectrum of thio bis (2-methyl 4-hydroxy 5-tertiar butyl)benzene; (c) difference between (a) and (b).

### Discussion

The procedure for testing food contact materials has been introduced gradually over the past few years and is continually being improved. The identification of an antioxidant, not mentioned on the positive list in the Food and Commodity Act, was described to show the power of combined techniques as GC-MS.

### Reference

Van Battum, D & van Lierop, J.B.H.; 1988. Testing of food contact materials in the Netherlands. Food additives and contaminants 5:381-395.

## OXIDATIVE STABILITY OF CHUFA AND OLIVE OILS

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### Summary

Oils of Chufa (*Cyperus esculentus*) and olive (*Olea europaea*) were compared in oxidative stability by peroxide values, electrical conductivity, GC-MS identification of oxidative degradation products and tocopherol analysis. Chufa oil is more sensitive to oxidative degradation than olive oil.

Descriptors: oxidative stability; chufa oil; olive oil.

### Introduction

Chufa is native to Africa and Southern Europe and has been cultivated from early times. It is a sedge-like plant with creeping rootstocks, which produce small sweet tubers, 1-2 cm in length, with an obtuse end of irregular form. The tuber has a taste typical of almond or hazelnut and for this reason the Chufa tuber is also known by names as Earthalmond and Earthnut (Heywood, 1978). The chufa tuber is used as a foodstuff. In 1932, Stampa described the industrial use of chufa and reported the use of roasted chufa tubers as coffee substitute, the feasibility of producing fermented chufa beverages and the nowadays most popular application 'Horchata de Chufa', which is a sweetened milky-like aqueous extract of chufa tubers.

Content of oil in chufa tubers is about 250 g/kg (Linssen et al., 1987), which could be of interest in food technology, because the yield of edible oil is twice that of soya (Frega et al., 1984).

In a previous study (Linssen et al., 1988), we have shown that the fatty acid composition and also the positional distribution of the fatty acids in the triglycerides of chufa and olive oils are similar. The aim of this study was to compare the oxidative stability of chufa oil and olive oil.

### Materials and methods

#### Sample and sample preparation

Chufas were kindly provided by Professor Dr B. Lafuente (Instituto de Agroquímica y Tecnología de Alimentos, Valencia, Spain). Chufa oil was extracted with a hydraulic press, IKA, Type IPH 2.5 and by extracting ground chufa with petroleum ether 40-60 °C. Before analysis, part of the chufa oil was filtered to remove small solid particles. Cold-pressed olive oil was bought in a local store.

### Peroxide value

The peroxide value was determined by the method of IUPAC (1979). A test portion solved in acetic acid/chloroform, was treated with a solution of potassium iodide. The liberated iodine was titrated with a standard solution of sodium thiosulphate.

### Conductivity measurements

Electrical conductivity was measured with the Rancimat instrument, which is based on the conductimetric determination of volatile products and features automatic plotting of the conductivity against time (Figure 1 shows a schematic view of a single test set-up of the Rancimat (Laubli & Bruttel, 1986)). The evaluation is performed graphically after the test. The temperature used in this test was 110 °C. The induction time ( $t_i$ ) obtained from the curves is a good indication of the oxidative quality of the oil.

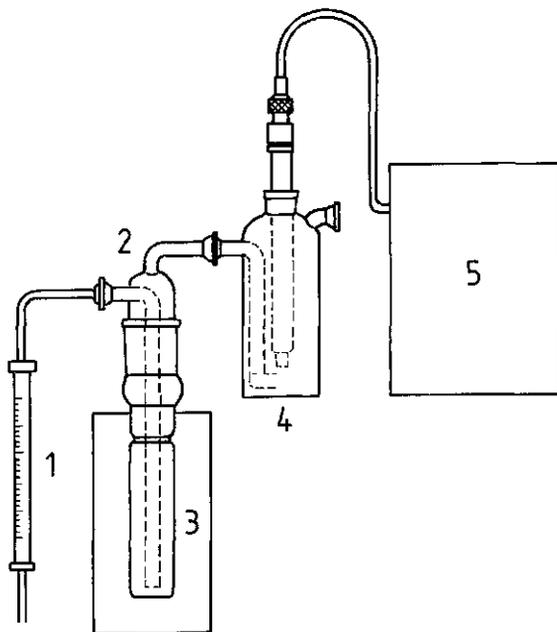


Figure 1. Schematic view of a single test set up in the Rancimat. 1. flowmeter, volume rate of air 12 l/h. 2. reaction vessel for oil samples. 3. aluminium heating block, temperature control to  $\pm 0.1$  °C. 4. absorption vessel, containing distilled water and double platinum foil electrode. 5. conductivity signal amplifier and recorder.

### Combined gas chromatography and mass spectrometry

2 g Oil was weighed into a vial, which was topped with a tube, containing Tenax, and placed in an oil bath at 130 °C. The volatiles of

degradation products were trapped on Tenax for 5 h by the method of Olafsdottir et al. (1985). The absorbed volatile products were identified on a combined gas chromatograph and mass spectrograph, using a heated desorption device to remove the volatile compounds from the Tenax and a cold trap to concentrate the volatiles before injection into the gas chromatograph, with a fused silica capillary column, Supelcowax 10, length 60 m, inner diam. 0.25 mm at an oven temperature of 30 °C for 4 min and a temperature rate of 2 °C/min to 250 °C (Badings et al., 1985).

#### Determination of tocopherols

The  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  tocopherols were determined by a IUPAC draft method (WG4/83) by HPLC, using a Lichrosorb SI 60 5  $\mu$ m column and a fluorescence detector with the excitation wavelength set at 290 nm and the emission wavelength at 370 nm (IUPAC 1985/1986).

#### Results and discussion

Table 1 shows the induction time ( $t_1$ ) of the various samples. The induction time of pressed chufa oil is lower than the induction time of extracted chufa oil. This effect is in agreement with the results of Gutfinger (1981), who reported that extracted oils contain more polyphenols, which have a certain antioxidant activity. A second explanation for this phenomenon could be the presence of prooxidants in the small particles left in the oils. In pressed oils, more small particles are left than in extracted oils, which is reflected in the measured induction time. This idea is supported by an increased induction time after filtration. The induction time of pressed olive oil is higher than the induction time of the chufa oils, which means that olive oil is more stable to oxidative degradation.

Table 1. Induction time ( $t_1$ ) of the oil samples at 110 °C.

	$t_1$ (h)
Chufa oil, pressed	4.30 $\pm$ 0.10
Chufa oil, pressed, filtered	9.35 $\pm$ 0.15
Chufa oil, extracted	10.40 $\pm$ 0.50
Chufa oil, extracted, filtered	13.35 $\pm$ 1.15
Olive oil, pressed	14.20 $\pm$ 0.40

Values are means of triplicates  $\pm$  SD

Figure 2 shows the conductivity curves of several oils, compared with their peroxide curves. The peroxide curves increased at an earlier stage than the conductivity curves. This effect can be explained by the peroxide values being based on measuring non-volatile hydroperoxides (primary fat oxidation), while the conductivity is based on the determination of polar volatile degradation products of secondary reactions of fat oxidation. Figure 2 confirms that chufa oil is more sensitive to lipid oxidation than olive oil.

