EDITORIAL

This issue of *Food Chemistry* is unusual in that all the contributions included are concerned with coffee, and represent papers which were presented at a Chemical Society symposium entitled 'Advances in Coffee Chemistry', held at the Scientific Societies Lecture Theatre, Savile Row, London, on 16 May, 1978. Previous proceedings of symposia organised by the Food Chemistry Committee of the Industrial Division of the Chemical Society have been published in *Chemical Society Reviews*. However, on this occasion it was felt that *Food Chemistry* should devote a single issue to this important subject.

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RECENT DEVELOPMENTS IN COFFEE CHEMISTRY OUTLINED IN THE ASIC MEETING, ABIDJAN, 28–30 NOVEMBER, 1977

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(Received: 16 May, 1978)

Chemical aspects of coffee research are related to green coffee composition and modifications brought about by hybridisation of varieties, fermentation, drying and roasting procedures. In the framework of the Scientific Association for Coffee Research (ASIC), an overall review of coffee was considered which included agronomical and technological aspects and also dealt with sensory evaluation of coffee brews and with physiological effects of coffee and its constituents on human health.

The present paper will be restricted to recent developments in coffee chemistry.

When the composition of green coffee beans is examined under the light of biochemical pathways, sequences of secondary metabolites, particularly those derived from (−) kaurene can be related together (Professor J. Poisson, Université Paris Sud, France): among these, mascaroside (Fig. 1) was recently identified in wild growing *Coffea vianeyi* and was related to the extreme bitterness of these beans, which present interesting prospects for hybridisation.

New histological techniques allow the location of specific constituents in the cellular structure of coffee beans (E. Dentan, Research Nestlé, Switzerland; H. V. Amorim, University of Queiros, Brazil). Localisations in reserve cells of enzymes, polysaccharides, proteins and lipids open up new ways of following changes occurring during fermentation, drying and storage of green coffee beans.

Biosynthetic pathways were also elucidated for caffeine by the team of Professor H. Wanner (Zurich University, Switzerland); a nucleotide, 7-Methyl-xanthosine, is the precursor of caffeine in tissue cultures of *Coffea arabica* (T. W. Baumann, P. S. Citroreksoko and P. H. Frischknecht).

The composition of a new hybrid, Arabusta, was compared with that of the Arabica and Robusta varieties. Lipids and caffeine contents (Professor J. Wurziger, Hamburg), chlorogenic acids contents (M. N. Clifford, Grimsby College, Great

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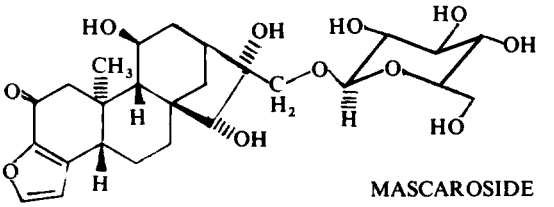

MASCAROSIDE

Fig. 1. Diterpene heteroside from *Coffea vianeyi*.

Britain), phenols (Professor R. Tressl, Technical University, Berlin), aroma patterns (O. Vitzthum, Hag, Bremen, DBR) and cup quality (J. Vincent, IFCC, Montpellier, France) of Arabusta beans occupy intermediary positions between the properties of the two varieties. A diterpene, khaweol, was identified in Arabica and Arabusta coffees and shown to be absent in Robusta species (Professor J. Wurziger, Hamburg University, West Germany). These facts show clearly an evidence for an hybridisation in the case of Arabusta coffee.

Analytical methods were refined, particularly for checking caffeine content (M. Quijano-Rico, LIQC, Bogota, Colombia), water contents (J. L. Multon, INRA,

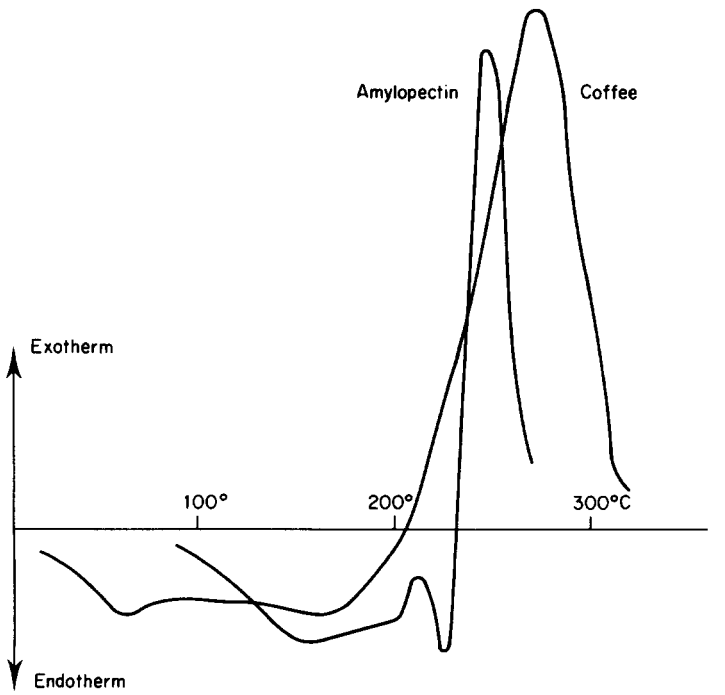

Fig. 2. Comparison of DTA diagrams of amylopectin and green Arabica coffee (both heated in closed vessels).

Nantes, France), chlorogenic acids (D. I. Rees, Research Lyons, London, Great Britain), tryptamides in waxes (P. Folstar, University of Wageningen, the Netherlands), trace elements (M. Quijano-Rico, LIQC, Bogota, Colombia) and for identifying phenols and furanic compounds (Professor R. Tressl, Technical University, Berlin).

Changes induced in coffee composition during roasting were reviewed by Professor W. Baltes (Technical University, Berlin); the role played by the Maillard reaction in flavour and colour production was outlined, showing the difficulty in characterising melanoidine formed during the primary steps of the thermally induced reaction. Thermal degradation patterns observed for amylopectin and green coffee (Fig. 2) show clearly the predominant role played by polysaccharides during coffee roasting. Evolution of given constituents during roasting was followed as production of Vitamin PP (R. Tchetche, University of Abidjan, Ivory Coast), denaturation of enzymes (H. Amorim, Université de Queiro, Brazil), rate of formation of organic acids (M. Blanc, Research Nestlé, Switzerland) and decomposition of chlorogenic acids (D. I. Rees, Research Lyons, Great Britain). Thermal degradation trials on ferulic acid (Fig. 3) give, for instance, an indication of traces of various diphenols found in roasted coffee (Professor R. Tressl, Technical University, Berlin, West Germany).

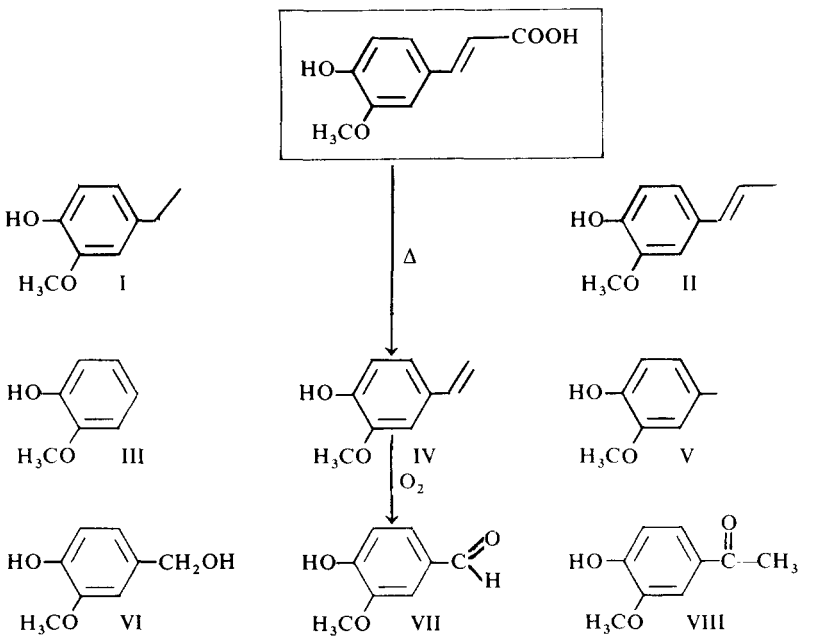

Fig. 3. Thermal fragmentation of ferulic acid. I. 4-Ethylguaiacol. II. Isoeugenol. III. Guaiacol. IV. 4-Vinylguaiacol. V. 4-Methyl-guaiacol. VI. Vanillinalcohol. VII. Vanillin. VIII. Acetovanillinon.

The physico-chemical properties of roasted coffee were reviewed by Professor R. Heiss (Food Packaging Inst., Munich, DBR) in his survey of coffee packaging; evolution of carbon dioxide from roasted coffee and preservation of coffee aroma against oxidation have to be mastered in order to obtain a proper packaging of roast coffee. Preservation of flavour volatiles during drying can be optimised by a careful determination of their sorption and evaporation behaviour (P. J. A. M. Kerkhof, University of Eindhoven, the Netherlands).

Advances in food engineering depend on a better knowledge of the physico-chemical properties of foodstuffs. Professor M. Loncin (Karlsruhe University, West Germany) reviewed, in general terms, roasting and extraction unit operations, pointing out the necessity of understanding better heat and mass transfers during these operations. Extraction patterns of various types of brewed coffees were determined; flavours of these brews were classified by applying a multidimensional mathematical analysis (G. Pictet, Research Nestlé, Switzerland). A general presentation outlined sensory analysis of coffee (Professor R. M. Pangborn, Davis University, California, USA) and coffee bitterness characterised by systematic checks (A. Voilley, Dijon University, France).

All these communications appear in the Proceedings published by the Association Scientifique Internationale du Café, 34, rue des Renaudes, Paris 75017, France.

IMPROVING THE COFFEE SHRUB IN WEST AFRICA

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(Received: 16 May, 1978)

INTRODUCTION

The Institut Français du Café et du Cacao (IFCC), which I had the honour to set up and which I have run for more than twenty years, is an organisation to provide technical help to the developing countries and specialises in agronomic research, in the widest sense of the term. It comes under the Ministry for Co-operation and enjoys under its statute great autonomy of administration and management, which gives it complete freedom of initiative and action.

The Institute currently has an annual budget in the region of 30,000,000 FF, or about £3,750,000, more than half of which is devoted to research on the coffee shrub and coffee. Approximately one-third of this amount is paid by the State of France while the remaining finance is provided by foreign States or by international organisations. The field of activity of the Institute is very wide since it covers agronomy, with all its research disciplines (improvement of plant stock, agrotechnics, phytopathology and entomology, etc.), chemistry, physiology and technology.

The IFCC provides permanent assistance to several French-speaking States: the Ivory Coast, Cameroon, Togo, Central African Empire, New Caledonia, etc. and plays an important part in many States, both French-speaking and otherwise, as an adviser by means of the technical support of its specialists who are posted there.

The headquarters of the Institute are in Paris where the Directorate-General and the Administrative Services are situated. The research laboratories, which work very closely with the Overseas Services, are at Montpellier. Overseas, the Institute runs, directly or indirectly, experimental stations and laboratories.

Since the subject of this paper is the improvement of the coffee shrub in West Africa, we shall deal with the following points in turn:

(a) The selection of the Robusta coffee shrub.
(b) The 'Arabusta' hybrid.
(c) Coffee shrubs which contain little or no caffeine.
(d) The prospects opened up for the improvement of coffee.

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At the beginning, some twenty years ago, the main objective of the work on improving the Robusta coffee shrub was to obtain coffee shrubs with a high yield. In fact, the African and Malagasy coffee plantations at that time consisted exclusively of indeterminate shrubs and their production per hectare was, at best, around 400/500 kg.

A significant selection process, considered to be a priority, was therefore undertaken which enabled remarkable results to be obtained fairly rapidly with selections likely to produce three to five times as much. It will be noted, however, that in the early stages the cross-fertilising nature of the Robusta meant that in order to propagate these selections (clones) only vegetative propagation (cuttings) could be used. Subsequently, new criteria were taken into account in respect of the selections: resistance to disease, resistance to drought, caffeine content, quality of the drink, etc.

At the present time the best selections of this coffee shrub are characterised by a potential production which currently reaches 2·5 metric tons/hectare and, in some cases, 3 and even 4 t/ha.

At the same time it was possible to collect in nursery plantations several selected clones with pollinic similarities in order to obtain hybrid seeds with potentially high genetic productivity.

This work is only described here because it is standard and it satisfied one requirement of the research—to bring about a noticeable improvement in productivity in a short time.

Its value, although important to the improvement in productivity, is, however, fairly limited in other respects and in particular as regards flavour.

This is why the IFCC lost no time in broaching the problem of interspecific hybrids which could perhaps be the answer to this problem.

The research therefore concentrated on crossing the two most common coffee shrubs in the world, the Arabica, known for its flavour, and the Robusta, less popular in this respect but with remarkable properties of productivity, hardiness, disease resistance, etc. and especially perfectly adapted to the climatic conditions of the low altitude, humid, tropical regions, i.e. the lower coastal areas of West Africa.

The research work which started in 1962 culminated in 1970 with the creation of the first hybrid coffee shrubs.

There were a great many difficulties. The major one resulted without doubt from the difference in the chromosomal make-up between the two species, the Arabica being tetraploid and having 44 chromosomes while the Robusta is diploid and only has 22.

The geneticists had two solutions available. (a) To obtain tetraploid hybrids by treating the diploid Robusta clones used as parent stock with colchicine. (b) To obtain hexaploid hybrids by doubling the chromosomes of the triploid hybrids (sterile). This combination offered the advantage of introducing 44 Arabica chromosomes compared with only 22 Robusta and offered the possibility that the

aromatic qualities of the hybrid product would turn out to be greater than in the tetraploid in which the presence of the two parents is of equal importance.

It was, however, the first solution which the IFCC experimented with in the beginning.

Among the difficulties to be overcome, the development of a practical technique for artificial pollination by hand should be mentioned, with all the incidental problems regarding the conservation of the germinative power of stored pollens.

It would be tedious to describe all the work which had to be carried out in order to obtain the present result: choice of Arabica and Robusta parent stock, study of the combinations *C. arabica* × *C. canephora* tetraploid and *C. canephora* tetraploid × *C. arabica*, propagation and study of hybrids, back-crossing with tetraploid Arabica and Robusta, studying the behaviour of the hybrids, etc., etc.

An idea of the scale of this work may be gained by the fact that the Genetics Division had to make individual observations on several tens of thousands of coffee shrubs and the development of the cultural techniques required the setting up of an experimental block of 500 ha.

The results, luckily, are remarkable. They may be summed up by saying that the Arabusta is the result of the favourable association of the characteristics of the Robusta coffee shrub, fertility, resistance to disease, particularly Coffee Rust Disease, with those of the Arabica as regards granulometry (larger beans than the Robusta) and the improved taste of the beverage.

It is particularly interesting to note that the caffeine content of the Arabusta is midway between that of its parents, i.e. about 2·0 % (Arabica 1·5 %; Robusta 2·5 %).

Taste tests and manufacturing trials for instant coffee which have been carried out have shown its organoleptic qualities.

The Arabusta also has the advantage of being suitable for climatic conditions which are very similar to those which are suitable for the Robusta. It therefore offers the States which produce Robusta—and particularly the Ivory Coast where this research work was undertaken and carried out—the possibility of cultivating an aromatic coffee shrub at low altitude, which is quite impossible with the Arabica which, in the tropics, needs a climate which can only be found in areas of altitude. This new coffee shrub is therefore destined for considerable expansion, not only in West Africa but in all countries with similar ecological conditions, and will probably, in certain cases, replace the Robusta.

It is of interest, expecially to chemists, that the work of Wurziger (1977) and Vitzthum *et al.* (1977) on the chemical constituents of Arabusta beans has shown that this coffee is closer to the Arabica than to the Robusta.

From an economic and commercial point of view, it should be pointed out that the later Arabusta selections have enabled the fertility rate to be increased to a yield of more than 2 metric tons/hectare which is comparable to that of the good Robusta selections.