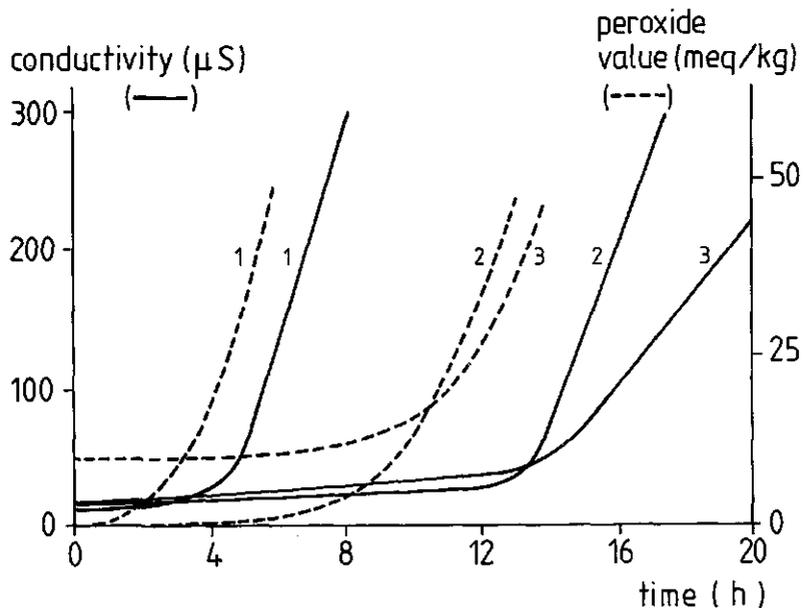


Figure 2. Conductivity curves and peroxide curves of different oils. 1. chufa oil, pressed. 2. chufa oil, extracted/filtered. 3. olive oils, pressed.

Table 2. Relative amounts (computer counts  $\times 10^{-2}$ ) of lipid-oxidation products of several oils, maintained for 5 h at 130 °C, analysed by GC-MS.

Component	Olive oil pressed	Chufa oil pressed	Chufa oil extracted
Butanone	2	8	6
Pentane		11	
2-Pentanone	1		
Pentanal	2	9	6
1-Pentanol or isomere	2	3	3
2-Hexanone or isomere	2	3	2
Hexanal	18	97	63
1-Hexanol	2	1	
4 OH-4-me-2 Pentanone		5	35
Heptanal	3	3	
Octane		4	
Octanal	5	5	4
6-me-5 Heptanone	7	3	
Nonane	2		5
Nonanal	18	13	65
1-Nonanol			2
Decane	2		3
Undecane	4		
Dodecene		2	

In the next test, the degradation products were concentrated on Tenax and identified by GC-MS. Table 2 gives a list of degradation products found in the GC-MS analysis and their relative amounts. In all samples, the amount of hexanal and nonanal is much higher than the amount of the other components. Frankel (1980) reported nonanal as the main degradation product as a consequence of oxidation of methyl oleate, but he did not reported the presence of hexanal. Chufa oil and olive oil contain oleic acid to 700-750 g/kg, which explains the large amount of nonanal. However other fatty acids, like linoleic acid, are present, which explain the presence of other degradation products.

Table 3 shows the amount of tocopherols in chufa and olive oils. There is no significant difference in the amount of  $\alpha$ -tocopherol between the two oils. Chufa oil contained a larger amount of  $\beta$ -tocopherol, but olive oil contained  $\gamma$ -tocopherol, while  $\gamma$ -tocopherol was not detected in chufa oil.  $\delta$ -Tocopherol was not found in either oils. The antioxidant activity of tocopherols increases from  $\alpha$  to  $\delta$  tocopherol. For this reason, it is not possible to explain the difference in oxidative stability of chufa oil and olive oil.

Table 3. Contents of tocopherols ( $\mu$ (Tc)) in chufa oil and olive oil (mg/kg)

	Tc $\alpha$	Tc $\beta$	Tc $\gamma$	Tc $\delta$
Olive oil*	396	5	26	-
Chufa oil**	415 $\pm$ 15	159 $\pm$ 5	-	-

\* means of duplicates

\*\* means of six determinations  $\pm$  S.D.

- not detected

Safwat Mohammed & Ahmed El-Fors (1959) and El-Difrawi et al. (1981) reported a strong antioxidant property of chufa oil, but they did not provide sufficient data to substantiate this.

None of the results in our experiments indicate a strong antioxidative property of chufa oil, when compared with olive oil. On the contrary, chufa oil was more sensitive to oxidative degradation than olive oil.

#### Acknowledgments

I am grateful to Ir N.W. Olieman and his coworkers at the Food Inspection Service Nijmegen (Netherlands) for estimating the tocopherols and to Dr M.A. Posthumus at the Organic Chemistry Department of the Agricultural University Wageningen (The Netherlands) for carrying out and interpreting the GC-MS.

#### References

- Badings, H.T.; de Jong, C.; Dooper, R.P.M.; 1985. Automatic system for rapid analysis of volatile compounds by purge-and-cold trapping/capillary gaschromatography. HRC & GC, 8(11):755-763.
- El-Difrawi; Entisar, A.; Moussa, M.M.; Hewela, Y.M.; Kandill, M.M.M.; 1981. Chufa tubers and safflower as potential sources of oils as cottonseed. Alex. Journal of Agricultural Research, 29:636-644.

- Frankel, E.N.; 1980. Lipid Oxidation (Review). *Progress in Lipid Research* 19:1-22.
- Frega, N.; Conte, L.S.; Lercker, G.; Carnacine A.; 1984. Composizione dei tubercoli di *Cyperus esculentus*. *Riv. Soc. Ital. Sci. aliment.* 13(3):211-214.
- Gutfinger, T.; 1981. Polyphenols in olive oils. *JAOCS*, 58:966-968.
- Heywood, V.M.; 1978. Flowering plants in the world. Oxford University Press, Oxford.
- IUPAC; 1979. Standard methods for the analysis of oils, fats and derivatives, 6th edition. Pergamon Press, Oxford.
- IUPAC WG 4/83; 1985/1986. Determination of tocopherols and tocotrienols by HPLC.
- Laübli, M.W.; Bruttel, P.A.; 1986. Determination of the oxidative stability of fats and oils: Comparison between the Active Oxygen Method (AOCS cd 12-57) and the Rancimat method. *JAOCS*, 63(6):792-795.
- Linssen, J.P.H.; van Olderen, J.D.; Pilnik, W.; 1987. *Cyperus esculentus*: Voedingsmiddel of onkruid. *Voedingsmiddelentechnologie* 20(5):24-26.
- Linssen, J.P.H.; Kielman, G.M.; Cozijnsen, J.L.; Pilnik, W.; 1988. Comparison of chufa and olive oils. *Food Chemistry* 28:279-285.
- Olafsdottir, G.; Steinke, J.A.; Lindsay, C.C.; 1985. Quantitative performance of a simple Tenax-GC adsorption method for use in the analysis of aroma volatiles. *Journal of Food Science*, 50:1431-1436.
- Pongracz, G.; 1986. Determination of edible fats by headspace gaschromatography. *Fette, Seifen, Anstrichmittel* 88(10):383-386.
- Safwat Mohammed, M.; Ahmed El-Fors, M.; 1959. A preliminary note. Chemical and technological studies on chufa tubers. *Alex. Journal of Agricultural Research* 7:119-127.
- Stampa, G.; 1932. Agricultural Industries. The edible *Cyperus* and its industrial uses. *International Agricultural Rev.* 7:T259-T270.

## APPLE PROTOPECTIN: PRELIMINARY STUDY OF ENZYMIC EXTRACTION<sup>1</sup>

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### Summary

The pectic material that could not be extracted from apple cell-walls with a chelating agent was solubilized with highly purified enzymes and their combinations. It was not possible to extract significant amounts of galacturonides without pectolytic enzymes. The most efficient combinations for extraction of pectins were not with arabinanases and galactanases, but with endo-glucanases.

Descriptors: apple; *Malus malus* L.; cell-wall; pectin; extraction; pectolytic enzymes; glucanase; arabinanase; galactanase

### Introduction

Pectic substances are a group of polysaccharides characterized by the presence of a high proportion of galacturonic acid. They have a backbone composed mainly of  $\alpha$ -1,4-linked D-galacturonic acid residues interspersed with a few 1,2-linked rhamnose residues. Some of the galacturonic acid residues are esterified by a methyl group. Neutral sugars side-chains, of which arabinose and galactose are the main components, are linked to that rhamnogalacturonic backbone. They are widely distributed in the tissues of dicotyledons, where they are one of the main components of the primary cell-wall. Pectic substances are found mainly in parenchymatous tissues, notably in apple parenchyma. They may cause problems in the pressing and concentrating of fruit juices, and influence cloud stability. More and more often enzyme mixtures containing pectolytic enzymes are used to extract fruit juices: these enzymes partially degrade the cell-walls and higher yields can be obtained with less energy expenditure. Pectins are

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<sup>1</sup>AIS alcohol insoluble solids  
AUA anhydrouronic acid  
CM carboxymethyl  
CyDTA cyclohexanediaminotetracetic acid (Complexon IV)  
CyDTA-IR CyDTA-insoluble residue  
DM degree of methylation  
FPLC fast protein liquid chromatography  
GLC gas liquid chromatography  
HM high methoxyl  
HPGPC high-pressure gel permeation chromatography  
mHDP metahydroxydiphenyl  
pnp paranitrophenyl  
UFR ultra filtration retentate.

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used also in food industry as an additive for their gelling properties in jams, jellies and some milk products.

The aim of our work was to study the pectic material that remains in the cell-wall after extraction with water, buffers and chelating agents and is often called protopectin. This protopectin can be extracted by harsher chemical treatments (hot acid, alkalis). However these methods bring about uncontrolled degradations that cause problems for structural studies.

Another possibility is to use pure and well characterized enzymes to split chosen glycosidic linkages and thus free some pectins. This gives access to a rhamnogalacturonic backbone that is not modified (by using non-pectolytic enzymes) or degraded in a defined way (by using pectolytic enzymes). This technique also provides information on the interrelations between pectin and other cell-wall polymers. Enzymes have often been used to study the structure of the cell-walls (Talmadge et al., 1973; Bauer et al., 1973; Keegstra et al., 1973; Knee et al., 1975; Voragen et al., 1980; Saulnier & Thibault, 1988). The main problem in this approach is the difficulty of obtaining pure enzymes in order to get unambiguous results.

Results of some enzymic extractions with highly purified enzymes prepared in the Laboratory of Food Chemistry of the Agricultural University (Wageningen, Netherlands) are presented here.

#### Material and methods

##### Apple cell-wall material

The substrate used was the partially depectinated cell-wall material (CyDTA-IR) obtained after extensive treatment of apple AIS with a chelating agent, CyDTA. This treatment removed about 45% of the pectic material present in the AIS.

##### Enzymes

The enzymes were purified in the Food Chemistry Department. The following enzymes were used:

- arabinofuranosidase B (Rombouts et al., 1988) (Exo-Ara)
- endoarabinanase (Rombouts et al., 1988) (Endo-Ara)
- endogalactanase (Voragen et al., to be published) (Endo-Gal)
- exoglucanase III (Beldman et al., 1985) (Exo-Glu)
- endoglucanase I (Beldman et al., 1985; active only on glucans) (Endo-Glu I)
- endoglucanase IV (Beldman et al., 1985; active also on xylans) (Endo-Glu IV)
- pectin lyase (van Houdenhoven, 1975) (PL)
- polygalacturonase (Versteeg, 1979) (PG)
- pectinesterase (Baron et al., 1979) (PE)
- rhamnogalacturonase (Geraeds et al., to be published) (RG).

The Endo-Ara and Endo-Gal, which still contained traces of PL activity, were successfully further purified with FPLC (results not shown).

Side-activities of the enzymes were detected after 24 h incubation with 0.1% of the substrates in sodium succinate/succinic acid 0.05 mol/l buffer pH 4.5 at 40 °C. Enzyme concentrations are given in table 1. The activities were estimated for paranitrophenyl sugars from absorbance at 400 nm in glycine buffer pH 9 and for polymers by determination of the reducing end-groups with the Nelson--Somogyi test, except for the HM

pectin, for which the changes in molecular-weight distribution were followed by HPGPC.

Table 1. Presence (+) or absence (0) of activities.

Substrates	EXO	ENDO	ENDO	EXO	ENDO	ENDO	PL	PG
	ARA	ARA	GAL	GLU	GLU I	GLU IV		
pnp $\alpha$ -D-glucopyranoside	0	0	0	0	0	0	0	0
" $\beta$ -D-glucopyranoside	+++	0	0	0	0	0	0	0
" $\alpha$ -D-galactopyranoside	0	0	0	0	0	0	0	0
" $\beta$ -D-galactopyranoside	0	0	0	0	0	0	0	0
" $\alpha$ -L-arabinofuranoside	+++	+/-	0	0	0	0	+/-	0
" $\alpha$ -D-xylopyranoside	0	0	0	0	0	0	0	0
" $\beta$ -D-xylopyranoside	+	0	0	0	0	0	0	0
" $\alpha$ -D-mannopyranoside	0	0	0	0	0	0	0	0
Microcrystalline cellulose	0	0	0	+/-	0	0	0	0
H <sub>3</sub> PO <sub>4</sub> -swollen cellulose	0	0	0	+	+	++	0	0
CM cellulose	0	0	0	0	++	++	0	0
Potato galactan	0	+/-	+++	0	0	0	0	0
UFR arabinan	+	++	0	0	0	0	0	0
Xylane ex oat spelts	0	0	0	0	0	+++	0	0
Polygalacturonic acid	0	0	0	0	0	+	0	+++
HM pectin (DM=93%)	0	0	0	0	0	0	+++	0
Enzyme concentration (U/l)	6	3	4	15	15	15	6	8

#### Incubation

Substrate suspension (CyDTA-IR, 10 g/l) was incubated in succinic acid/sodium succinate buffer 0.05 mol/l, pH 4.5 for 24 h at 40 °C with end-over-end mixing. Enzyme concentrations are indicated in Table 1. These concentrations were calculated as the amounts of enzymes that would theoretically degrade the amount of the corresponding polymer present in the CyDTA-IR in 24 h. The buffer was freshly prepared and sterilized by boiling for 10 min. Glass beads were added to each tube to help mixing. After incubation the suspension was centrifuged for 10 min at 3000 gn. The enzymes were inactivated by boiling the supernatants for 5 min and the pellets were washed with distilled water and freeze-dried.

#### Analytical methods

Yields: the concentrations of neutral sugars and uronic acid in the supernatants were estimated by automated orcinol and mHDP assays as described by Thibault et al. (1988).

Molecular weight distribution: the molecular weight distribution was obtained by HPGPC on combined Biogel TSK 20XL, 30XL and 40XL columns, eluted with acetic acid/acetate buffer (0.4 mol/l, pH 3) at 30 °C with a flow of 0.8 ml/min. The eluted material was detected with a Shodex SE61 reflective index detector.

Sugar composition: the neutral sugar composition of the extracts was estimated by GLC of their alditol acetates prepared according to Englyst

& Cummings (1984). The extracts and dialysed extracts were hydrolysed for 3 h in 1mol/l sulfuric acid at 100 °C. The column used (OV 275) gave separate peaks for rhamnose and fucose. The AUA content was measured in the hydrolysates.

## Results

### Yields

The concentrations of uronides and neutral sugars in the extracts are given in Figure 1.

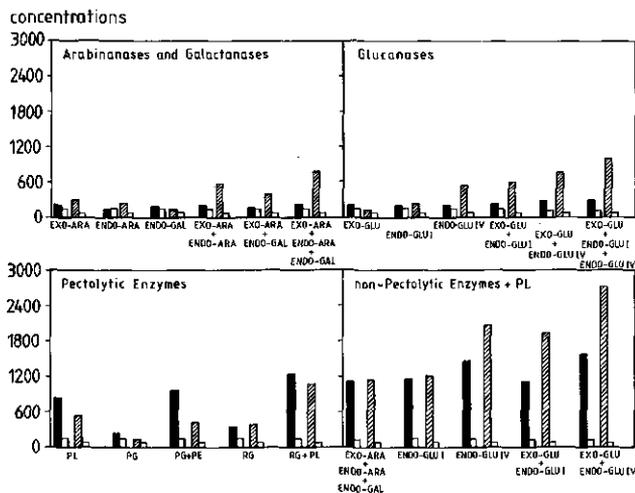


Fig. 1. Concentrations (mg/l) in the extracts. AUA: sample; blank. Neutral sugars: sample; blank.