The Ivory Coast has already started to popularise this coffee shrub, especially in

the west of the country, and it is anticipated that the first exports of this coffee on a substantial scale will be after 1982.

The considerable scope of the programme for improving the coffee shrub led us, about ten years ago, to ask for the assistance of the geneticists of another French organisation which specialises in tropical research, ORSTOM. A joint programme was drawn up with a logical division of the work.

This was how the second method of improvement mentioned above, obtaining hexaploid hybrids, was explored in the Ivory Coast by that organisation's researchers.

The results obtained to date are not very positive, to be sure. A considerable improvement in the taste qualities of the hybrid has been recorded, which was to be expected, but the rate of fertility and the granulometry are not satisfactory. Commercial exploitation is therefore ruled out, at least at the present stage of the work, since, we must admit, there are, genetically speaking, possibilities for improvement.

In addition to these hybrid associations Arabica × Robusta, research is being actively pursued to study other associations between species. Particular attention is being paid to the use as parent stock of a native African coffee shrub, *C. eugenioides*, which is characterised by its caffeine content of much less than 1 %.

To complete this paper, mention should now be made of the very important work done in Madagascar by the IFCC and ORSTOM, working together, on coffee shrubs native to that island and characterised by the absence of caffeine in the beans.

It is known that the Malagasy flora—and more generally the flora of the Islands of Madagascar, Reunion, Mauritius and the Comoro Islands—contains a number of native species of coffee shrubs, most of which have been known about for half a century but not exploited. These coffee shrubs, which number about fifty species or maybe more, are found in various parts of Madagascar in very varied spontaneous plantations; they all have the same characteristic: the absence of caffeine in their beans. Agricultural exploitation of these shrubs is not possible for the moment, however, particularly in view of the low quality of the drink. This is the reason for the lack of interest in them.

However, these shrubs constitute a stock of exceptional genetic value for improving the coffee shrub, in particular to offer a genetic solution to the production of coffees containing little or no caffeine.

This is why the IFCC has attempted to gather in Madagascar several collections of these coffee shrubs and, with ORSTOM, has begun to study them. This work was unfortunately interrupted five years ago for political reasons. It is very regrettable because the two research organisations had become convinced that using this vegetative stock could sooner or later result in obtaining usable coffee shrubs producing coffee containing little or no caffeine.

It is needless to stress the commercial interest in such products nowadays when the

consumption of natural products is much sought after. We must hope that such research may be continued.

This paper only gives a general view of the problems encountered in West Africa in the improvement of the coffee shrub. It must be stressed that some very impressive results have been obtained in a relatively short time from the selection of the Robusta and especially with the Arabusta. The countries which produce Robusta coffee in Africa, and elsewhere in the world, have been given the opportunity to cultivate high-yielding clones which are likely, in traditional cultivation, to double and even triple the yield per hectare and also, with the Arabusta, to supply the big consumer markets with a coffee of much higher quality than the classic Robusta. There is also the possibility with this higher profitability of maintaining or even of increasing, if necessary, the production potential of the States while releasing large areas of land for other export or food crops.

These results, however, spectacular as they are, do not put an end to the improvement of the coffee shrub. The research possibilities opened up by associations between species have not yet been fully explored and, as we have seen, nor have the extraordinary resources of the Malagasy coffee plants. So there are still high hopes in that field.

Considerable progress may also be expected in the more distant future with the completely new genetic process of using haploids and tissue cultures, to which the IFCC is fully committed. But it will be several years before a fair verdict can be reached on these new techniques which have been raised here in order to give a full picture of the many and very diverse methods used by geneticists in order to improve the coffee shrub and its very precious product, coffee.

I will have achieved my purpose if I have also managed to give an idea of the work which has been carried out on the coffee shrub in the field of genetics and to prove that nothing has been neglected in this branch of agronomy, as in chemistry, to improve this very important product.

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THE CHEMISTRY OF COFFEE EXTRACTION IN RELATION TO POLYSACCHARIDES

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(Received: 16 May, 1978)

ABSTRACT

Technically produced extracts from roasted Arabica and Robusta coffees contain, just like the infusions prepared in the home, 20–36 % *carbohydrates, depending on the degree of extraction. They are composed predominantly of mannan and galactan in about the same proportions, the share of glucan and araban making up only* 1–3 % *of the extracts. With dialysis a group of polysaccharides with a molecular weight of more than* 10 000 *can be separated. They make up about half of the carbohydrates of the extracts. Their composition corresponds to that of the latter. Finally, one can obtain yet another group of almost intact high polymeric carbohydrates as copper complexes. However, they consist only of mannan and galactan, mannan predominating significantly. Arabica and Robusta coffees showed differences in this respect. Whereas Arabica coffee was able to release only a certain amount of these very high-polymeric carbohydrates, Robusta coffee delivered ever greater amounts of these polysaccharides with increasing extract yields.*

INTRODUCTION

The polysaccharides of raw and roasted coffee have been investigated from time to time for about a century. For a long time no attention at all was paid to the carbohydrates of the coffee extracts, although these have been produced for over 40 years. It was apparently obvious that with higher extract yield, sufficient polysaccharides are dissolved in order to bind the aroma substances. Thus, the previously necessary addition of carbohydrate mixtures from other sources became superfluous. This perception apparently satisfied people; the question of what kind

Fd. Chem. (4) (1979)—
Printed in Great Britain

of polysaccharides are released by the roasted coffee bean during extraction did not seem of particular interest.

Thaler (1957) was able to isolate from coffee extracts and also later (Thaler, 1959) quantitatively determine a galactomannan as a copper complex. Later, Wolfrom & Anderson (1967) obtained, by means of another method, an arabinogalactan and a galactomannan from a coffee extract. However, the latter was not the same substance as the polysaccharide isolated by Thaler. Since his method, the oxidation of Maillard products, peptides, etc., using chlorine dioxide, also proved useful in subsequent investigations of the carbohydrates of green and roasted coffee beans (Thaler & Arneth, 1968, 1969; Thaler, 1970, 1974, 1975; Asante & Thaler, 1975). This procedure was also finally used on coffee extracts. The results are described below.

TABLE 1
RESEARCH MATERIAL

Columbia Arabica Coffee		*Angola Robusta Coffee*	
Roasting loss:	17·0%	Roasting loss:	18·7%
Extraction yield (%):	36·4	Extraction yield (%):	38·8
	38·0		39·5
	39·8		45·2
	43·6		48·7
	53·2		58·0

Extracts from a roasted Columbia Arabica and an Angola Robusta coffee were investigated which were produced with increasing extraction yields in a pilot plant. The extract with the lowest yield was acquired only with water at a temperature of 100 °C. In addition, in the laboratory an extract was prepared from each of the two roasted coffees by extraction using boiling water in a 1:25 ratio. The extraction yields are shown in Table 1. There were now available five extracts of each of the two coffees.

CONSTITUENTS AND TOTAL AMOUNT OF CARBOHYDRATES

The first question was: Which polysaccharides and how much of each—in the event that there should be more than one—are, in fact, present in the extracts?

In order to demonstrate carbohydrates qualitatively, the extracts were hydrolysed with sulphuric acid and the solutions examined by paper chromatography. We found, as expected, mannose, galactose, glucose and arabinose. Xylose and rhamnose were only present in traces and were not taken into further consideration. Quantitative determination of the sugars was also undertaken, initially by means of paper chromatography which is quite laborious. Later, enzymic analysis was used.

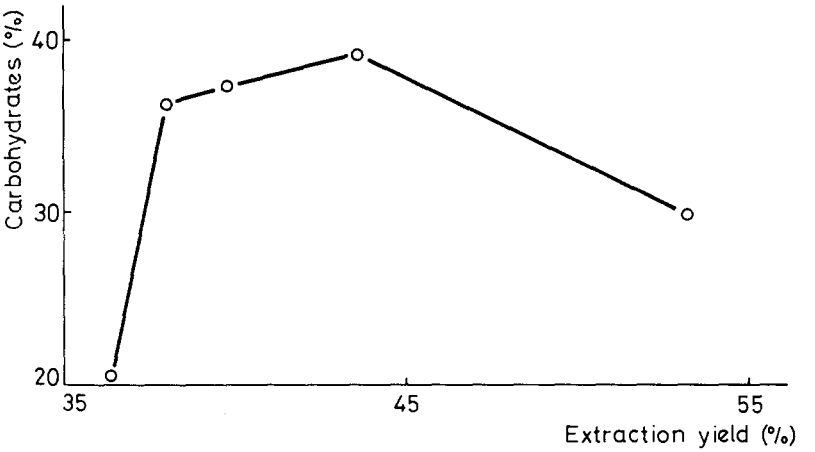

Fig. 1. Total carbohydrates in extracts of Arabica coffee.

In each of the ten extracts, mannose and galactose were the main ingredients; glucose and arabinose were present only in considerably smaller amounts. The results for Columbia Arabica coffee are shown graphically in Fig. 1: as the yield increases, the extracts at first show a strong increase in the entire carbohydrate content; this slows down, however, and finally the amount of carbohydrates in the extracts decreases sharply. For Angola Robusta coffee, the polysaccharide content also increases sharply at first, but remains stable between 40 % and 45 % yield, only to increase again slowly at higher extraction (Fig. 2).

However, such curves are not particularly informative due to the irregularly increasing extraction yields. They become more interesting when one recomputes

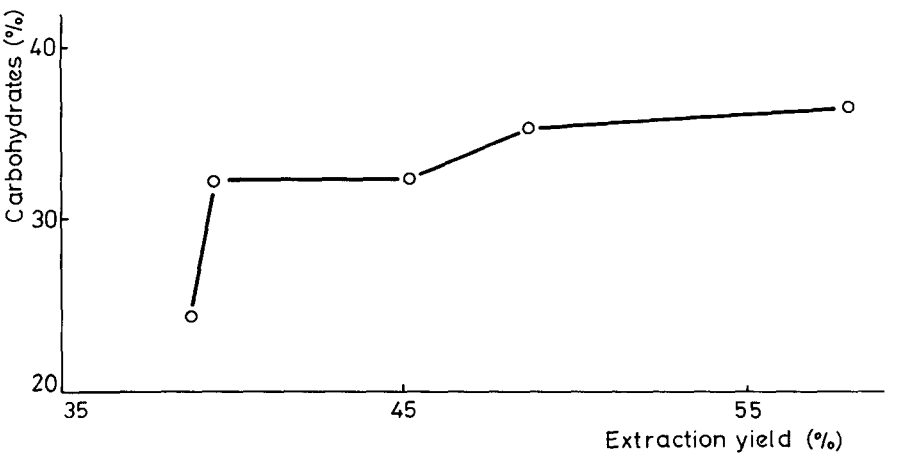

Fig. 2. Total carbohydrates in extracts of Robusta coffee.

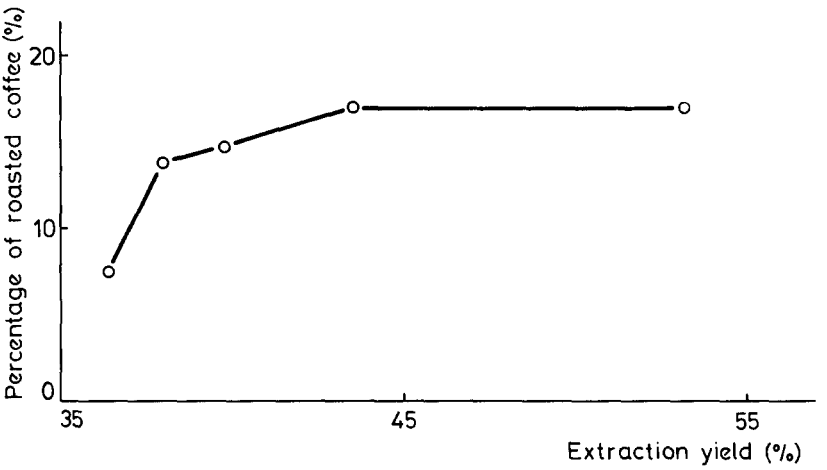

Fig. 3. Delivery of carbohydrates from roasted Arabica coffee to the extracts.

the figures for roasted coffee. The question is no longer: How much mannan, galactan, etc. is present altogether in the individual extracts? but rather: How much carbohydrates altogether has the roasted coffee delivered to the extracts? One then recognises (Fig. 3) that the Arabica coffee has delivered increasing amounts of carbohydrates up to a yield of 43·6% but that, from then on, no further release of polysaccharides occurred. One could say that the supply of polysaccharides which can be extracted from Arabica coffee is exhausted at this point. In the case of the Robusta coffee (Fig. 4), however, polysaccharides are released continuously, even at the highest yield of 58%.

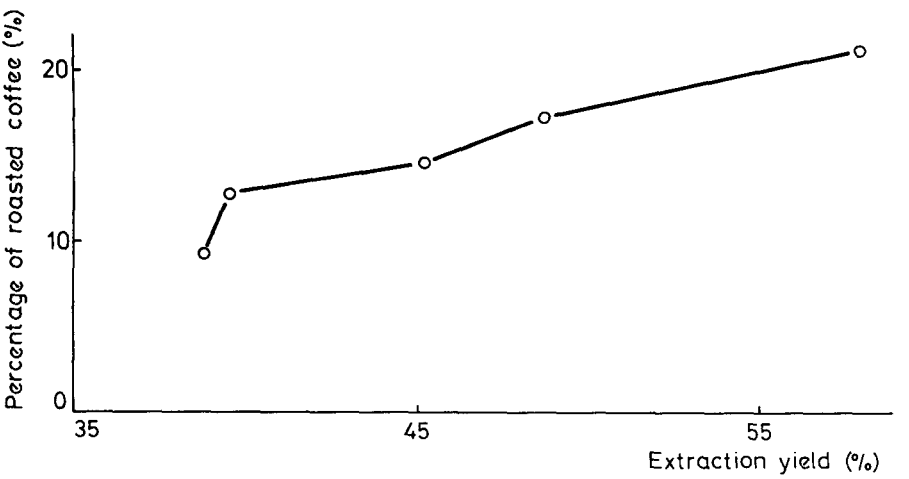

Fig. 4. Delivery of carbohydrates from roasted Robusta coffee to the extracts.

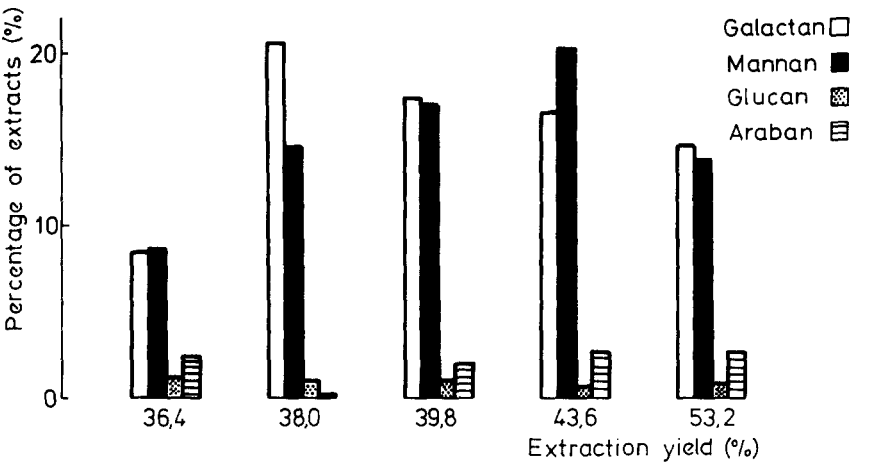

Fig. 5. Total carbohydrates in extracts of Arabica coffee.

Up to now we have been dealing only with the entire carbohydrates. The question arises now of how this mixture is composed. The quantitative determination of the individual ingredients had shown, as previously stated, that mannan and galactan are the main ingredients. Again this becomes more obvious when the relations are represented graphically (Fig. 5). For the Arabica coffee, somewhat more galactan than mannan is present up to a yield of 40%; from that point on more mannan is present and at the end nearly equal amounts of mannan and galactan are found. The Robusta coffee shows a basically similar picture (Fig. 6). Here, too, mannan and galactan predominate, although there is always somewhat more of the latter present.