Though the blanks had low concentrations, still part of the CyDTA-IR was solubilized merely by the buffer. Incubation with the buffer released 7 to 9% of the uronides and 1 to 2% of the neutral sugars. The hemicellulases (arabinanases and galactanase) and cellulolytic enzymes failed to extract significant amounts of uronic acid from the cell-wall material, but they extracted low concentrations of neutral sugars. The synergisms between exo and endo enzymes were clearly visible for the arabinanases and the glucanases. The PG had a limited effect, due to the high degree of methylation of the pectin in the CyDTA-IR. The presence of an efficient pectolytic system (PL, PG+PE) was necessary for extraction of pectins. The rhamnogalacturonase released a fraction containing polymeric uronides accompanied by a high proportion of neutral sugars. When non-pectolytic enzymes were combined with the PL, the highest synergistic effects were obtained with the endoglucanases, notably Endo-Glu IV. The arabinanases and galactanase led to a slight increase in the quantities of extracted material. The most efficient of these combinations was Exo-Glu + Endo-Glu IV + PL. There was no liquefaction,

contrary to what was reported by Voragen et al. (1980), but smaller amounts of enzymes were used.

#### Molecular weight distribution

The HPGPC patterns of some of the extracts on TSK 20XL + 30XL + 40XL are shown in Figure 2. The excluded volume of the combined columns corresponded to a molar mass of  $10^6$  Da for dextrans and  $10^5$  Da for pectins.

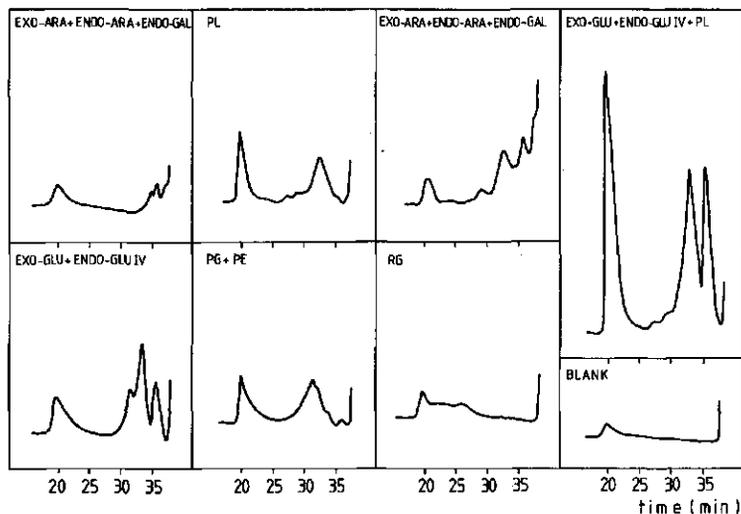


Fig. 2. HPGPC patterns of the extracts on combined Biogel TSK 40XL + 30XL + 20XL columns.

The hemicellulases (arabinanases and galactanase) and cellulases released mostly small oligomers and monomers. The PL (and PG+PE) freed oligomers but also some high-molecular-weight polymers that eluted at the excluded volume of the combined columns. The RG extracted a whole range of high-molecular-weight polymers. When the hemicellulases were combined with PL, the area of the excluded peak diminished compared to PL alone, but the area of this excluded peak was increased by the addition of endoglucanase. The combination Exo-Glu + Endo-Glu IV + PL gave the largest excluded peak, together with high small oligomers peaks.

#### Sugar composition

The sugar compositions of the supernatants extracted by the PL and the combinations Exo-Ara + Endo-Ara + Endo-Gal + PL and Exo-Glu + Endo-Glu IV + PL are shown in Figure 3. Dialysis was used as a quick way to remove the oligomeric reaction products of the enzymes.

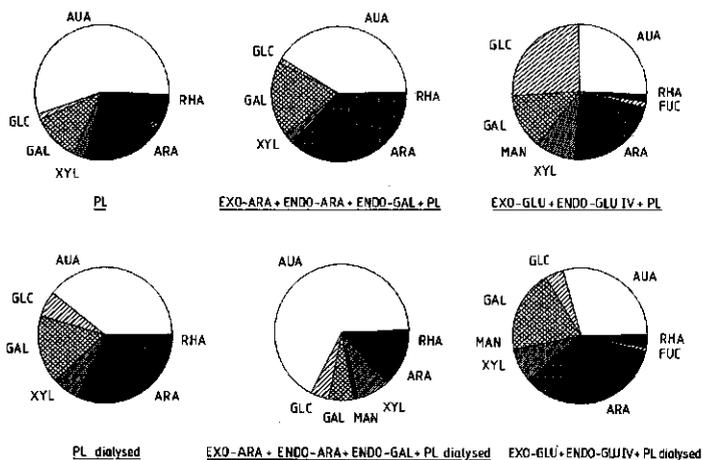


Fig. 3. Substance fractions of some of the extracts, before and after dialysis.

The PL extracted mostly AUA, arabinose, galactose and rhamnose. A large amount of the AUA was present as oligomers, eliminated during dialysis. The non-diffusable material contained a higher proportion of neutral sugars. Addition of non-pectolytic enzymes led to extraction of more neutral sugars. The endoglucanase extracted glucose but also xylose and fucose. A significant proportion of these neutral sugars, however, were oligomers that could be dialysed. This was specially marked for Exo-Ara + Endo-Ara + Endo-Gal + PL: the non-diffusable material was poorer in arabinose and galactose than that extracted with PL alone. The arabinanase and galactanase had caused some additional degradation. With the glucanases, the non-diffusable material -poorer in glucose- contained high proportions of arabinose and galactose. However in contrast to the situation with the arabinanases and galactanase, the proportion of glucose in the non-diffusable material was not noticeably lower than in the PL-extracted material.

#### Discussion and conclusion

Though the soluble pectins had already been removed from the CyDTA-IR substrate, some uronides and neutral sugars were still extracted by the buffer alone. The amounts released, however, were lower than reported by Voragen et al. (1980).

It was not possible to extract significant amounts of pectins without

the presence of an efficient pectolytic system (PL or PG+PE), as was already noted by Talmadge et al. (1973), Knee et al. (1975), Voragen et al. (1980) and Thibault et al. (1988). The arabinanases and galactanase had little effect on pectin extraction, as was noted by Thibault et al. (1988). The most efficient combinations for extraction of pectins were obtained with endoglucanases. These results correspond to the data reported by Keegstra et al. (1973) and Bauer et al. (1973) for enzymic extractions of cell-walls from suspension cultured sycamore cells. They also agree with what is known in the fruit juice industry: a combination of pectinases and cellulases is necessary for any major degradation of the cell-walls; the cellulases alone have limited effects and the hemicellulases play practically no role in liquefaction.

The material extracted with the PL, and even more so by with the glucanases in combination with the PL, contained a high proportion of non-diffusible arabinose and galactose. This is in agreement with the results of Saulnier et al. (1988) that PL extracts pectins with a higher proportion of side-chains than chemical methods.

The rhamnogalacturonase extracted high molecular weight fragments with a high proportion of neutral sugars. It seems that some degradation of the rhamnogalacturonic backbone is necessary to obtain fragments small enough to be extractable. The rhamnogalacturonic backbone and the cellulosic network thus appear to have multiple connections in the cell-wall. Xyloglucans seem to have an important role in these connections (extractions with PL and endoglucanases), whereas the arabino- $\beta$ (1,4)galactans (though they are extracted together with the pectin) play a minor role. They seem to be present as side-chains with loose ends on the rhamnogalacturonic backbone.

#### References

- Baron A., Rombouts F.M., Drilleau J.F. and Pilnik W., 1980. Purification et propriétés de la pectine-esterase produite par *Aspergillus niger*. *Lebensmittelen-Wissenschaft und -Technology* 13:330-333.
- Bauer W.D., Talmadge K.W., Keegstra K. and Albersheim P., 1973. The structure of plant cell walls II: The hemicellulases of the walls of suspension-cultured sycamore cells. *Plant Physiology* 51:174-187.
- Beldman G., Searle-v. Leeuwen M.J., Rombouts F.M. and Voragen A.G.J., 1985. The cellulases of *Trichoderma viride*: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and  $\beta$ -glucosidases. *European Journal of Biochemistry* 146:301-308.
- Englyst H.N. and Cummings J.H., 1984. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* 109:937-942
- v. Houdenhoven F.E.A., 1975. Studies on pectin-lyase. Ph. D. thesis, Agricultural University Wageningen, The Netherlands.
- Keegstra K., Talmadge K.W., Bauer W.D. and Albersheim P., 1973. The structure of plant cell walls III: A model of the walls of suspension cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiology* 51:188-196.
- Knee M., Fielding A.H., Archer S.A. and Laborda F., 1975. Enzymic analysis of cell wall structure in apple fruit cortical tissues. *Phytochemistry* 14: 2213-2222.
- Rombouts F.M., Voragen A.G.J., Searle-v. Leeuwen M.F., Geraeds C.C.J.M., Shols H.A. and Pilnik W., 1988. The arabinanases of *Aspergillus niger*- Purification and characterization of two  $\alpha$ -L-arabinofuranosidases and

- an endo-1,5- $\alpha$ -L-arabinanase. *Carbohydrate Polymers* 9:25-47.
- Saulnier L. and Brillouet J.M., 1988. Structural studies on pectic substances from the pulp of grape berries. *Carbohydrate Research* 182: 63-78.
- Talmadge K.W., Keegstra K., Bauer W.D. and Albersheim P., 1973. The structure of plant cell walls I: The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. *Plant Physiology* 51: 158-173.
- Thibault J.F., Dreu de R., Geraeds C.C.J.M. and Rombouts F.M., 1988. Studies on extraction of pectins from citrus peels, apple marks and sugar-beet pulps with arabinanase and galactanase. *Carbohydrate Polymers* 9:119-131.
- Versteeg C., 1979. Pectinesterases from the orange fruit: their purification, general characteristics and juice cloud destabilizing properties. Ph.D. Thesis, Agricultural University, Wageningen, The Netherlands, p.25.
- Voragen A.G.J., Heutink R. and Pilnik W., 1980. Solubilization of apple cell walls with polysaccharide degrading enzymes. *Journal of Applied Biochemistry* 2:452-468.

## SENSORY EVALUATION OF ENZYMIC SOYA PROTEIN HYDROLYSATES PRODUCED WITH ANTIOXIDANTS

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### Summary

The solubility of soya protein in foodstuffs is improved by enzymic hydrolysis. However hydrolysis generates unpleasant bitter and beany flavour compounds, which minimize the utilization of the hydrolysate. Bitterness originates from hydrophobic peptides formed and beany flavour is fortified by hydroperoxide degradation of fat and release of aldehydes and ketones. Several combinations of antioxidants were studied for their ability to reduce formation of beany flavours in enzymic protein hydrolysates. Defatted soya flakes were acid-washed ( $\text{pH} \approx 4$ ) and then hydrolysed by different proteolytic enzymes, of which molsin gave a low bitterness. That enzyme was used throughout the work on beany flavour with several combinations of antioxidants. Differences in the peak patterns of gas chromatograms were analysed by mass spectrometry, which mainly shows a decrease of hexanal upon addition of an antioxidant mixture. A favourable effect on flavour was found for the addition of a mixture of antioxidants during hydrolysis (butylated hydroxyanisole 0.1 g/kg, butylated hydroxytoluene 0.1 g/kg and sodium dihydrogen pyro-phosphate 4 g/kg). Attributes of the hydrolysate like musty and nutty are changed into sour and pungent, which constitute the first principal component in the Procrustes analysis. The second component is the fruity-beany axis of the two-dimensional perceptual space of the analysis. Descriptors: sensory evaluation, enzymic hydrolysis, antioxidant, soya protein.

### Introduction

Soya proteins are mainly globulins, which are water insoluble at their isoelectric points ( $4 < \text{pH} < 6$ ) and so of limited adaptability to foodstuffs. To improve their application, the proteins are hydrolysed by proteolytic enzymes, with which the nutritional value is unchanged because of modest pH and temperature. Use of enzymes with pH optima  $< 3.5$  has the advantage of microbial stability and slow Maillard reactions (Roozen & Pilnik, 1974). Hydrolysis of soya proteins enhances solubility, but releases unpleasant bitter and beany flavours. The bitter taste is caused by hydrophobic peptides and its intensity increases with higher degrees of hydrolysis (Adler-Nissen, 1984). The amino acid composition of the substrate proteins and the specificity of the endopeptidases of an enzyme preparation are both major factors in formation of bitter peptides (Ney, 1971; Luning, 1987). Hydrolysis makes the beany flavour more apparent because of release of volatiles (Huang, 1979) or promotion of the secondary reaction of fat oxidation. The volatiles can easily form hydrophobic interactions with soya proteins, especially aldehydes and ketones (Kinsella & Damodaran, 1981), in which condition they have no influence on the flavour.

Recently student projects of our department have tackled the problem of the unpleasant beany flavour of soya protein hydrolysates in three ways: prevention (Ansenwoude, 1985; Ridder, 1987; Kranen, 1987), removal and masking (Helsper, 1985; Klein Essink, 1985; Miltenburg, 1985). Most successful has been the work on prevention by using a mixture of antioxidants (Bovee, 1988; Herz, 1988).

In sensory evaluation, free-choice profiling differs from conventional profiling in two ways: there is no need for elaborate training of assessors to develop a common set of product attributes, and assessors do not have to agree upon the meaning of the attributes (Williams & Langron, 1984). Each assessor uses his own vocabulary developed by the repertory grid method (Kelly, 1955) and then the data of the panel are analysed for interrelationships between the samples by generalized Procrustes analysis (Gower, 1975). The dimensions extracted can be made explicit because of the use of expressions to assess the samples.

This paper will establish the effect of antioxidants on the beany flavour of enzymic hydrolysates of soya protein concentrate using free-choice profiling.

#### Materials and methods

Oligosaccharides were removed from defatted soya flakes (Cargill, Amsterdam) by acid-washing five times (mass ratio of sample to acidified water, 1:8) at pH 4.5. The protein concentrate was hydrolysed with acid-proteolytic enzymes described in Table 1. Their activity was determined according to Merck (1970) using a solution of soya protein isolate 10 g/l (Ralston Purina PP660). Protein content of the hydrolysates was determined from absorbance at wavelength 280 nm and by biuret assay (Legget-Bailey, 1969), and amino acid content by ninhydrin colorimetric analysis (Rosen, 1957). Aldehydes by the method of Sawicki et al. (1961) with formaldehyde as a reference. Volatiles in the hydrolysates were concentrated on Tenax GC with a dynamic head-space technique (Herz, 1988) and measured by GC-MS (Dr M.A. Posthumus, Department of Organic Chemistry).

Assessors for the comparison tests were 14 semitrained students selected for their sensitivity to bitterness and beany flavour. Assessors for free-choice profiling were recruited from the workers in the Biotechnion Building of the University, of whom 75 were interviewed for availability, health, eating habits and knowledge of food products. Of them, 36 were chosen for criteria like creativity, verbal expression and motivation for participation. Further selection for smell and taste sensitivity, smell recognition and scoring on two-point line scales resulted in a trained panel of 9 men and 9 women with ages of 20-45 years. These panel members were asked to use Kelly's repertory grid method (Kelly, 1955) for generation of individual lists of objective attributes, which described the sensory properties of the soya protein hydrolysates. At each profiling session, assessors in individual booths were presented with six randomly coded samples of hydrolysates in plastic cups. Assessments were made for the intensity of attributes generated on a two-point continuous linear scale. The data were subjected to generalized Procrustes analysis. The computer program was a slightly modified version of the Rothamsted Experimental Station's version and was kindly made available by Ir. H.C.M. van Trijp (Department of Marketing and Marketing Research).

## Results

Soya protein concentrate obtained after acid-washing was enzymically hydrolysed as shown in Table 1. The effect of antioxidants on the formation of aldehydes during hydrolysis is demonstrated in Table 2. The hydrolysates treated with SDP, BHA/BHT/SDP and the control were sensorially evaluated by a paired-comparison test.