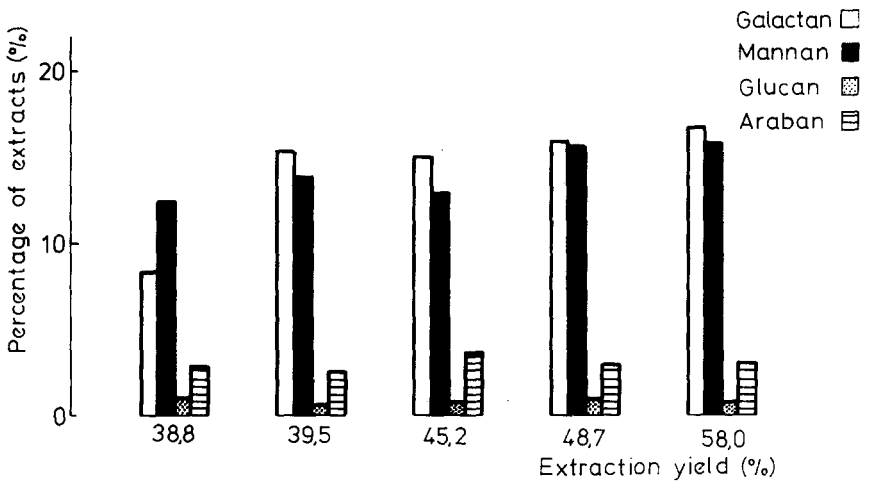

Fig. 6. Total carbohydrates in extracts of Robusta coffee.

It is noteworthy that glucan and araban do not show any changes with increasing extract yield. That remains the case when one converts the figures for the roasted coffee. For glucan, fragments of the cellulose totalling less than 0·5 % are released from both Arabica and Robusta coffee. This is remarkable since cellulose is definitely more sensitive to chemical attack than mannan, especially to hydrolysis. Moreover, the extraction residue of both coffees, the coffee-grounds, still contains about 20 % cellulose even after the most intensive extraction. On the other hand, araban is apparently already completely extracted at a relatively low extract yield. It is no longer to be found in the coffee-grounds, or only in traces of less than 0·1 %.

This answers the question of which carbohydrates are present in the extracts and in what quantities. Carbohydrate is, however, a broad concept, and the 'polysaccharides' discussed here have very dissimilar molecular weights. One portion is certainly high polymeric, another low polymeric. Yet there is a possibility of separating such mixtures through dialysis. Of course this is completely arbitrary and the result is dependent only upon the permeability of the membrane. In this case a tube of regenerated cellulose was used with an exclusion limit of 10 000 Dalton.

AMOUNT OF HIGH POLYMERICS

In order to dialyse the carbohydrates, however, they must first be released from the extracts. In the extract they are tightly bound to Maillard products. It has up to now been impossible to separate the polysaccharides from the Maillard products and from proteins or peptides without destroying these associated materials. One would imagine that polysaccharides which carry a carbonyl function at one end of their chain of hexose or pentose units could react with terminal or laterally positioned amino groups of proteins. At this location a Maillard reaction occurs. However, the hexose chain of the polysaccharide and the peptide chain of the protein remain unaffected and now form, so to speak, lateral chains which emanate from the location of the Maillard reaction. This entity represents a giant molecule. If one could destroy the Maillard compounds, the polysaccharide chains would be set free. This is, of course, a hypothesis.

In order to destroy the Maillard compounds and set the polysaccharides free, oxidation by means of chlorine dioxide according to the method of Wise *et al.* (1946) was employed. Chlorine dioxide destroys Maillard products, proteins, etc., but does not attack carbohydrates of any kind.

The coffee extract solution was therefore treated with chlorine dioxide and then dialysed. The content of the dialysis tube, which contained the high polymeric carbohydrates, was precipitated with alcohol in a ratio of 1:9 and the sediment was washed and dried. With some difficulty, all this could also be accomplished quantitatively.

However, one does not obtain pure polysaccharides in this manner. With

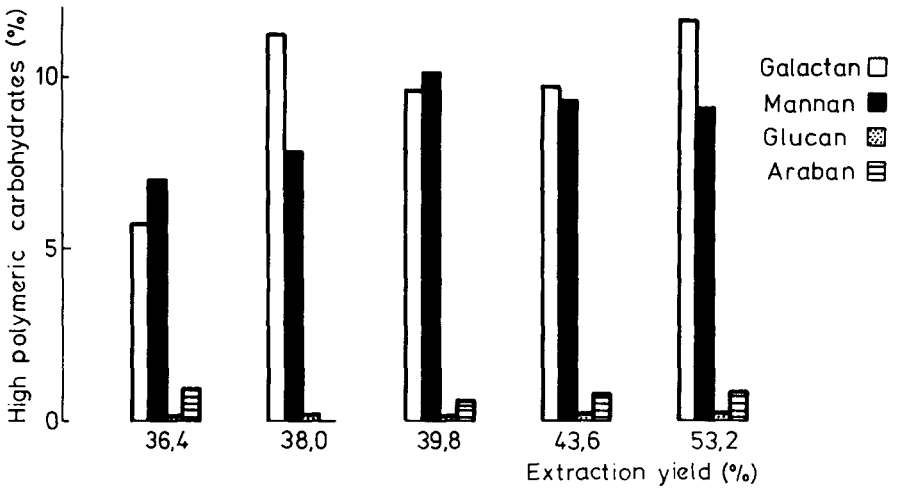

Fig. 7. High polymeric carbohydrates in extracts of Arabica coffee.

hydrolysis the monosaccharides always make up only 70%–80% of the amount theoretically to be expected. This holds for all polysaccharides obtained from roasted coffee and not only for those of the extracts. The remainder are presumably transformation products of pectin, as well as anhydrides. Such anhydrides, which arise by means of the separation of water between two hexose or pentose molecules, are known in connection with dextrins won by heating dry starch.

In the case of the Arabica extracts, the following results were obtained: in the high polymeric carbohydrates, mannan and galactan also greatly predominated over

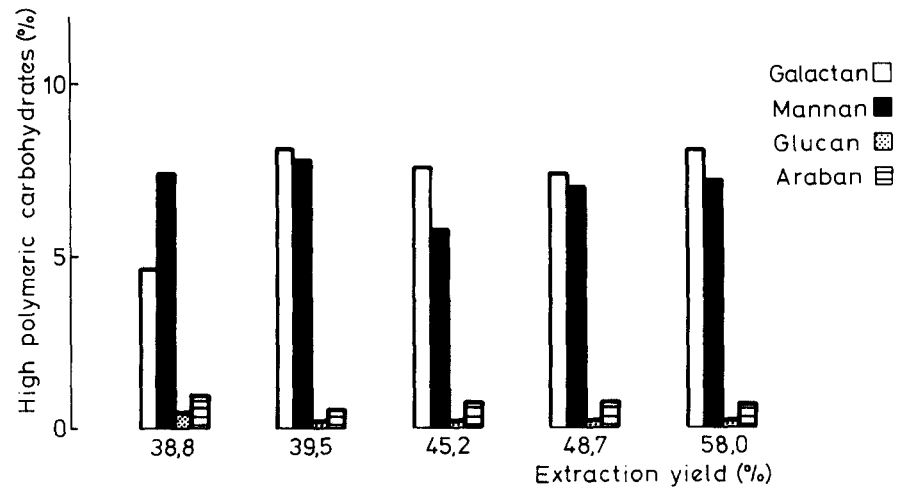

Fig. 8. High polymeric carbohydrates in extracts of Robusta coffee.

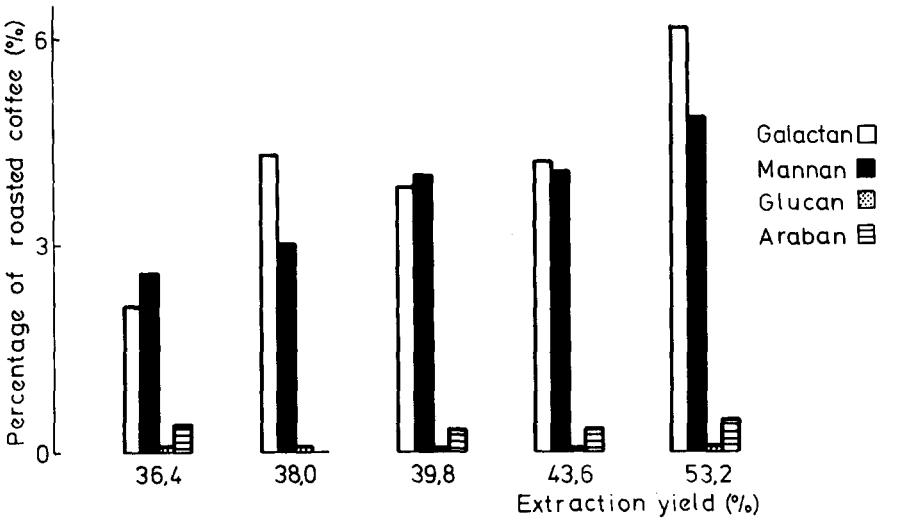

Fig. 9. Delivery of high polymeric carbohydrates from roasted Arabica coffee to the extracts.

glucan and araban (Fig. 7). Once it was galactan and once mannan which was present in a moderate surplus. A rule for this could not be established. The case was about the same for the Robusta extracts (Fig. 8). Here the reduction in the share of mannan at an extraction yield of 45% is particularly striking.

The amount of high polymeric carbohydrates was relatively high. With Arabica coffee it always came to something more than half of the total polysaccharides.

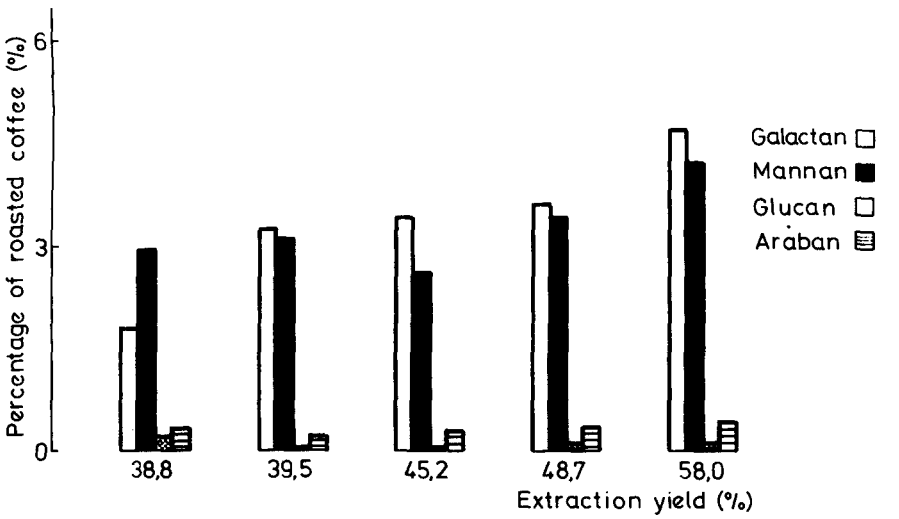

Fig. 10. Delivery of high polymeric carbohydrates from roasted Robusta coffee to the extracts.

With Robusta coffee, on the other hand, there were, at low yields, the same amounts of low and high polymeric carbohydrates. With yields of more than 40 %, however, the share of low polymers increased rapidly. Their ratio to the high polymers was about 4:3.

This again becomes clearer when one recomputes the figures for the roasted coffee. It is then obvious (Fig. 9) that the roasted Arabica coffee releases more galactan up to an extraction yield of 40 % but a cessation then occurs and only at above 44 % yield is galactan again released. The irregularities with galactan can only be substantiated but not explained. The Robusta coffee behaved quite similarly, except that here variations in the release of mannan were to be observed (Fig. 10).

The lower polymeric carbohydrates can only be computed from the difference between the entire content of carbohydrates in the extracts and that of the high polymers. They were composed exactly as the latter.

COPPER PRECIPITABLE POLYSACCHARIDES

However, there exists another possibility of at least separating the high polymers into two groups. Very high polymeric carbohydrates such as cellulose (or in other words, almost unaltered cellulose, which is soluble in water), mannan or xylan produce, with alkaline copper or iron solutions—e.g. Fehling solution—complexes which are insoluble in alkalis. A complex of this type consists of the polysaccharide concerned, copper(II) or iron(III), the alkali metal and the organic hydroxy acid. Such a complex was obtained 20 years ago from solutions of coffee extracts with Fehling solution in the form of brown flakes (Thaler, 1959). If one first destroys the Maillard products, etc., with chlorine dioxide, a light blue copper complex precipitates. From treatment with methanol-acetic acid one obtains the colourless polysaccharide or a mixture of polysaccharides. It was not particularly difficult to work out a quantitative method of determination. The amount of polysaccharides which can be precipitated in this manner depends upon the degree of roasting of the coffee. With increasing roasting, the content in the extract of this precipitable polysaccharide increases somewhat (Ara & Thaler, 1977).

In contrast to the previously discussed more or less high polymeric carbohydrates, the polysaccharide precipitated with alkaline copper solution is composed only of mannan and galactan. Usually a little glucan is also present. In the case of normally roasted Arabica coffee the polysaccharide contains approximately 94 % mannan and 6 % galactan while Robusta coffee contained 88 % mannan and 10 %–12 % galactan. There is usually less than 0·5 % glucan and, at the most, 1 %. These figures apply only to the polysaccharide content of the precipitates, for here, too, there is always a certain amount of substances present which cannot be hydrolysed to monosaccharides.

The quantity of precipitable polysaccharides in the extracts amounted to

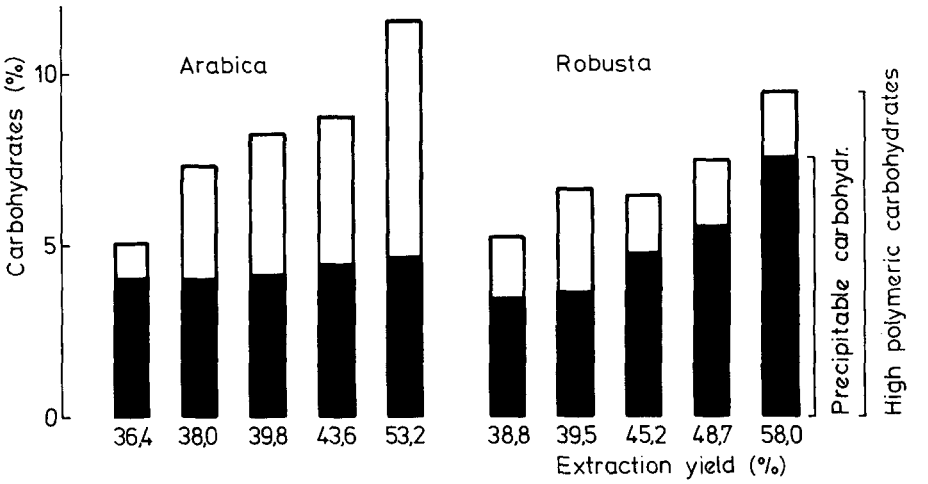

Fig. 11. Delivery of high polymeric and copper precipitable carbohydrates from roasted Arabica and Robusta coffee to the extracts.

8·4%–9·8% of the extracts with Columbia coffee and 7·7%–9·5% with Angola Robusta coffee. If each of these figures is again recomputed for the roasted coffee and compared with the amount of high polymeric carbohydrates, the result is quite interesting (Ara & Thaler, 1977). One sees that the roasted Arabica coffee has always released about the same amount of precipitable polysaccharides for all extract yields (Fig. 11). In addition, one recognises that they represent approximately half the high polymeric carbohydrates. This is different with the Robusta coffee: here ever greater amounts were delivered from the roasted coffee with increasing extract yields. Their share of the total high polymeric carbohydrates always amounted to more than half—by 58% yield even to four-fifths. Perhaps this is interesting in view of the property of such high polymeric carbohydrates to bind aroma substances.

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THE EFFECT OF DEWAXING OF GREEN COFFEE ON THE COFFEE BREW

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(Received: 16 May, 1978)

ABSTRACT

The two commercially most important mild treatments for green coffee are the steam treatment and the dewaxing process. In the former treatment the green coffee is just steamed. In the dewaxing process the waxy layer is extracted from the green coffee with an organic solvent, after which this coffee is also steamed to remove solvent residues. Some of the main constituents of the waxy layer are the carboxy-5-hydroxytryptamides (C-5-HT). What happens to these components during the roasting and brewing of the coffee is discussed. During roasting part of the C-5-HT decomposes, but during brewing most of the C-5-HT remains in the spent grounds due to its insolubility.

Literature reports about physiological effects of both types of coffee in human and animal experiments are summarised briefly. For both types of treated coffee additional evidence seems to be needed before hard conclusions can be drawn about their digestibility.

INTRODUCTION

For many years cases of indigestibility of coffee have been an issue of continuing interest. The complaints expressed most frequently are (Stieve, 1965; Sziegoleit *et al.*, 1972*a*,*b*):

A feeling of pressure or fullness in the stomach.
Heartburn.
Internal uneasiness.
A whole series of other complaints, mostly localised in the gastro-intestinal system.

Fd. Chem. (4) (1979)—
Printed in Great Britain

Starting with the Lendrich process (Lendrich, 1933), according to which the green coffee is subjected to a steam treatment before roasting, several processes have been developed to improve the digestibility of the coffee brew. However, apart from steam treatment only the dewaxing treatment has achieved real commercial importance. This dewaxing consists of the removal of the waxy layer from the green coffee with an organic solvent followed by steaming in order to desolvetise the coffee.

During recent years also some development work has been done on processes designed to reduce the content of chlorogenic acids before roasting.

To date the following groups of components have been indicated to be responsible for the indigestibility of coffee:

Irritating roast products.
Ether-extractables.
Waxy components (such as C-5-HT).
Phenolcarboxylic acids.

Many of these components have not been chemically identified satisfactorily. Of these components, the waxy components—and particularly the carboxy-5-hydroxytryptamides (C-5-HT)—have received much attention during the last ten years.

This paper is divided into four parts:

(i) A mass balance of C-5-HT over the steps of dewaxing, roasting and brewing of the coffee and the consequences thereof for the chemical composition of the brew.
(ii) The chemical consequences of the steaming or desolvetising step.
(iii) The results reported in the literature of the physiological testing of these types of treated coffee.
(iv) Some suggestions for working models for some types of indigestibility relating to treatments of green coffee.

EVALUATION OF C-5-HT

The C-5-HT content of the coffee is used as an analytical parameter for treatments of the waxy layer. Sometimes the C-5-HT themselves have been linked with the indigestibility of the coffee brew, mostly with reference to the pharmacologically active 5-hydroxytryptamine (serotonine). This serotonine is, however, inactive when administered orally. Even oral administration to humans of 60 mg has been reported to be without noticeable effects. In addition, fruits, such as bananas, tomatoes and avocados, contain considerable amounts of serotonine (Udenfriend *et al.*, 1959). It may therefore be concluded that 5-hydroxytryptamine in the coffee brew does not provoke indigestibility.

TABLE 1
C-5-HT CONTENTS OF COFFEE SAMPLES (PPM ON DRY BASIS)

	Untreated	*DCM extracted*
Green coffee	735 ($s = 21$)	266 ($s = 14$)
Roasted coffee	604 ($s = 26$)	189 ($s = 7$)
Spend grounds	699 ($s = 23$)	274 ($s = 13$)

The fact that there is no effect of C-5-HT via serotonine does not mean that C-5-HT has no effect at all. For instance, roast products of C-5-HT could be physiologically active. A method of checking this is to draw a mass balance in order to see how much C-5-HT in any form could get into the brew. Therefore, for the different process steps: dewaxing, roasting and brewing a mass balance was drawn to see what happens to this major component of the waxy layer.

Green coffee, roasted coffee and spent grounds have been analysed with the results shown in Table 1.

TABLE 2
CHANGES IN C-5-HT CONTENTS DURING COFFEE PROCESSING (PPM ON GREEN COFFEE DRY BASIS)

	Untreated	*DCM extracted*
Removed by DCM	—	469
Decomposed on roasting	161	86
Extracted or emulsified on brewing	63	−11[a]

[a] Not significantly different from zero.

If one recalculates the figures shown in Table 1 for dry material loss during roasting and extraction yield at brewing the figures on a green coffee dry material base can be obtained. The changes in the absolute amounts of C-5-HT are, by then, clear (Table 2).

What happened to the amount of C-5-HT which disappeared upon brewing was

TABLE 3
C-5-HT CONTENTS IN COFFEE BREWS

	Untreated	*DCM extracted*
Brewed in the pot (without filter)	2·3 mg/litre	0·5 mg/litre
Filtered (paper filter)	ND	ND

ND = Not detectable.

TABLE 4
PERCENTAGE DISTRIBUTION OF C-5-HT

	Untreated (%)	*DCM extracted* (%)
Removed by DCM		64
Decomposed on roasting	22	12
Spent grounds	70	26
In brew/retained by paper filter	6–9	0–1
Total	98–101	102–103

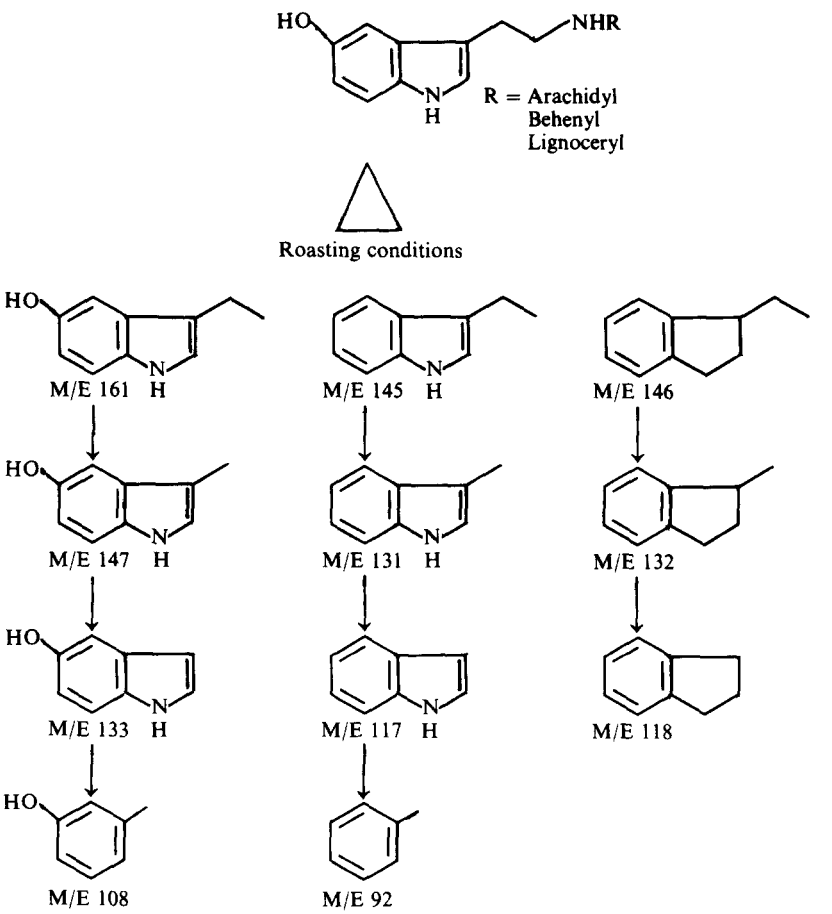

Fig. 1. Thermal decomposition of serotonin amides (Viani *et al.*, 1975).

checked in an experiment where the C-5-HT contents of two brews—one made with, and one without, a paper filter—were measured (Table 3).

These figures are summarised in the percentage distribution shown in Table 4. From these figures two things are clear. First, that a small part of the C-5-HT may pass into the brew. However, if a paper filter is used no C-5-HT is detectable in the brew. This could indicate that the C-5-HT which passes into the brew does so via the emulsified oil phase.

In addition to these intact C-5-HT, the possibility of roast products of the C-5-HT passing into the brew has to be considered. Secondly, the amount of C-5-HT decomposed by roasting is considerably reduced by removal of the waxy layer.

With regard to the identity of the products formed at roasting, Viani *et al* (1975) (Nestlé) has reported some results concerning the roast products of C-5-HT (Fig. 1). Some of these products, especially the phenolic ones, are water soluble and thus may be expected to pass into the brew. Removal of the waxy layer before roasting will thus result in a reduction in the content of phenolic roast products of C-5-HT in the brew.

CHEMICAL EFFECTS OF THE STEAM TREATMENT

The chemical effects of the steam treatment (either in the Lendrich process or as the desolvetising step in the dewaxing process) have been reported in the literature in a number of cases. Mentioned in this respect have been the ether-extractables (Albanese, 1964; Mülhens, 1972), sulphur compounds (Albanese, 1964; Kaden, 1962) and polysaccharides (Albanese, 1964; Meyer, 1978). Conflicting results have been reported about what happens to the chlorogenic acids during steaming (Lendrick, 1933; Albanese, 1964; Kaden, 1962; Kröplein, 1963; Werner *et al.*, 1965; Windeman, 1974). Possibly the most evident effects are those from the work of Windeman (1974), Gal *et al.* (1976) and Meyer (1978).

The results of Windeman (1974) are especially interesting. He reported the presence of 3-methoxy-4-hydroxystyrene in the steam condensate. This phenolic component is formed by decomposition of feroylquinic acid which belongs to the group of chlorogenic acids. Whereas Windeman found in a closed autoclave only a low degree of formation of 3-methoxy-4-hydroxystyrene, this formation might be increased in a system with constant steam or condensate discharge and more optimal conditions for temperature, moisture content of the coffee and time (as is approached by desolvetising conditions).

Summarising the discussed chemical effects of dewaxing and steaming one may expect that both treatments contribute to a reduced content of phenolic components in the brew. Dewaxing does so via a reduction of the phenolic roast products of C-5-HT and steaming via the removal of the 3-methoxy-4-hydroxystyrene.

PHYSIOLOGICAL TESTS ON TREATED COFFEE

In order to study the digestibility of treated coffees several types of test have been reported, as follows:

Using pigeons or dogs to measure the emetic activity of the brew (Behrens & Malorny, 1940; Kudsi, 1964; Malorny *et al.*, 1967).
With rats having stress ulcers induced by immobilisation in narrow cages—the effect of coffee on the healing of these ulcers has been studied (Frommolt *et al.*, 1972).
Bile secretion has been measured with rats and intestinal motility by ink transportation upon mice (Czok & Lang, 1963).
In humans the experiments are, apart from X-ray studies, usually limited to acid secretion and pH measurements in the stomach (Stieve, 1965; Rösner *et al.*, 1971; Finterlman *et al.*, 1978) and scoring of the different types of complaint (Sziegoleit *et al.*, 1972*a*,*b*).

The following test results for treated coffee have been reported.

Dewaxed coffee was found to give less retardation in the healing of stress ulcers in rats than regular coffee (Frommolt *et al.*, 1972). In humans dewaxed coffee was found to give a lower secretion of gastric acid than regular coffee (Rösner *et al.*, 1971).

The steam treatment was found to reduce the stimulation of bile production and of intestinal motility (Czok & Lang, 1963). The steam treatment was also shown to reduce the emetic activity of the brew (Behrens & Malorny, 1940; Kudsi, 1964; Malorny *et al.*, 1967). In these studies the brew was also fractionated in order to search the components responsible for the emetic activity. This activity was localised in the group of components that are extractable with ether from an acidified brew. This method of isolation suggests that the group consists of weakly acidic components such as, for example, phenols and pyrrols. Indeed, phenolic components have been identified in this fraction (Högl & Mosimann, 1958) and these phenolics were also found to be emetic (Malorny *et al.*, 1967). In tests on the effects of steamed coffee on gastro-enterological patients it was found that steamed coffee provoked less complaints than regular coffee in liver and gall bladder patients (Sziegoleit *et al.*, 1972*a*,*b*). In this respect it is interesting to know that feroylquinic acid has been indicated as one of the cholinergic factors of the brew (Czok, 1977). It was recently reported that a coffee subjected to a steam–hot air treatment showed reduced changes of the gastric juice pH (Finterlman *et al.*, 1978).

SUGGESTIONS FOR WORKING MODELS

Summarising the discussed chemical differences due to the treatments and the physiological effects of the treated coffees, it can be stated that the physiologically

active components of the brew, other than caffeine, are still not fully chemically identified. Nevertheless, it could be interesting to consider some working models on the basis of the known facts. These models are, however, still very speculative.

Both dewaxing and steaming were found to contribute to a reduced phenolics content in the coffee brew, dewaxing via phenolic roast products of C-5-HT and steaming via the removal of 3-methoxy-4-hydroxystyrene. Such a reduction of phenolics—and thus of ether-extractables—could positively contribute to the digestibility of the coffee via a reduction of the emetic activity of the brew. But chemical elucidation of the ether-extractables and identification of the main emetic factor is still needed.

Another interesting combination of results is:

(a) The decomposition of feroylquinic acid during steaming or desolvetising and the removal of the decomposition product 3-methoxy-4-hydroxystyrene (Windeman, 1974; Gal *et al.*, 1976).
(b) The finding that feroylquinic acid is one of the cholinergic factors of the brew (Czok, 1977).
(c) The reduced frequency of complaints after consumption of steamed coffee by a group of liver and gall bladder patients (Sziegoliet *et al.*, 1972*a*,*b*).

As feroylquinic acid has cholinergic activity it is logical that decomposition of this compound by steaming reduces the frequency of complaints from liver and gall bladder patients. At least for this type of indigestibility this could be a plausible explanation.

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RECENT DEVELOPMENTS IN THE MANUFACTURE OF INSTANT COFFEE AND COFFEE SUBSTITUTES

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(Received: 16 May, 1978)

ABSTRACT

This paper describes modern production methods resulting from the developments that have been taking place recently in the field of instant coffee and coffee substitutes. Such developments involve new processing techniques to achieve higher extraction yields and improve extract concentration, and more efficient spray drying. A tabulated comparison of operating conditions when spray drying chicory, pure coffee and chicory/coffee mixtures is given.

INTRODUCTION

Modern instant coffee production is well known and well recorded. Cleaning, roasting and grinding of coffee beans is followed by a split extraction and concentration to achieve a high solids concentrate for a low energy spray drying operation. Using two-stage continuous extraction techniques, yields of up to 60% are obtained (Kjaergaard, 1971). Developments in this field involve improved extraction and more economical spray drying techniques.

In spite of the optimisation of coffee processing, it is no secret that instant coffee today resembles less and less the original, home-brewed percolated coffee. This is a result of the higher extract yields presently achieved during industrial processing and the more widespread use of the high yield Robusta coffee variety which, unfortunately from a taste point of view, is not the best. Such trends, combined with the increased prices of green coffee, have led to an increasing world demand for instant coffee substitutes, the manufacture and current developments of which are described in this paper in some detail.

A further area of increasing interest is in decaffeinated instant coffee due to its reported effect of not creating possible sleeplessness.