Table 1. Soya protein concentrate (suspension 200 g/l) hydrolysed with acid-proteolytic enzymes and characterization of hydrolysates (Luning, 1987).

Enzyme preparation		Hydrolysate		
sample (supplier)	activity units <sup>a</sup>	yield (g/l) <sup>b</sup>	ratio exo/ endo <sup>c</sup>	bitterness Friedman (Ri) <sup>d</sup>
Acid fungal protease (Takamine)	1.1	9.0	2.0	33
Fungal protease (NOVO Ferment)	0.3	7.0	3.2	24
Molsin (Seihin Seiyaku)	1.3	9.0	2.3	27
FAAN type A (NOVO Ferment)	0.9	7.8	4.4	25
Saure protease (NOVO Ferment)	0.9	5.5	2.7	25
Pepsin (Biochemica Merck)	0.8	6.9	1.0	nd

<sup>a</sup> Merck, 1970

<sup>b</sup>  $A * 1.116 \text{ g/l}$ , where A is absorbance at wavelength 280 nm

<sup>c</sup> ninhydrin (mg/l) / biuret (g/l)

<sup>d</sup> triple-comparison test: no significant values (Ridder, 1987);  
nd = not determined

Table 2. Addition of antioxidants during hydrolysis for 6 h of soya protein concentrate with molsin: effect on aldehyde concentration of the hydrolysate (Kranen, 1987).

Antioxidant <sup>a</sup>	Aldehyde concentration (mg/l)	
		difference <sup>b</sup>
Control (self-made flakes)	1.2	-
CA (4000)	2.6	1.4
AA/CA (1000/4000)	9.4	8.2
BHA/BHT/CA (100/100/4000)	3.4	2.2
Control (commercial flakes)	5.1	-
SDP (4000)	4.8	-0.3
AA/SDP (1000/4000)	7.7	2.3
BHA/BHT/SDP (100/100/4000)	4.6	-0.5

<sup>a</sup> Abbreviations used: CA citric acid; AA ascorbic acid; BHA butylated hydroxyanisole; BHT butylated hydroxytoluene; SDP sodium dihydrogen pyrophosphate; numbers in parenthesis are contents in mg/kg.

<sup>b</sup> concentration in sample - concentration in control.

The data were analysed according to Thurstone (Swanborn, 1982) and plotted in Figure 1. The effect on volatile composition is shown in Figure 2. The results of free-choice profiling are shown in Figure 3, in which two principal components explain 77% of the variance in the consensus configuration. From examination of Table 3 it would appear that the first principal component is the contrast between "sour" (left) and "musty" (right), and the second component between "beany" (top) and "fruity" (bottom).



Figure 1. Sensory continuum of beany flavour in Molsin hydrolysates of soya protein concentrate: effect of antioxidants added (codes explained in Table 2). Least significant difference is 0.67 (Ridder, 1987).

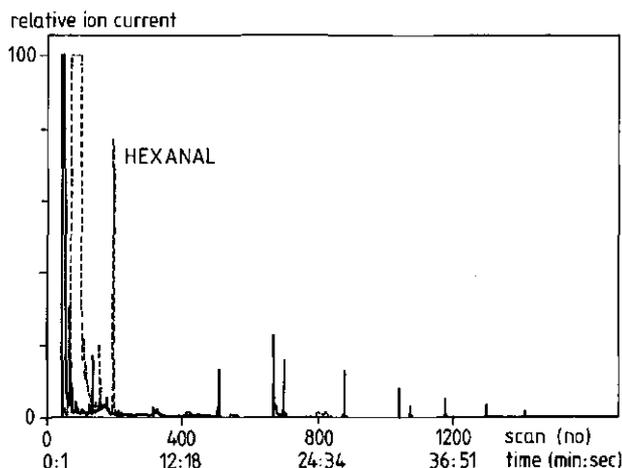


Figure 2. Combined direct-sampling capillary gas chromatography and mass spectrometry of volatiles from enzymic soya protein hydrolysates (Herz, 1988). - - - - control, ——— antioxidants added (BHA/BHT/SDP, Table 2).

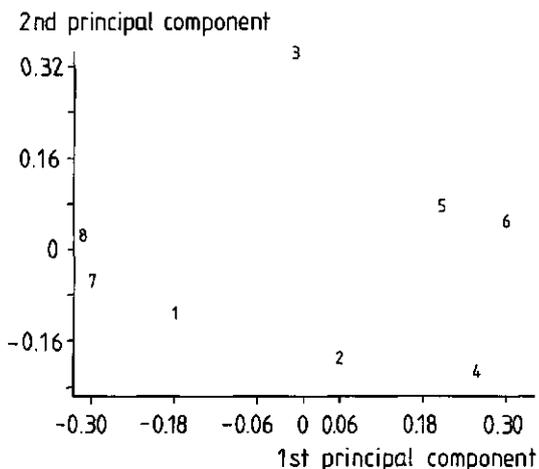


Figure 3. Consensus configuration of the assessments of eight soya protein hydrolysates by free-choice profiling after generalized Procrustes analysis (Bovee, 1988). Explanation of codes (also Table 2):

- 1-BHA/BHT/SDP + citric acid 0.25 g/kg 5-control  
 2-BHA/BHT/SDP 6-control + dialysate (1:3 v/v)  
 3-control + citric acid 0.25 g/kg 7-as 1 with nose clip  
 4-BHA/BHT/SDP + dialysate(1:3 v/v) 8-as 3 with nose clip

Table 3. Definition of first two principal components in terms of assessors' original descriptions (vector loadings for individual assessors > 0.7; Bovee, 1988).

First component		Second component	
sour (left)	musty (right)	fruity (bottom)	beany (top)
sour	musty	fruity	beany
pungent	nutty	apple	nutty
refreshing-sour	beany	apple juice	musty
sweet-sour	sickly	sour	sickly
tart	cardboard	sweet	mealy
bitter	woody	refreshing	oily
	mealy	almond	tart
	rancid		dry
	apple		bitter
			rubber
			acid

## Discussion

Acid-proteolytic enzyme preparations from micro-organisms have much higher exopeptidase activities than pepsin (Table 1), and so are more suitable for making low bitter protein hydrolysates (Clegg & McMillan, 1974). Contrary to previous work with soya protein isolate (Roezen & Pilnik, 1974), hydrolysis of soya protein concentrate by these bacterial enzymes does not show a significant difference in bitterness between the hydrolysates (Table 1). Molsin has been chosen for further work on beany flavour because of its combination of high activity and yield, and low bitterness.

The addition of an antioxidant to a concentrated suspension of soya protein can have the effect of a prooxidant, as shown in Table 2 for citric and ascorbic acid. The latter promote the formation of aldehydes, while SDP and BHA/BHT/SDP are effective in diminishing the concentration of aldehydes (Table 2). A change in aldehyde composition can result in quantitative (less beany) or qualitative differences in flavour. Figure 1 demonstrates a significant decrease in beany flavour, when BHT/BHA/SDP is added to the suspension. The GC-MS analysis of volatiles (Figure 2) shows that the addition of this antioxidant mixture lowers the total amount of volatiles with a striking difference in hexanal content. This aldehyde is formed mainly by oxidation of linoleate and has a green-fruity smell (Grosch, 1987). Probably hexanal promotes the beany flavour impression of soya protein hydrolysates.

Addition of BHA/BHT/SDP changes also the flavour of the hydrolysates (Figure 3). Attributes of the control sample are, for instance, musty, beany and rancid, while the treated sample is fruity, apple or sour (Table 3). There is a shift to the left in Figure 3, when citric acid is added to both hydrolysates, and in opposite direction for beany-flavour additions of dialysate. This confirms the nature of the two principal components, as described in Table 3. The volatiles responsible for beany flavour are no longer detectable when nose clips are used. The latter does not hinder the detection of sour taste, so the codes of Samples 7 and 8 are near to each other and utmost left in Figure 3. The assessors know how to get along with free-choice profiling. They are free to use their own words, but they might have difficulties in finding the right expressions for their observations.