Fd. Chem. (4) (1979)—
Printed in Great Britain

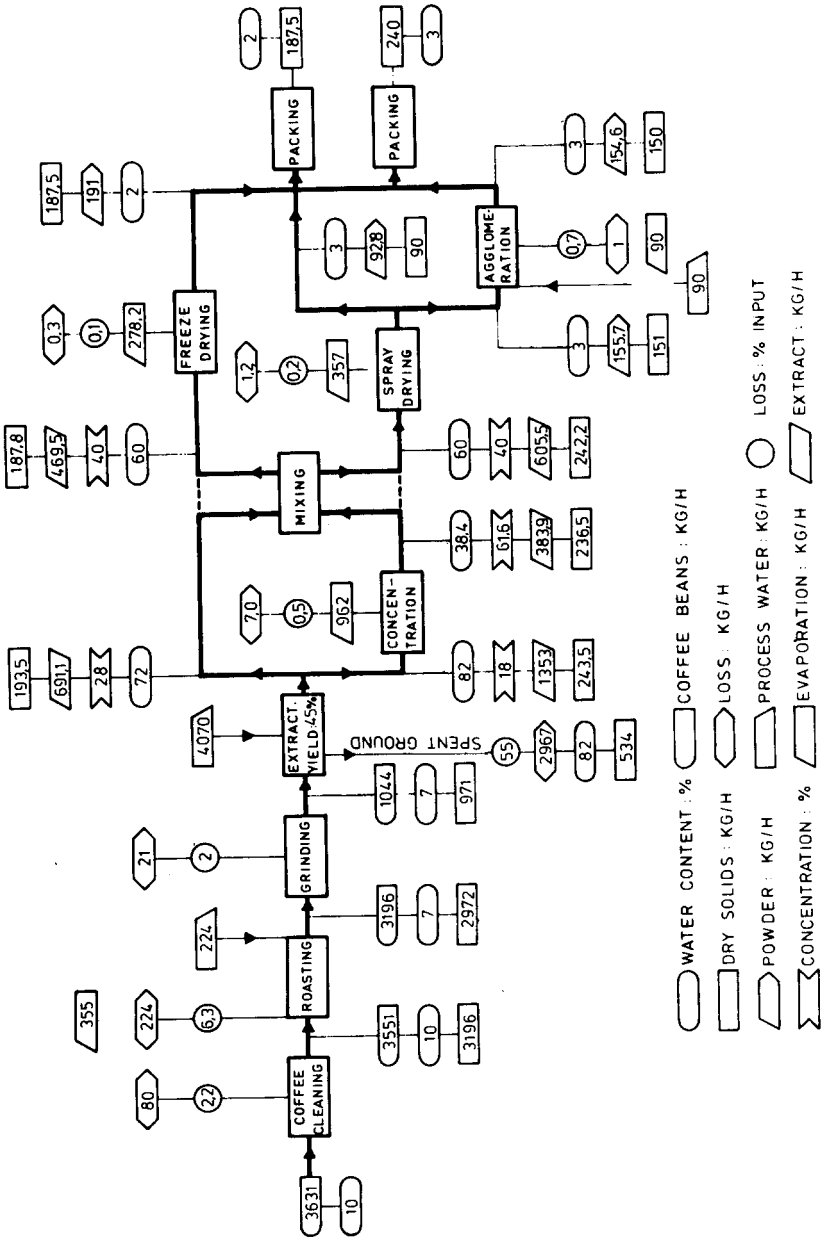

Fig. 1. Flow sheet for a modern instant coffee plant.

MODERN PRODUCTION OF INSTANT COFFEE POWDER

A flow sheet for a modern instant coffee plant is shown in Fig. 1 for a processing line of approximately 400 kg/h of instant coffee powder. Following the conventional coffee cleaning, roasting and grinding, the prepared coffee is extracted in a counter-current column extraction battery with split extraction, under strictly controlled conditions that enable yields of over 45 % to be achieved. The first extraction stage produces a prime quality extract (28 % solids) which is held separately in a concentrate tank pending final pretreatment and spray drying. The second stage achieves the high overall extract yields but the extract (hydrolysate) is of a lower quality (18 % solids) and is concentrated in a falling film or a rotary thin-film concentrator to over 60 % solids. This high concentrate is then mixed with prime quality extract from the first stage to give a 40 % solids concentrate mix for spray drying. Spray drying takes place in a co-current nozzle tower design. Inlet drying temperatures of 300 °C are used and, due to the high solids concentrate feed, drying is achieved in an economic, low energy consumption operation. A specific heat

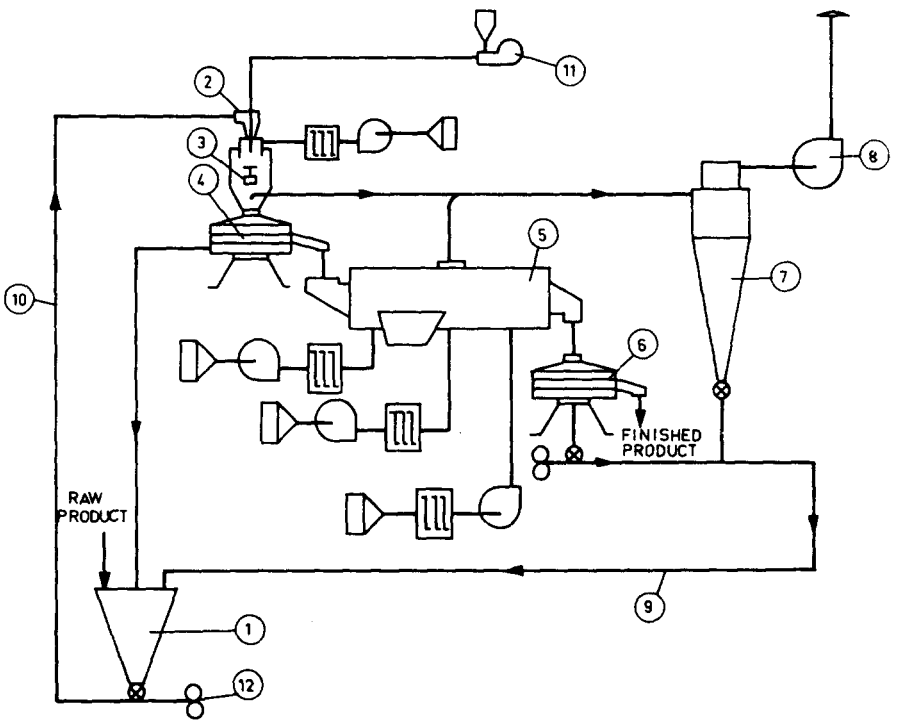

Fig. 2. Rotating disc agglomerator. 1. Feed hopper. 2. Powder feeder. 3. Rotating disc. 4. Coarse sieve. 5. Vibro fluidiser. 6. Sieve. 7. Cyclone. 8. Fan. 9. Pressure conveying system for fines. 10. Pressure conveying power feeding system. 11. Liquid feed pump. 12. Blower.

consumption in the range of 2800 kcal/kg of dried powder produced is possible with present-day spray drier designs.

Powder leaving the spray drier can be packed directly in bulk or agglomerated for packing in tins or jars as a prime consumer instant coffee. Much development has taken place in coffee agglomeration and today's established technique involves agglomeration on a rotating disc and after-drying in a vibrated fluid bed. This modern agglomerator has recently been described by Sivetz (1976). The flow sheet is shown in Fig. 2. The agglomerator features a fast rotating disc on which coffee powder falls after being moistened. The flow and distribution of powder feed plus water and steam are accurately controlled for optimum moistening. In addition, the quantity and temperature of warm air flowing through the agglomeration chamber provides an environment conducive to the agglomeration process.

Powder is superficially moistened both by water from a spray nozzle and by steam. Agglomeration takes place due to the collision of moistened particles on the rotating disc. The agglomerates are ejected from the disc edge by centrifugal forces. The agglomerates pass to a vibrated fluid bed which acts as a combined drier and product cooler.

The principle of the rotating disc has proved very successful for agglomerating instant coffee and several plants have, in the past few years, been put into full scale operation in South Africa, the Philippines, Belgium, Germany, Switzerland and Spain. Important advantages of this type of agglomerator feature particle size which can be effectively controlled to produce a uniform, attractive reddish-brown particle, closely resembling freeze-dried or even-ground coffee granules. A processing advantage is that there is minimum powder recycling and thermal treatment of the powder is virtually undetectable by taste.

MODERN PRODUCTION OF COFFEE SUBSTITUTES

For several generations in mid and southern Europe there has been a strong market for coffee substitutes based upon mixtures of coffee, chicory, barley, rye, wheat and sugar beets. However, today's interest is no longer solely restricted to those areas. There is an increasing world demand for instant coffee substitute based upon the above raw materials which are much cheaper than coffee. This increase in world interest is due to several factors including, as already mentioned, the sharp increase in coffee prices on the world market, increased use of less suitable coffee varieties in instant coffee manufacture and the economic necessity of higher processing yields achieved at the expense of high quality. An additional, indirect factor is possibly the so-called health promoting component 'inulin' found in chicory. Whatever the reasons, producing an instant beverage based upon a mixture of pure coffee and coffee substitute or pure coffee substitute makes sound economic sense. All products

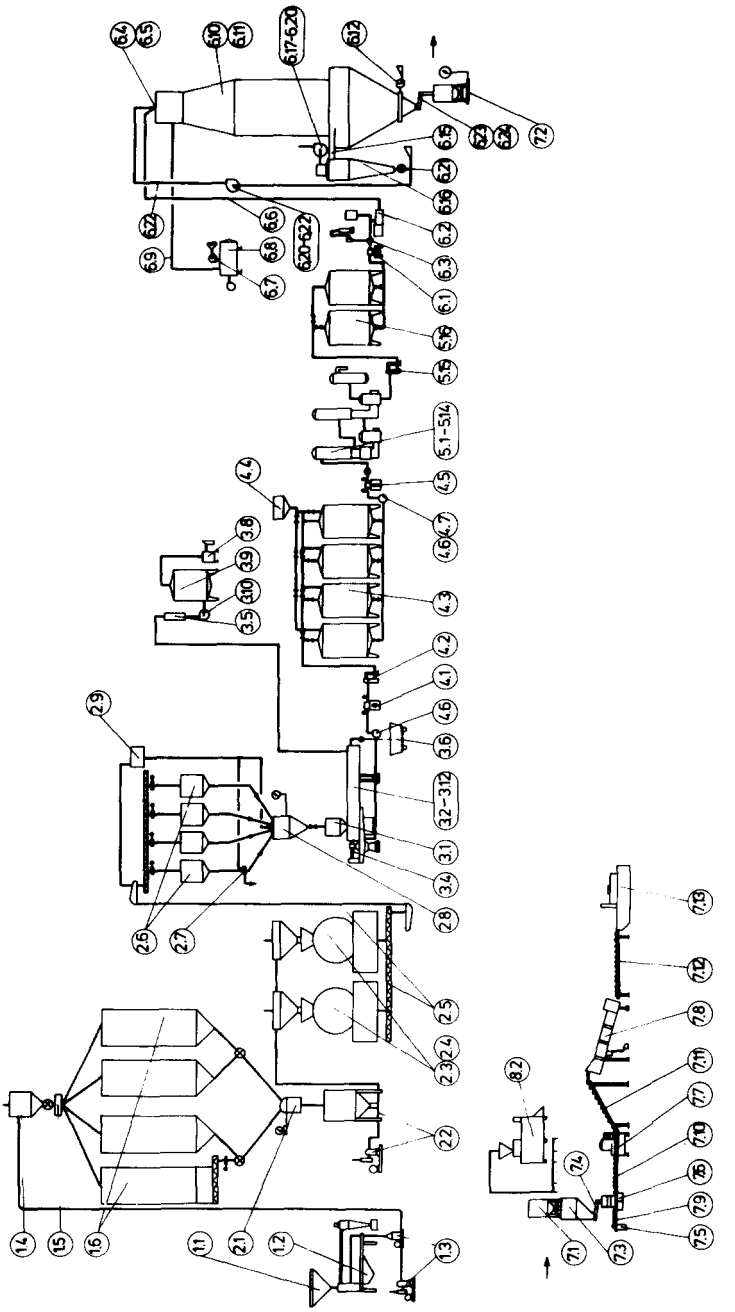

Fig. 3. Flow sheet for a modern coffee substitute plant.

used in coffee substitutes are easier to extract than pure coffee. Even at 100 °C, yields for most cereals are 40–50 %, based upon roast raw material weight. Chicory gives an overall 60–70 % yield. The extraction of pure roasted chicory can take place in a continuous extractor (Stoltze *et al.*, 1973) operating under atmospheric pressure. For mixtures of coffee, corn and chicory, a continuous pressurised extractor is preferred. Another alternative is to use a batch extractor for coffee and a continuous extractor for chicory. This type of set up gives the greatest plant flexibility.

Roasted chicory contains up to 70 % carbohydrates and thus the instant powder is considerably more hygroscopic and thermoplastic than instant coffee. This means that spray drying of chicory is more difficult than spray drying of coffee and special drier designs and drying conditions are essential for continuous operation. A flow sheet of a modern coffee substitute plant for the manufacture of instant powder is shown in Fig. 3.

Storage and cleaning of the raw material

The raw material is supplied in bags or in bulk and the grain, preferably graded in uniform sizes, is discharged directly into the discharge system (1.1) built into the floor above the cleaning machine (1.2).

The cereals are fed to the grain cleaner (1.2). After cleaning, the raw material is pneumatically conveyed directly into the silos for batchwise discharge to the roasters.

Roasting

The individual ingredients are roasted separately. Charging of the roaster (2.3) takes place by means of the weighing equipment (2.1). The roasting time and temperature are manually controlled during the roasting period which varies from 1 to $1\frac{1}{2}$ h, depending on the type of raw material. The combustion products and gases from the roasting are cleaned in the chaff-separating centriclone and burning cyclone.

After cooling, the roasted material is gently transported by means of screws and a cup conveyor (2.5) towards the storage silos (2.6). The roasted cereals are now ready for extraction while the roasted chicory passes a sifter (2.7) for separation of the fines prior to the extraction.

The various ingredients for the extraction are fed by gravity into the entirely automatic scale (2.8) according to the pre-set composition. For separation of dust developing during the treatment of the roasted material, a central de-dusting plant (2.9) reduces the development of dust around the various discharge points.

Extraction

A fixed charge of the roasted cereals, sugar beets and chicory is batchwise supplied to the feeding device (3.1) of the continuous extraction unit ((3.2) to (3.12)). The supply of material takes place automatically in accordance with the setting of the

continuous extractor capacity. The softened feed water from the ion exchanger (3.8) is supplied to a buffer tank (3.9) from where the feed water is pumped into the extraction trough by means of the pump (3.10). The continuous plugflow extraction takes place under atmospheric pressure.

The spent material is automatically discharged into a container mounted on wheels. The material can thus be dumped directly into a lorry for further transportation.

The extract leaves the extraction unit continuously through an automatically controlled discharge pump.

Extract treatment

The relatively concentrated extract leaving the continuous extraction unit with a concentration of approximately 18 % is filtered in one of the two filters (4.1) and led through a plate cooler (4.2).

The extract is collected in one of the four calibrated and well insulated storage tanks (4.3). The pH value of the extract is controlled and adjusted with a range of 4·6 to 5·2 by adding a buffering agent, e.g. sodium carbonate or similar from the mixing tank (4.4). Now the enzymic treatment (Pazola *et al.*, 1974) can take place under slow agitation by adding the enzyme directly into the storage tank. The duration of the enzyme process is approximately 4 h during which the viscosity changes from an initial value of 8 cP to 2 cP.

The extract is prepared for further concentration in a vacuum evaporator. The evaporator ((5.1) to (5.14)) is a two-stage vacuum falling film evaporator, especially designed for the food industry. The extract, which has a concentration of 45 %, is cooled in a plate cooler (5.15) and collected in one of the two storage tanks (5.16).

Spray drying

From the extract storage tank, the concentrate comes through a filter (6.1) to the high pressure feed pump (6.2) which feeds the pressure nozzles (6.4) situated on top of the drying chamber ((6.10) and (6.11)).

In the feed pipe, a special in-line sparging system (6.3) provides the possibility of adjusting the bulk density and colour of the final instant soluble beverage.

The spray drier is a co-current drier, especially designed for the production of instant coffee-like powders. The drying air is heated in an indirect gas-fired air heater (6.8). A direct fired heater is an alternative. By means of an air filter and a fan (6.7) the drying air is forced through a heat exchanger to the drying chamber (6.10). The combustion gases are thus not in contact with the drying air. The combustion gases leave the air heater through a flue gas stack.

The powder leaves the drying chamber after having passed the cooling zone (6.12) and a vibration sifter (6.23) at the bottom of the conical chamber. The moisture content of the powder is 2–3 %.

The drying air is cleaned through a set of cyclones (6.16) eventually leaving the

drier through the exhaust fan (6.17) to (6.19). The fine powder is separated in the cyclone and is fed back by the conveying system ((6.20 to (6.22)) into the wet zone at the spray nozzle where an agglomeration of the fine particles takes place.

When processing pure chicory extract special care has to be taken in order to handle the very thermoplastic and hygroscopic powder. In order to have uniform production conditions it is normal to have a dehumidifying unit installed prior to the air heater for conditioning the drying air to a fixed humidity level. This unit operates throughout the year and plant operation is then not influenced by seasonal atmospheric humidity changes.

The drying takes place under lower temperatures than for other coffee substitutes (for example, chicory/coffee mixtures, see Table 1).

TABLE 1

TYPICAL OPERATING CONDITIONS FOR SPRAY DRYING CHICORY, COFFEE AND CHICORY/COFFEE MIXTURES

Basis: Drying air
Ambient 10 °C 760 mm Hg
Indirect air heating

	Pure chicory	*Pure coffee*	*Chicory/coffee mixture* (40/60)
Feed solids (%)	26	36–40	33–36
Powder residual moisture (% H_2O)	~2	~3	~2·5
Inlet temperature (°C)	215	300	240
Outlet temperature (°C)	~120	~115	~115
Powder output	~305	~1265	~625
Atomisation	----------	Nozzle ----------	
		(40–60 kP/cm²)	
Specific heat consumption (approx.) kcal/kg powder produced	7400	2800	3800

Besides the above-mentioned conditioning of the drying air, special care must be taken regarding the cooling of the powder where a large amount of cooling air with an extremely low relative humidity is required.

Packing

The powder discharged from the drier is filled into bulk containers (7.1) situated on a floor-mounted scale (7.2) (packing arrangement). The filled container acts as a buffer storage for the two types of packing line where tins from 50 to 250 g can be packed.

Because of the hygroscopic character of the instant product and in view of the delicate packing machinery, it is necessary that this arrangement works in a conditioned room with a relative humidity not exceeding 45 % at 20 °C. The powder can, however, be kept in an unconditioned room in the hermetically closed containers for an unlimited period of time.

When the powder is going to be packed the container is connected to a discharge tube. A butterfly valve, permanently fixed on the container, is opened. The powder flows into an intermediate silo (7.3) just above the packing machine.

The filling machine is supplied with empty tins from a combined unscrambler and a dust cleaning unit (7.5) in which possible dust is removed by means of compressed air.

Filled tins leave the volumetric filling machine with the desired quantity of instant soluble powder and are conveyed into the automatic tin closing machine (7.7). The closed tins are transferred by means of a conveyor belt out of the packing room on to a case packer (7.8) from whence the carton is rolled into a batch closing machine (7.13) in which the filled cases are sealed by means of a one-sided gum tape.

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CHANGES IN CARBOHYDRATES DURING THE PRODUCTION OF COFFEE SUBSTITUTE EXTRACTS, ESPECIALLY IN THE ROASTING PROCESS

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(Received: 16 May, 1978)

ABSTRACT

The changes in the content of starch, dextrin and reducing sugars in rye and barley, and inulin and fructose in chicory roots, during the roasting process, were investigated. The analysis of carbohydrates was connected with the estimation of changes in extract content, colour of the roasted materials, pH and the sensoric evaluation of the brew. The changes in the content of the respective carbohydrates in the basic stages of the whole process of production of coffee substitute extract were also investigated.

In the case of roasted rye and barley the high quality of the brew was connected with the highest content of dextrin and reducing sugars. In the case of roasted chicory the highest fructose content (~13%) *and the relation between inulin and fructose may be recognised as parameters for 'optimum' quality.*

As a result of the specific technology of the enzymatic treatment of the extract, a very characteristic carbohydrate composition in the final product, with a high content of dextrins (above 30%) *and of reducing sugars, could be observed.*

INTRODUCTION

The production of soluble cereal coffee on a large scale was developed in Europe much later than the extract production of natural coffee, namely after 1955. This was true especially of those countries where the consumption of cereal coffee was already a tradition, for example, in the German Federal Republic the production of soluble cereal coffee increased more than ninefold in the years 1956–64 but later, due to the lower prices of natural coffee, this tendency towards growth was stopped (Beitter &

Fd. Chem. (4) (1979)—
Printed in Great Britain

Schroeder, 1970). The present situation of high natural coffee prices may bring new trends to the market.

Cereal coffee extract, introduced into Poland at the beginning of the 1970s, was a real 'hit' on the food market. It began to supplant the traditional cereal grain coffee and in the following years there was an enormous growth in the production capacity of extracts (Pazola *et al.*, 1968, 1971). The soluble cereal coffee achieved a great success due to its very pleasant taste and high instant properties (Pazola *et al.*, 1971; Pazola & Pordab, 1975*a*,*b*; Masters & Stoltze, 1974).

The applied process consists of the following basic stages:

(1) Pre-treatment of the raw material.
(2) Roasting.
(3) Extraction.
(4) Enzymic treatment and concentration.
(5) Spray drying.
(6) Packaging.

During the production extension of soluble cereal coffee it was found necessary to modify the roasting process and there was a conversion from the roasting of watersoaked cereal grains to roasting of dry grains in modern contact convection roasters. The aim was the application of a short (rapid) roasting process and to obtain roasted cereal grains with a required quality (taste, flavour, acidity) which would facilitate the extraction. The basic raw materials were roasted rye and barley grains and chicory roots (Pazola *et al.*, 1971; Pazola & Pordab, 1975*a*). The object of this research was to investigate the changes in the content of individual carbohydrate fractions during the roasting of rye and barley and the changes of inulin and fructose during the roasting of chicory roots. The analyses of carbohydrates were connected with the estimation of changes in extract content, colour of the roasted materials, pH of the extract and sensoric evaluation of the brew.

Similar investigations were also carried out in the basic stages of the whole process of cereal coffee extract production.

METHODS

The experimental procedures

Roasting: With a laboratory roaster, Probat type LP-5 with a loading capacity of 4 kg of grain or 5 kg of chicory roots. In the case of cereal grains 0·5 kg of water was added at the end of the roasting process. In the case of chicory 50 ml of vegetable oil was added at the beginning of the roasting.

Extraction: In a pilot-plant extraction battery (NIRO Atomizer) consisting of six

extractors with a volume of 8·5 litres. The load of one extractor was 1·6 kg of roasted material. In one experiment 96 kg of roasted material (60 extractors) were used.

Evaporation: With a falling film vacuum evaporator, type Luva, with an evaporating capacity of 50 kg water an hour.

Spray drying: With a NIRO Atomizer spray drier type Production Minor, nozzle atomisation/pressure nozzle: inlet air temperature—210–230 °C; outlet air temperature—105–110 °C. Chicory extract was atomised by a rotating disc; inlet air temperature—150–165 °C, outlet air temperature—75–77 °C.

Analysis

The contents of the following carbohydrates were estimated: reducing sugars (before and after inversion)—calculated as glucose, reducing sugars calculated as fructose and inulin, 'dextrins' and starch.

Further analysis: Extract content, pH, colour in reflected light (Universal–Messeinkeit UMB 3), colour of the brew in UV (286 nm) and sensoric evaluation.

RESULTS AND DISCUSSION

Comparison between soaked and dry grains

Beitter & Schroeder (1970) have already noted that in the case of the roasting of dry cereal grains, their extract content was markedly higher. However, the problem is whether the grains roasted in the dry state have a suitable structure (porous fracture) and whether they give a brew with a high quality of taste and flavour. The results are shown in Table 1.

In the roasting of dry grains the process was shorter (*ca.* 40 %). A lower loss of dry

TABLE 1
A COMPARISON OF ROASTED MATERIALS FROM SOAKED AND DRY CEREAL GRAIN

	Roasted rye		*Roasted barley*	
	Soaked	*Dry*	*Soaked*	*Dry*
Roasting time (min)	95	55	100	60
Loss of dry substance (%)	11·4	8·0	9·4	8·4
Extract content (%/dry substance)	42·1	53·8	58·0	67·9
pH of the brew	5·1	5·0	5·0	4·8
Sugars, calculated as glucose (%)				
total, after hydrolysis	77·5	77·4	72·8	67·5
reducing	1·4	1·3	1·5	2·1
reducing (after inversion)	2·6	2·8	2·6	4·5
Dextrin	20·5	27·5	26·0	42·5
Starch	40·0	36·7	24·3	15·2
Sensoric evaluation (taste and flavour) on a five-point scale	4·01	4·33	4·21	4·50

substance was obtained and the extract content was markedly increased. The distinct differences are in the changes of polysaccharides: in roasted material from dry grains the 'dextrin content' considerably increased, especially in the case of barley and similarly the starch content is lower—due to a faster dextrinisation process. The pH value of the brew is slightly lower and the sensoric evaluation score is higher. The roasted material from soaked grains had a glassy fracture. The roasted dry grains had a porous and uniform coloured fracture; the degree of expansion was *ca.* 26% for rye and *ca.* 24% for barley.

Roasting of rye grains

The changes of carbohydrates and some quality parameters during the roasting process were investigated. The grains were loaded to a heated roaster, maximum temperature being 200–220 °C. The changes in the most important parameters are shown in Fig. 1. The scales are adjusted to individual parameters, shown on the left and right of the figure.

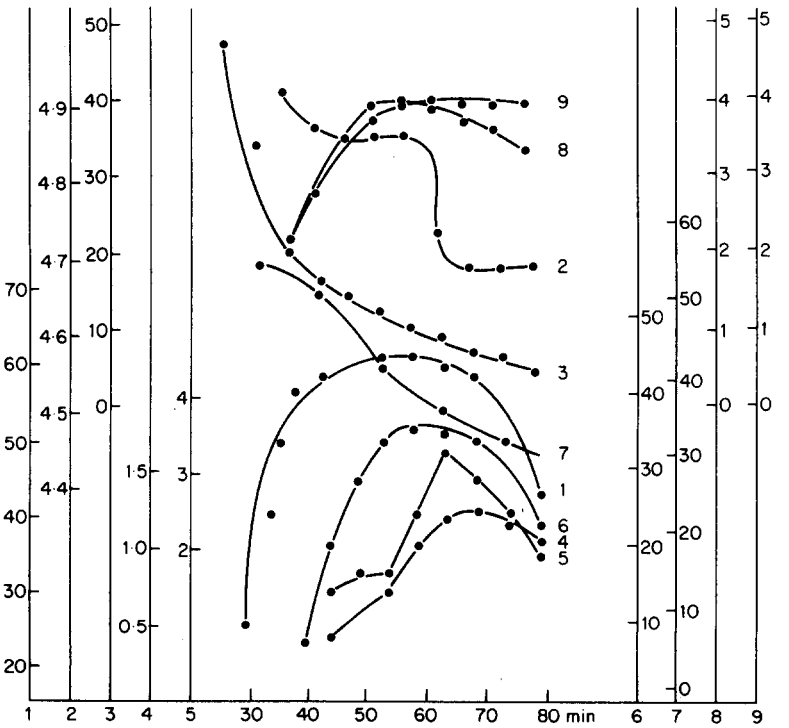

Fig. 1. Physical, chemical and sensoric parameters during the roasting of rye grains. 1. Extract content, % dry weight. 2. pH of the extract. 3. Colour in reflected light, %. 4. Reducing sugars, % dry weight. 5. Reducing sugars, after inversion, % dry weight. 6. Dextrins, % dry weight. 7. Starch, % dry weight. 8. Sensoric evaluation. 9. Colour of the brew, in five-point score.

The extract content (curve 1) reaches a maximum value in the roasting time range 35–70 min. The savouriness—taste and flavour taken together—is the most favourable in the range 55–65 min (curve 8). With the lowering of savouriness, a reduction in the pH value of the brew (curve 2) may be observed. In the 'optimum' range of roasting time for the rye grains the maximum contents of reducing sugars (curves 4 and 5) and dextrins (curve 6) appear.

These results were obtained during one continuous roasting cycle in which the samples were taken from the roaster at appropriate times. Next, batches were roasted for three different periods, near to the 'optimum' roasting time.

The following products were obtained:

	Roasting grade		
	Light	*Medium*	*Dark*
Time of roasting (min)	45–50	55	65–70
Total weight loss (%)	17	19	21
Loss of dry substances (%)	7·5	8·2	11
Colour in reflected light (%)	17	14	10

The values of the individual physical and chemical characteristics of the roasted materials are given in Table 2. All three roasted grain products indicated different

TABLE 2
THE EFFECT OF ROASTING GRADES ON RYE

	'Light roast'	*'Medium roast'*	*'Dark roast'*
Extract content (%)	62·1	61·5	61·0
pH of the brew	5·0	5·0	4·9
Colour, in reflected light (%)	17·0	14·0	10·0
Reducing sugars (%)	0·93	1·48	1·29
As above, after inversion (%)	2·16	3·16	3·2
Dextrin (%)	33·2	36·4	36·7
Starch (%)	40·3	35·9	33·1
Sensoric evaluation of the brew			
taste	4·0	4·28	3·76
flavour	3·52	4·0	3·37

sensoric scores, that may be used for the development of differentiated (in taste and flavour) cereal coffee extracts.

Roasting of barley grains

Similar experiments were carried out during the roasting of barley grains. The set of plotted curves is shown in Fig. 2. The dextrinisation and further decomposition of barley starch is more rapid than that of rye grains. The maximum amount of dextrin is *c.* 50%. The highest values of extract content (curve 1) appear in 35–60 min of

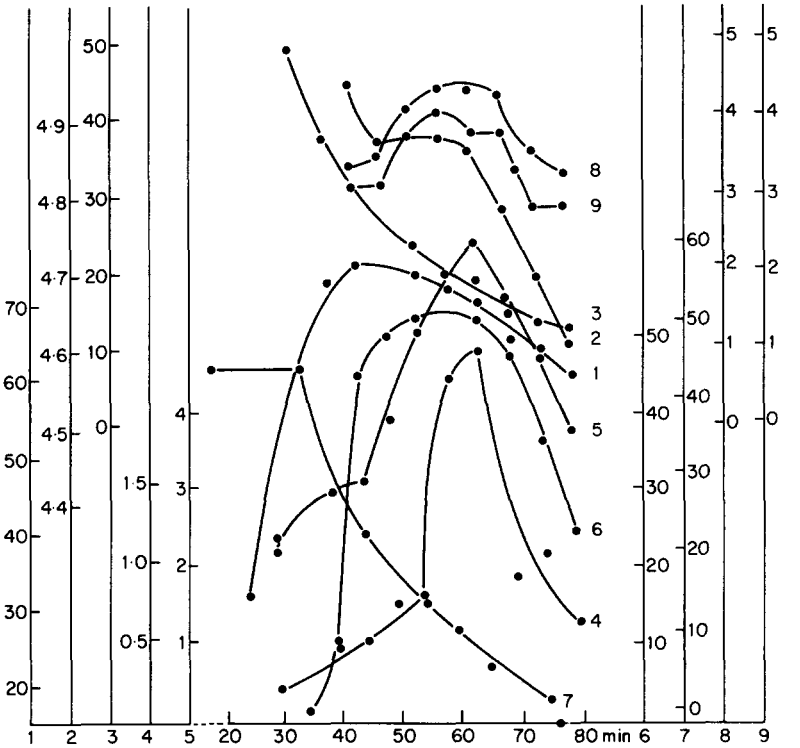

Fig. 2. Physical, chemical and sensoric parameters during the roasting of barley grains. 1. Extract content, % dry weight. 2. pH of the extract. 3. Colour in reflected light, %. 4. Reducing sugars, % dry weight. 5. Reducing sugars, after inversion, % dry weight. 6. Dextrins, % dry weight. 7. Starch, % dry weight. 8. Sensoric evaluation. 9. Colour of the brew, in five-point score.

roasting but the best quality parameters (the taste and colour of the brew—curves 8 and 9) are in the range 60–70 min. In addition, a steady pH value of the brew may be observed in this range. Maximum contents of reducing sugars (curves 4 and 5) occur in the range 60–65 min. The time range is similar for the highest values of dextrin. During the prolonged roasting a reduction in the contents of starch, dextrin and reducing sugars may be observed and the taste of the brew deteriorates. Three batches of barley grain for different roasting grades were prepared:

	Roasting grade		
	Light	*Medium*	*Dark*
Roasting time (min)	50	60	70
Total weight loss (%)	18	19	20
Loss of dry substance (%)	8·2	9·6	10·7
Colour in reflected light (%)	16	10	8

TABLE 3
THE EFFECT OF ROASTING GRADES ON BARLEY

	'Light roast'	*'Medium roast'*	*'Dark roast'*
Extract content (%)	75·1	67·5	66·1
pH of the brew	4·5	4·4	4·4
Colour in reflected light (%)	16·0	10·0	8·0
Reducing sugars (%)	1·56	1·80	4·17
Reducing sugars, after inversion (%)	3·73	4·17	5·19
Dextrin (%)	50·2	52·6	45·9
Starch (%)	17·3	10·0	7·7
Sensoric evaluation of the brew			
taste	4·08	4·46	4·16
flavour	4·0	4·3	4·3

The respective data for the estimated parameters of the above-mentioned roasted materials are given in Table 3.