In conclusion, free-choice profiling is suitable for analysing the effect of additions of antioxidants during enzymic hydrolysis of soya protein concentrate. BHA/BHT/SDP has a favourable effect on the flavour of soya protein hydrolysates.

## References

- Adler-Nissen, J., 1984. Control of the proteolytic reaction and the level of bitterness in protein hydrolysis processes. *Journal of Chemical Technology and Biotechnology* 34B: 215-222.
- Ansenwoude, G.H.M.G. van, 1985. Verbetering van de smaak van een proteolytisch hydrolysaat van ontvet sojaschroot. *Doktoraalverslag, LU levensmiddelenchemie, Wageningen.*
- Bovee, E.H.G., 1988. Sensorische analyse naar de invloed van anti-oxydantia op de bonensmaak van soja-eiwit hydrolysaat. *Doktoraalverslag, LU levensmiddelenchemie, Wageningen.*
- Clegg, K.M. & A.D. McMillan, 1974. Dietary enzymic hydrolysates of protein with reduced bitterness. *Journal of Food Technology* 9: 21-29.
- Gower, J.C., 1975. Generalized Procrustes analysis. *Psychometrika* 40: 33-51.

- Grosch, W., 1987. Reactions of hydroperoxides-Products of low molecular weight. In: H.W.-S. Chan (Ed): Autoxidation of unsaturated lipids. Academic Press Inc., London. p. 95-139.
- Helsper, J.W.W., 1985. Smaakverbetering van een enzymatisch verkregen soja-eiwithydrolysaat. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Herz, I.F., 1988. Instrumentele analyse naar de invloed van antioxidantia op de bonensmaak van soja-eiwithydrolysaat. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Huang, A., 1979. Identification of objectionable flavor components in soybean protein products. Dissertation Rutgers University, New Jersey.
- Kelly, G.A., 1955. The psychology of personal constructs, volume 1. W.W. Norton & Company Inc., New York. p. 219-266.
- Kinsella, J.E. & S. Damodaran, 1981. Interaction of carbonyls with soy protein: thermodynamic effects. Journal of Agricultural and Food Chemistry 29: 1249-1253.
- Klein Essink, G., 1985. Een methode ter beperking van de bonensmaak in een soja-eiwithydrolysaat. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Kranen, B., 1987. Vermindering van de bonensmaak van een enzymatisch verkregen soja-eiwithydrolysaat: toevoeging van antioxidantia en het gebruik van azetropische extractie. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Leggett Bailey, J., 1969. Techniques in protein chemistry. Elsevier Publishing Company, Amsterdam.
- Luning, P.A., 1987. Bitterheid van sojaschroot hydrolysaat verkregen door inwerking van zure proteasen. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Merck, 1970. Biochemica Merck: Enzyme. E.Merck, Darmstadt.
- Miltenburg, S.M.S., 1985. Beperking van bonensmaak in hydrolysat van soja-isolaat. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Ney, K.H., 1971. Voraussage der Bitterkeit von Peptiden aus deren Aminosäurezusammensetzung. Zeitschrift für Lebensmittel-Untersuchung und -Forschung 147: 64-68.
- Ridder, M.A.M., 1987. Sensorisch onderzoek naar de bittere smaak en de bonensmaak van soja-eiwithydrolysat. Doktoraalverslag, LU levensmiddelenchemie, Wageningen.
- Roozen, J.P. & W. Pilnik, 1974. Improvement of the acid solubility of soy proteins by enzymic hydrolysis and its influence on their taste properties. Deutsche Lebensmittel-Rundschau 70: 280-283.
- Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. Archives Biochemistry and Biophysics 67: 10-15.
- Sawicki, E, T.R. Hauser, T.W. Stanley & W. Elbert, 1961. The 3-methyl-2-benzothiazolone hydrazone test. Analytical Chemistry 33: 93-96.
- Swanborn, G., 1982. Schaaltechnieken: theorie en praktijk van acht eenvoudige procedures. Uitgeverij Boom, Meppel.
- Williams, A.A. & S.P. Langron, 1984. The use of free-choice profiling for the evaluation of commercial ports. Journal of the Science of Food and Agriculture 35: 558-568.

# POLYSACCHARIDE CHEMISTRY: THREE EXAMPLES OF BASIC RESEARCH FOR TECHNOLOGICAL PROGRESS IN THE FOOD AREA

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## Summary

The thesis that polysaccharide chemistry can result in technological progress in food production is illustrated by three recent examples from the author's work. The first example describes enzymic degradation studies of pectin; the second treats the use of periodate oxidation in the characterization of carrageenans; and the third deals with the effect of starch modification on its freeze/thaw stability. Descriptors: pectins; carrageenan, modified starch, chemical structure, gelling properties, freeze/thaw stability.

## Introduction

Polysaccharide chemistry has resulted in substantial food-technological progress, as is shown by the following examples: production of starch sugars like dextrose, maltose and high-fructose-maize syrups, production of sorbitol, production of modified starches and celluloses (cross-linking, esterification and etherification), production of low-methoxyl pectins. These arbitrarily chosen examples are only a few from a wide range of technologies based on polysaccharide chemistry. Since part of the work of Professor Pilnik has been dedicated to polysaccharide chemistry, let me discuss here some activities going on in this field.

Does polysaccharide chemistry still lead to technological progress in the food sector? Certainly, since we do not fully understand the behaviour of polysaccharides in food systems. But, like all science, food chemistry must now more than ever face the question of technological relevance. This paper does not answer this question in a comprehensive way but takes three examples from my recent work. Though not the most striking examples, they illustrate the thesis that polysaccharide chemistry is likely to result in technological progress.

## Enzymic degradation of pectins

Pectin is one of the few hydrocolloids used as a gelling agent in the food industry, specially in acidic foods.

The laboratory of Professor Pilnik has paid much attention to the structure of pectins. It has shown that pectin is constructed of huge "repeating units" in the cell wall (de Vries, 1988). A polygalacturonan chain and a rhamnogalacturonan chain (with arabinogalactan and xylose side-chains) together form such a repeating unit ("smooth" and "hairy" regions).

However in commercial pectins, sugars linked to the galacturonan chain

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are almost absent because of the rather severe conditions of extraction. Neutral sugar side-chains are not likely to play a role in pectin gelation.

Also, a possible insertion of rhamnose units in the main chain (causing a kink in the molecules) probably does not function as a "junction-zone-terminating factor", because the rhamnose units are concentrated on certain small regions in the molecule and these regions are degraded during commercial pectin extraction.

The main factor governing the functional properties is the DE, degree of esterification (of the galacturonic acid residues with methanol). For this reason, commercial pectins are divided into low-methoxyl and high-methoxyl, slow-set and rapid-set according to their DE. However not only the overall DE determines the behaviour of a pectin. The distribution of the ester groups is equally relevant. So the problem of the distribution of methoxyl groups has received considerable attention in recent years. So far, no methods existed to describe this distribution. Pectolytic enzymes were shown to be viable tools in the description of the distribution of the ester groups (de Vries et al., 1986).

Table 1. Degradation of pectin (DE = 80%) by pectin lyase (methods as in de Vries et al., 1986).

	Computer simulation	Transesterified pectin	Natural pectin
Proportion of bonds broken (%)	12	18	25
Mass fraction of dimer* (g/kg)	60	40	30
Mass fraction of trimer* (g/kg)	50	190	210
Mass fraction of tetramer* (g/kg)	0	180	160
Mass fraction of pentamer* (g/kg)	30	130	220
Mass fraction of hexamer* (g/kg)	60	110	160
Mass fraction of heptamer* (g/kg)	50	40	130
Total dimer-heptamer (g/kg)	260	600	900

\* present in the PL-digest of the pectin

Table 1 shows the results of degradation of pectin by pectin lyase, an enzyme only able to degrade high-methoxyl pectin. The degraded pectins were analysed by HPLC (de Vries et al., 1986). Pectins with a natural distribution were compared to "transesterified" pectins (which were first esterified up to 90-95% and subsequently de-esterified with cold alkali, in order to induce a random distribution of ester groups). The experimental results are compared with the results obtained by computer simulation. The programme generated a chain with a random distribution of ester groups and assumed that the enzyme attacked the chain at random but could only to split the central bond in a sequence of 4 esterified residues:



Simulation is hampered by our lack of knowledge the exact requirements for the enzyme to degrade a certain bond. Transesterification probably does not result in a completely random distribution. Another draw back is that transesterification also causes some chain degradation.