Roasting of chicory roots

In this case the estimation of inulin—and fructose—changes was introduced. Maximum roasting temperature did not exceed 170 °C.

The values for the major parameters (the roasting time, range 30–75 min) are shown in Fig. 3.

The characteristic progressive decrease in extract content (curve 1) and in pH value is observed, and the same applies to the inulin content (curve 5).

The content of fructose (curve 4) increases from the start of the investigated roasting time and reaches its maximum in the range 55–65 min, in a further phase of the process an intense reduction of this component takes place.

The optimum sensoric evaluation was achieved in the range 55–60 min.

Three separate batches were prepared in different roasting times:

	Roasting grade		
	Light	*Medium*	*Dark*
Roasting time (min)	40–45	50	60
Total weight loss (%)	14	16·3	18·1
Loss of dry substances (%)	6·3	7·0	8·1
Colour in reflected light (%)	12	9	7

The results obtained for individual parameters are specified in Table 4. The highest content of fructose (*ca.* 13%), the relationship between inulin and fructose (2·5:1) and the pH value of the brew (about 4·3–4·4) may be recognised as parameters for the 'optimum' quality of roasted chicory. The exceeding of the 'optimum roasting time' results in a decline in both the inulin and fructose contents and a further lowering of pH below 4.

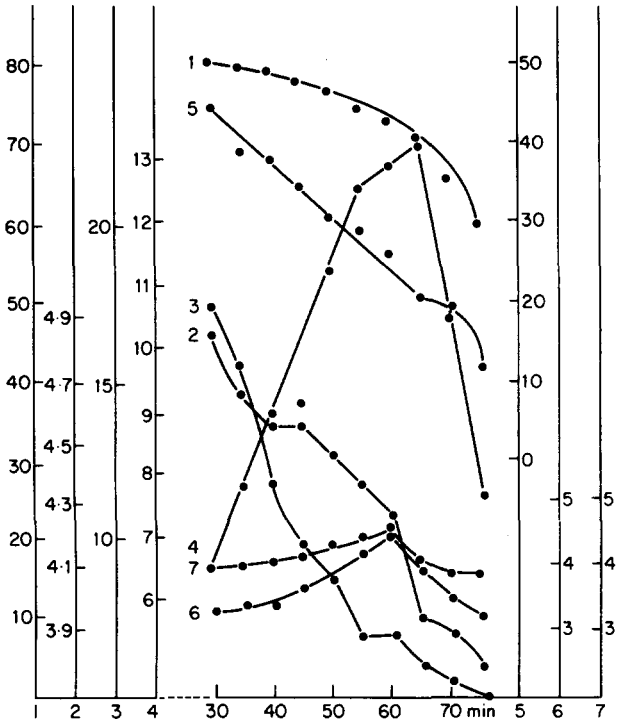

Fig. 3. Physical, chemical and sensoric parameters during the roasting of chicory roots. 1. Extract content, % dry weight. 2. pH of the extract. 3. Colour in reflected light, % dry weight. 4. Fructose, % dry weight. 5. Inulin, % dry weight. 6. Sensoric evaluation. 7. Colour of the brew, in five-point score.

TABLE 4
THE EFFECT OF ROASTING GRADES ON CHICORY ROOTS

	'Light roast'	*'Medium roast'*	*'Dark roast'*
Extract content (%)	83·9	80·5	76·3
pH of the brew	4·5	4·4	4·3
Colour in reflected light (%)	12·0	9·0	7·0
Reducing sugars, after inversion	58·2	52·3	44·4
Fructose	8·9	9·7	13·7
Inulin	44·4	38·4	29·0
Sensoric evaluation:			
taste	3·66	4·00	4·83
flavour	3·75	4·00	4·66

Changes in carbohydrates during the production of cereal coffee extracts

In a pilot-plant production, coffee substitute extracts were obtained from roasted rye, barley and chicory, as well as from a mixture of these components. The industrial roasted grains and chicory of a relatively dark roast were used as raw materials. The changes in formerly estimated carbohydrate groups (or inulin and

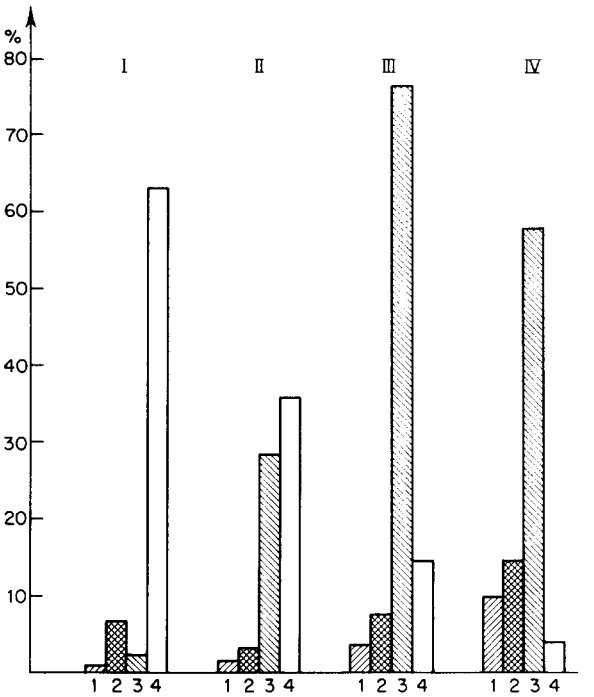

Fig. 4. Changes in carbohydrates in the production of roasted rye extract. I. Raw material. II. Roasted grains. III. Powdered extract without enzymic treatment. IV. Powdered extract with enzymic treatment. 1. Reducing sugars, % dry weight. 2. Reducing sugars, after inversion, % dry weight. 3. Dextrins, % dry weight. 4. Starch, % dry weight.

fructose in the case of chicory) were investigated. This is illustrated graphically in Fig. 4 for rye, Fig. 5 for barley, Fig. 6 for chicory and Fig. 7 for the mixture.

As may be quickly noticed, the highest starch and dextrin content is typical for the roasted rye extract. Especially interesting changes appear during the production of coffee substitute extract obtained from the raw materials mixture.

In this process the starch is decomposed step by step to dextrin, and then to reducing sugars which occur first in the roasting process and in later stages of the process (extraction, enzymic degradation, dehydration). The final product has a characteristically high dextrin content and about the same content of reducing

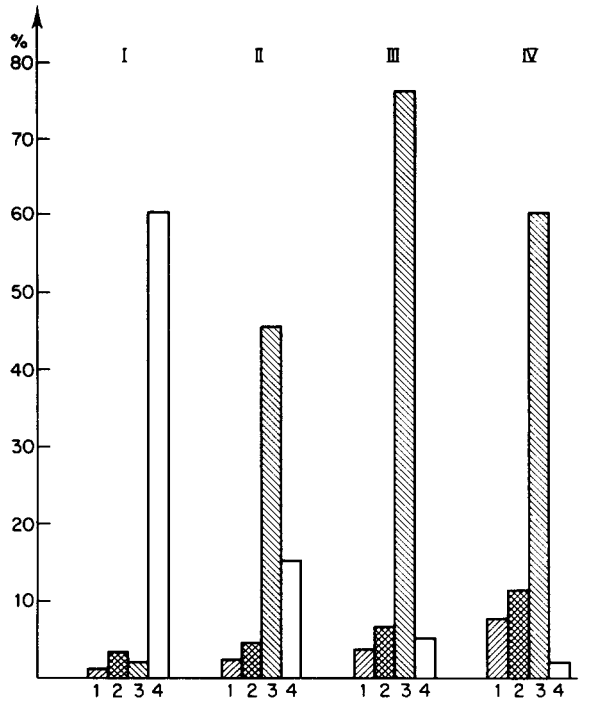

Fig. 5. Changes in carbohydrates in the production of roasted barley extract. I. Raw material. II. Roasted grains. III. Powdered extract without enzymic treatment. IV. Powdered extract with enzymic treatment. 1. Reducing sugars, % dry weight. 2. Reducing sugars, after inversion, % dry weight. 3. Dextrins, % dry weight. 4. Starch, % dry weight.

sugars (after inversion). Such a high content of dextrin compared with the reducing sugars cannot be detected in other foreign commercial products on the European food markets. The comparison of analyses shown in Table 5 illustrates the above statement.

Similar results were obtained by Beitter & Schroeder (1970) in the case of seven German products. In addition, the soluble cereal coffee obtained with this process has very good instant properties: quick penetrability (wettability) and solubility. However, these were the subject of another investigation.

CONCLUSIONS

During the production of soluble cereal coffee the changes in individual carbohydrate groups (or compounds) are very distinct and characteristic.

These changes may be taken into account as an additional test for the evaluation

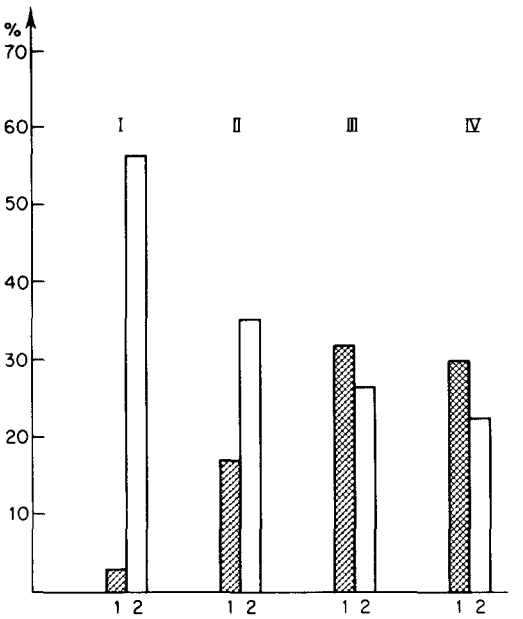

Fig. 6. Changes of inulin and fructose in the production of roasted chicory extract. I. Raw material. II. Roasted chicory. III. Extract. IV. Powdered extract. 1. Fructose, % dry weight. 2. Inulin, % dry weight.

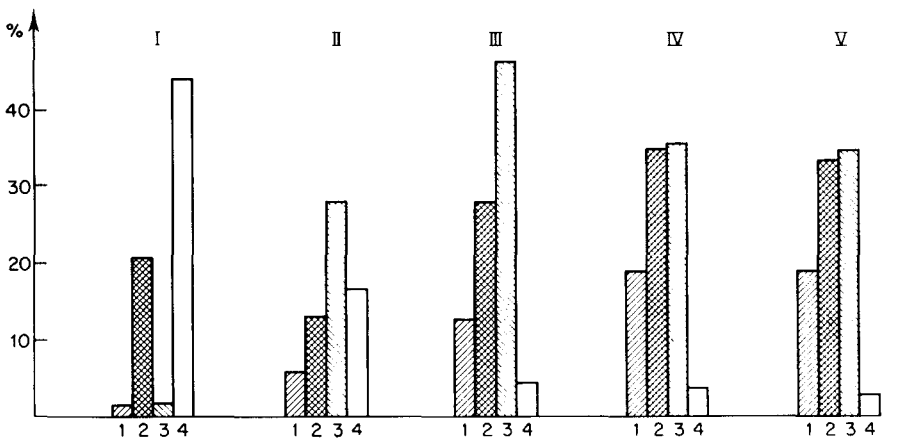

Fig. 7. Changes in carbohydrates in the production of coffee substitute extract. I. Raw material. II. Mixture of roasted material. III. Extract. IV. Extract after enzymic treatment. V. Powdered extract. 1. Reducing sugars, % dry weight. 2. Reducing sugars, after inversion, % dry weight. 3. Dextrins, % dry weight. 4. Starch, % dry weight.

TABLE 5
THE CARBOHYDRATE CONTENT IN VARIOUS COMMERCIAL COFFEE SUBSTITUTE EXTRACTS

Kind of product	*pH*	*Sugars, calculated as glucose (%)*			*Dextrin*
		Reducing	*Reducing after inversion*	*After hydrolysis*	
Product No. 1 (GFR)[a]	4·15	11·9	16·2	60·5	16·5
Product No. 2 (GFR)	4·22	14·6	19·4	66·0	18·1
Product No. 3 (Czekoslovakia)	4·40	14·8	29·8	62·7	11·1
Product No. 4 (Poland)	4·90	18·5	33·3	74·3	34·5

[a] German Federal Republic.

of the roasting process, the kind of technological parameters used and the quality of final product. In the roasting of cereal grains—rye and barley—the most characteristic changes were found in dextrin and reducing sugars content and, in the case of chicory, in the inulin and fructose contents and their relationship. The content of the various components mentioned is closely related to such parameters as extract content, colour of roasted material, sensoric evaluation and pH of the brew.

During the production of powdered extracts the enzymic treatment of an extract leads to further changes in carbohydrates which result in a final product with a very characteristic carbohydrate composition.

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DIFFUSION PHENOMENA DURING THE DECAFFEINATION OF COFFEE BEANS

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(Received: 16 May, 1978)

ABSTRACT

The coffee bean behaves with respect to the diffusion of caffeine almost as perfectly as a spherical model system. This behaviour is a consequence of at least two opposite effects—the taking up of solvent and the water loss. The overall change of the diffusion coefficient caused by these factors remains, however, small compared with the shifts brought about by the parameters investigated: water content and temperature. It is therefore reasonable to express the effects of these variables by using a single quantity, namely, the average apparent diffusion coefficient which, for Columbia and Robusta coffee and at optimal experimental conditions, lies in the range $0{\cdot}5\text{–}1{\cdot}3 \cdot 10^{-6}$ cm^2 sec^{-1}.

INTRODUCTION

The coffee story is a rather old story. Throughout the whole evolution of coffee technology the stimulant properties of the coffee beverage constituted the driving force. Many liked it very much, some hated and banned it and quite a few could not tolerate it very well because of the physiological side-effects of the caffeine.

This leads us to the starting point of the decaffeination business: at the beginning of this century, in 1905, the HAG Company took a German patent (No. 198 279) which turned out to be the foundation stone of a huge building. Thousands and thousands of tons of coffee have been decaffeinated since then. The technological procedure has been improved and improved, with the result that the consumer now gets a high quality caffeine-free coffee whose flavour can hardly be distinguished from an untreated coffee.

According to our knowledge the refinements of the decaffeination process are essentially based on a large technical experience and very few scientific approaches

Fd. Chem. (4) (1979)—
Printed in Great Britain

have been published up to now. At least no attempt has been made to formulate the diffusion of the caffeine in the beans using the mathematical tools of diffusion kinetics. In other words, our objective was to determine the diffusion coefficient of caffeine in the coffee beans during the extraction with chlorinated solvents as a function of coffee provenance, type of solvent, temperature and water content of the beans, respectively.

MATERIALS AND METHODS

It is well known that the decaffeination procedure basically consists of the following four operations:

(1) Swelling the beans.
(2) Extraction of the caffeine with an organic (mostly chlorinated) solvent.
(3) Driving off the solvent by steam to a tolerable level.
(4) Drying the beans.

We simulated this procedure on a laboratory scale. For practical reasons all extraction experiments were carried out at atmospheric pressure and we chose the degree of swelling at three levels (high, medium and low) and the extraction temperature at three levels (high, medium and low) as experimental variables. These variables were investigated according to a 3 × 3 factorial experimental plan with several replications.

The swelling of the beans was carried out at 90 °C and in such a way that the slowly added water was readily absorbed in order to avoid the extraction of water-soluble compounds from the beans and to guarantee a homogeneous swelling degree.

I would like now to give a somewhat detailed description of our caffeine extraction procedure and the analytical methods involved. Then we will have a look at the diffusion model system and the mathematical treatment of the experimental values.