But even so the natural distribution of methanol ester groups in pectin is likely to be a regular one (Table 1). So pectin molecules do not

possess configurations specially fit as "junction zones". The enzyme polygalacturonase (PG) can be used to estimate the low-methoxyl part in the molecules (de Vries, 1988).

Degradation with PG shows that most pectins have "blocks" of non-esterified residues, most likely the result of pectin esterase action.

In summary, degradation with PG and PL yields structural information that facilitates selection of pectins for functional properties like Ca sensitivity and stabilizing power.

As a side-effect of these research efforts, the production of enzyme-treated pectins is being investigated.

#### Periodate oxidation of carrageenan

A second example of technologically relevant polysaccharide is also taken from the area of gelling agents. Carrageenans are isolated from red seaweeds. They can be divided into (roughly) kappa, iota and lambda carrageenan. For a better understanding, the reader is referred to relevant textbooks.

Commercial carrageenans are almost always a mixture of two or more types. Major sources are Chondrus, Euchema and Iridaea species. The hydrocolloids extracted from Euchema cottonii, Chondrus crispus and Iridaea species are all kappa type carrageenans. Nevertheless, the functional properties of these products are widely different, and this raises the question whether these differences can be explained by the structural characteristics of each type.

Relevant parameters are molecular weight distribution, counter-ion composition and ratios of kappa, iota and lambda. These features do not differ strikingly between Chondrus crispus and Iridaea carrageenans; nevertheless their behaviour, especially in milk, differs. A possible explanation is that Chondrus is a mixture of kappa and lambda carrageenans, whereas Iridaea is a hybrid (kappa and iota or lambda regions in one molecule). This hypothesis was tested by periodate oxidation followed by reduction and mild acid hydrolysis (20 °C, pH = 1.5, 18 h; these conditions are rather critical, because carrageenan itself is acid-labile).

This (famous) sequence of reactions is called the Smith degradation. In carrageenans, only the mu structures can be degraded by this procedure. They are only present in carrageenans that have not been treated with alkali (as is usual in commercial carrageenan). During this treatment, mu structures are converted into kappa structures.

Therefore carrageenans extracted in neutral environment were subjected to the Smith degradation procedure. The results were analysed by high-pressure gel permeation chromatography (Bondapack E 1000 column run at 60 °C in LiCl 0.1 mol/l).

If the samples were a mixture of kappa (or mu) and the other types, only some of the molecules should be degraded, whereas in a hybrid, all the molecules should be degraded (as can be observed in the HPLC plots as a decreased peak area because the small fragments disappear in the excluded volume).

Table 2 shows the results for some carrageenan types.

As expected, Euchema spinosum (iota type) and alkaline-extracted carrageenan cannot be degraded by the Smith procedure.

In the case of E. cottonii, almost all the molecules can be degraded: neutrally extracted E. cottonii carrageenan is mainly mu type, the 20% of undegradable material is probably of the iota type as can be deduced from the IR spectrum.

Table 2. Smith degradation of carrageenans extracted in a neutral environment.

Carrageenan type	$M_w$		Recovery (%)*
	after Smith degradation	before Smith degradation	
<u>E.spinosum</u>	310 000	450 000	96
<u>E.cottonii</u>	90 000	280 000	20
<u>Iridaea</u> sp.	215 000	500 000	35
<u>Iridaea</u> alkaline extract	240 000	500 000	95

\* Expressed as area relative to that of the chromatograms of untreated carrageenans

Iridaea species contain about 33% of lambda carrageenan (extracted by KCl). The remaining 67% can be degraded completely by the Smith procedure (Table 2). Since the IR-spectrum show that iota structures are present, Iridaea carrageenan molecules are of the kappa/iota hybrid type rather than a mixture.

Periodate oxidation can be used for structural characterization of carrageenans.

A practical application is the detection of mu structures as a parameter of the efficiency of the alkali treatment.

The proportion of mu structures in a kappa-carrageenan is crucial to the gelling properties, because these structures prevent the molecules from forming a helix. In the sample of alkaline-treated Iridaea, only about 1 out of 1000 sugar rings is of mu type.

#### Effects of substituents on freeze/thaw stability of potato starch solutions

Starches used in foods that are subjected to freeze/thaw cycles or long storage periods are usually esterified with acetic acid or etherified by propylene oxide to give starch acetates and starch hydroxypropyl ethers. This is done to prevent the occurrence of retrogradation (i.e. all phenomena related to the aggregation of mainly amylose molecules, like syneresis, decreased gel strength and turbidity).

For use in food, only three substituents can be chosen: phosphate esters and the two mentioned above. Legislation is the main factor influencing the actual choice. Hardly any studies have been published on the comparison of the three substituents for functional properties. For this reason, some research was done to describe the effect of the nature of the substituent on the freeze/thaw stability of starch solutions. To compare samples in this way, they should have roughly the same viscosity and also the same "state of gelatinization" of the granules: when the starch granules are only partially swollen, no freeze/thaw stability can be expected.

Freeze/thaw stability is influenced by many factors: temperature and temperature fluctuations, rate of freezing, size of the ice crystals, viscosity, water activity, ingredients of the testing medium, method of preparation of the starch solution.

The following method of testing was chosen:

potato starch derivatives (not cross-bonded) are gelatinized by stirring

for 5 min after the temperature reached 95 °C at 2000 min<sup>-1</sup> in a beaker (in a 100 °C - thermostat) at 30 and 40 g/l starch concentration (dry matter based) in deionized water containing NaCl 1 g/l and sugar 50 g/l. After cooling to 20 °C in a water bath, the samples are frozen in 100 ml portions in sealed plastic cups at -20 °C. After 0, 1 and 3 freeze/thaw cycles (18 h at -20 °C, 6 h at +20 °C) syneresis is measured by putting the frozen mass in a paper filter funnel placed on top of a volumetric cylinder. The effect of the substituents can be seen in Table 3.

Table 3. Effect of substituent on freeze/thaw stability of modified potato starch solutions (for methods, see text).

Starch solution (30 g/l)	Synaeresis (% of volume expelled)		
	blank	1st cycle	3rd cycle
Potatostarch unmodified	0	52	61
Acetate DS* = 0.06	0	7	46
Acetate DS = 0.09**	0	3	51
HP-ether** DS = 0.10	0	0	21
HP-ether DS = 0.15	0	0	6
HP-ether DS = 0.24***	0	0	0
phosphate ester DS = 0.02***	5	39	40

\* DS, degree of substitution

\*\* HP, hydroxypropyl

\*\*\* represents the maximum permitted by law

There is not much difference in effectivity between acetate groups and hydroxypropyl groups: the latter perform slightly better at the same DS perhaps by their more hydrophilic nature or their higher "bulkiness". Increasing DS yields - not unexpectedly - increased stability. Although the phosphate ester is a charged group and thus supposed to be quite influential, phosphated starches are not freeze/thaw stable in the DS range tested.

Multicycle freeze/thaw stability requires a rather high DS of about 0.2 which can - for rather illogical reasons of law - only be achieved with difficulty by propylene oxide etherification.

Although complete freeze/thaw stability cannot be achieved with potato starches in these model systems, in food items such as sauces and bakery cream the other ingredients and the limited mobility through higher gel strength can result in a good performance even without highly substituted starches.

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### References

- de Vries, J.A.; Hansen, M.; Söderberg, J.; Glahn, P.-E.; Pedersen, J.K; 1986. Distribution of the methoxyl groups in pectins. Carbohydr. Polymers 6:165-176.
- de Vries, J.A.; 1988. Repeating units in the structure of pectin. Proc. 4th International Conference: Gums and stabilizers for the food industry, Wrexham, UK, 1987