The extraction apparatus (Fig. 1) consisted of a 1 litre cylindrical flask in which the temperature was kept constant by means of an ultrathermostat. The solvent, previously saturated with water, was kept flowing through a flow meter and through the flask. The mixture was constantly stirred during the extraction process.

The batch was initially made up of 500 ml of solvent and 300–370 g of swollen beans. The flow rate of the solvent was generally 2 litres an hour at which the maximum possible rate of extraction was maintained. In other words, the extraction was carried out at sink conditions.

Our main interest was to know what happened to the coffee beans during the extraction and particularly what time function followed the caffeine content of the beans. We soon realised that during the extraction process the beans were subject to a constant change in composition, not only as far as the caffeine content was concerned but also with regard to the water content and the solvent uptake. The

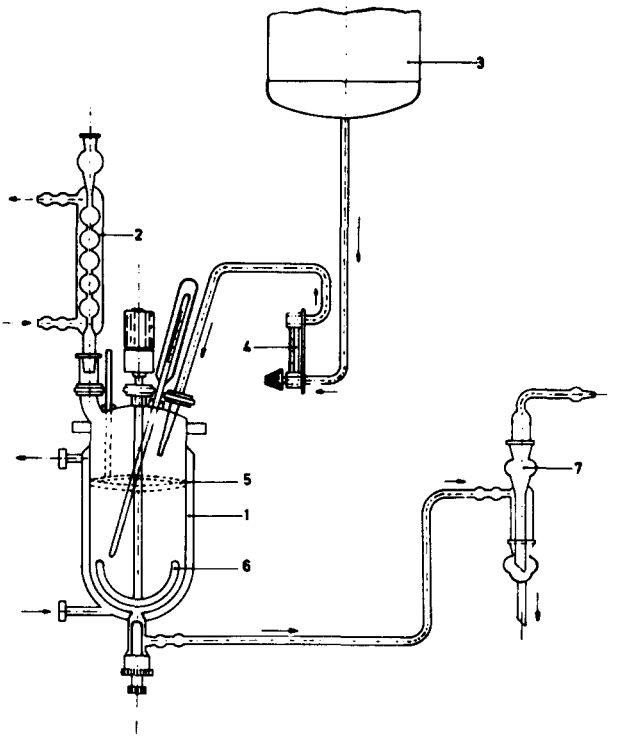

Fig. 1. Extraction apparatus: 1. Thermostatic reaction vessel. 2. Cooler. 3. Solvent reservoir. 4. Flow meter. 5. Ring. 6. Stirrer. 7. Niveau control.

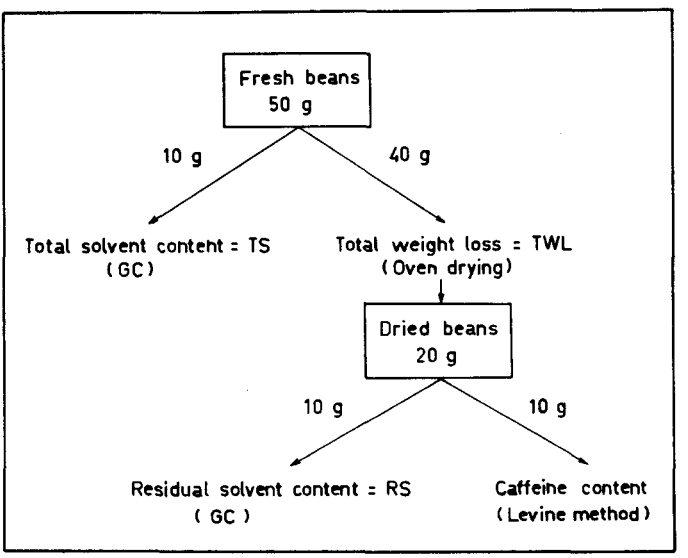

Fig. 2. Methods of analysis. Water content = TWL − (TS − RS).

analytical methods had therefore to be worked out with special care. Samples were withdrawn by opening the flask for 2–3 min and taking out the 50 g of beans necessary for the analysis which was carried out according to the scheme shown in Fig. 2.

The 50 g sample was divided into two parts: 10 g were used to determine the total solvent content (TS) by a gas chromatographic method and 40 g were dried overnight at 105 °C. As during oven drying, the beans lost the whole of their water content but only an unknown part of the solvent content; the dried, water-free beans had to be re-analysed for the residual solvent content (RS). The water content could then be calculated by this indirect method according to the equation:

$$\text{Water content} = \text{TWL} - (\text{TS} - \text{RS}) = \text{TWL} + \text{RS} - \text{TS}$$

One part of the dried beans was subject to the modified Levine method for the determination of the caffeine content.

The mathematical treatment of the results obtained by these techniques had to be based on a model system. The basic equation we used was Fick's second law of diffusion. Applied to a spherical diffusion space and with the assumptions that:

(1) the coffee beans are spheres
(2) the diffusion in the beans is radial, isotropic and independent of the concentration of the caffeine in the beans
(3) at the beginning of the experiment ($t = 0$) the caffeine is evenly distributed in the beans

the following equation gives the caffeine content of the beans as a function of time:

$$\frac{M_\infty - M_t}{M_\infty} = 1 - \frac{M_t}{M_\infty} = 6/\pi^2 \sum_{n=1}^{\infty} 1/n^2 \exp\left[-Dn^2\pi^2 t/r^2\right] \tag{1}$$

where: M_t = mass of the caffeine diffusion out of the beans at time, t; M_∞ = total mass of the diffusing species (= initial caffeine content of the beans); D = diffusion coefficient; r = radius of the bean-volume equivalent sphere (spherical diffusion space) and n = integer.

If we put $D/r^2 \equiv 1$ and $t \equiv x$, then the right-hand side of eqn. (1) becomes identical to the well known sigma-function except for the factor 3:

$$\sigma(x) = 2/\pi^2 \sum_{k=1}^{\infty} 1/k^2 \exp\left[-\pi^2 k^2 x^2\right] \tag{2}$$

If $3 \cdot \sigma(x)$ is plotted against $\log x$ one obtains an S-shaped curve.

In Fig. 3 the experimental values of $1 - M_t/M_\infty$ are plotted against $\log t$. This also results in a sigmoidal curve which, however, is shifted parallel to the $3\sigma(x)$ curve by the amount of $d = \log(D/r^2)$.

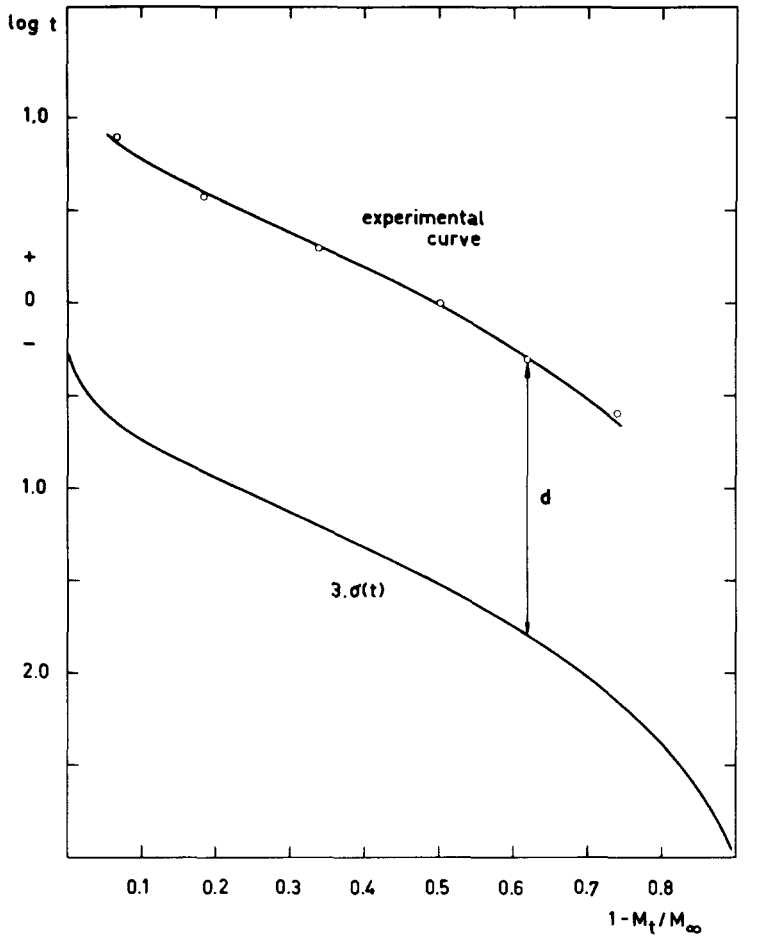

Fig. 3. Sigmoidal graph of the caffeine content.

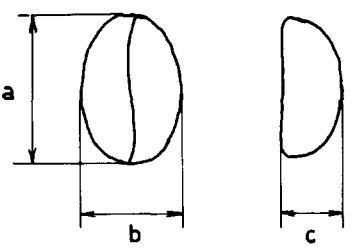

Fig. 4. Geometrical form of coffee beans.

The diffusion coefficient can be calculated by the equation:

$$D = r^2 10^{-d} \tag{3}$$

d is obtained simply by measuring the distance between the sigma function and the experimental curve on the $1 - (M_t/M_\infty)/\log t$ plot.

Now, what about r?

We based our mathematical treatment on a spherical diffusion model and r is simply the radius of the sphere. This sounds very simple but, in fact, it is quite complicated because of the geometrical form of the coffee bean which is everything except a sphere.

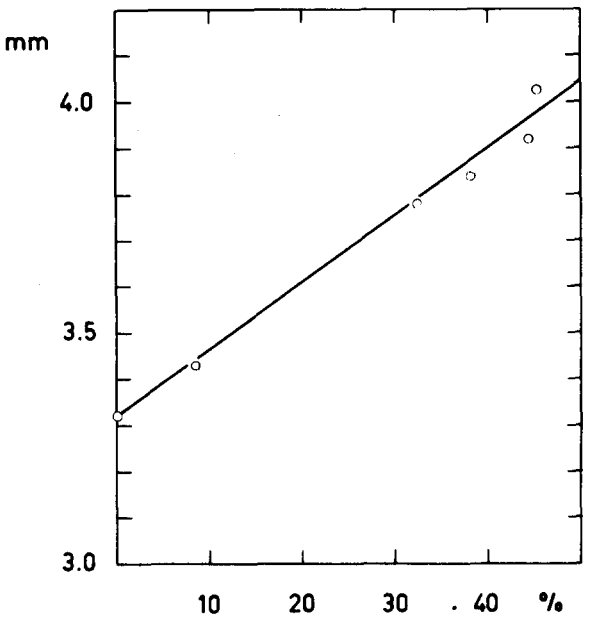

Fig. 5. Sphere radius as function of water content.

We had to make a rough approach by considering the bean as one half of an ellipsoid having the half axes $a/2$, $b/2$ and c, respectively (Fig. 4) and by calculating the volume equivalent sphere radius, r, which is a function of the water content (degree of swelling) of the beans (Fig. 5).

RESULTS AND DISCUSSION

With all these elements ready for use we could now start our caffeine extraction experiments and calculate our diffusion coefficients. However, prior to doing so I

TABLE 1
PARTITION COEFFICIENTS OF CAFFEINE IN THE SYSTEM WATER–CHLOROFORM–CAFFEINE

Temperature (°C)	*Total caffeine content (mmol/50 ml)* 4·8	1·2	0·31	0·077	0·019	0·0048
20	18·7	21·4	22·4	22·6	24·1	22·8
30	15·5	17·3	19·5	21·0	20·2	19·4
40	13·9	17·1	16·8	17·4	17·4	17·4
50	12·1	13·9	14·4	14·4	15·2	14·6
55	11·5	11·4	13·3	13·2	13·5	13·2

would like to give the results of two of our previous studies which might be of general interest: the partitioning of caffeine between water and two chlorinated hydrocarbons on the one hand and the diffusion of caffeine in pure water and in pure methylenechloride on the other.

The partition coefficients $C = c_{solvent}/c_{water}$ are shown in Tables 1 and 2.

The figures show that in both systems (water–chloroform–caffeine and

TABLE 2
PARTITION COEFFICIENTS OF CAFFEINE IN THE SYSTEM WATER–METHYLENECHLORIDE–CAFFEINE

Temperature (°C)	*Total caffeine content (mmol/50 ml)* 10·3	5·15	1·29	0·33	0·0824	0·0206
20	4·85	6·87	9·44	9·54	9·69	8·40
25	5·00	6·87	8·28	9·18	9·43	8·91
30	5·18	6·91	8·39	9·13	9·09	8·50
35	5·48	6·85	7·89	8·86	9·00	8·68

water–methylenechloride–caffeine) the partition coefficient increases with decreasing caffeine concentration. In addition, the chloroform system shows a reduction in partition coefficient with increasing temperature.

At comparable experimental conditions (e.g. 20 °C/0·3 mmol/50 ml) the 'chloroform values' are 2–3 times greater than the 'methylenechloride values'.

Without any further comment on these results, I would like to switch over to the diffusion of caffeine in pure water and pure methylenechloride. Unfortunately, the interferometric technique we used was not applicable to chloroform as a solvent. We

TABLE 3

Solvent	*Temperature* (°C)	*Diffusion coefficient* ($cm^2\ sec^{-1}$)	*Relative standard deviation* (%)
Water	21·5	$5{\cdot}3\ 10^{-6}$	6·0
CH_2Cl_2	21·5	$13{\cdot}8\ 10^{-6}$	5·0

TABLE 4

Time (t)	*Caffeine content* $\bar{c}(t)$	M_t/M_∞	$1-M_t/M_\infty$	*log* t	|d|
0·00	1·36	0·000	1·000	$-\infty$	—
0·25	1·00	0·265	0·735	−0·602	1·56
0·50	0·84	0·382	0·618	−0·301	1·49
1·00	0·68	0·500	0·500	0·000	1·52
2·00	0·46	0·662	0·338	0·301	1·50
3·75	0·25	0·816	0·184	0·574	1·48
8·00	0·086	0·934	0·066	0·903	1·55

$M_t = \bar{c}(t)$ mean: $|\bar{d}| = 1{\cdot}52$
$M_\infty = \bar{c}(t=0)$ relative standard deviation: $s/|\bar{d}| = 0{\cdot}021$

First column: Extraction time.
Second column: Caffeine content of the beans.
Third–fifth columns: Mathematical transformations.
Last column: Distance, d, of the sigmoidal curve from the standard σ function.

TABLE 5

Provenance	*Solvent*	*Degree of swelling* (% H_2O)	*Temperature* (°C)	*Diffusion coefficient* (10^{-6} cm^2 sec^{-1})	*Relative standard deviation* (s/$\bar{x}$)
		44·8	50·0	1·31	0·21
		44·8	50·0	1·34	0·08
	$CHCl_3$	44·6	50·0	1·35	0·16
		44·4	40·0	0·82	0·17
		44·8	30·0	0·57	0·09
		44·7	37·0	1·06	0·12
		45·2	37·0	0·79	0·10
		44·7	36·5	0·68	0·27
		44·2	36·8	1·02	0·10
Arabica from Columbia		45·4	33·5	0·53	0·16
		44·8	30·0	0·51	0·10
		43·9	30·0	0·46	0·10
		43·9	30·0	0·46	—
		44·2	30·0	0·46	—
		44·4	30·0	0·46	—
	CH_2Cl_2	44·2	30·0	0·48	—
		39·2	36·8	0·72	0·06
		39·4	33·5	0·50	0·14
		39·2	30·0	0·42	0·05
		34·2	37·0	0·38	0·14
		33·1	33·5	0·31	0·11
		33·8	30·0	0·24	0·15
		32·6	30·0	0·25	0·07
Robusta	CH_2Cl_2	44·2	37·0	0·57	0·24

therefore only got results in water and in methylenechloride which, again, I would like to present (Table 3) without further comment.

Let us return to the diffusion of caffeine in the coffee beans. Table 4 shows the results of a typical extraction experiment.

As already mentioned, we can calculate the diffusion coefficient D of the caffeine in the beans by using the d value according to the formula:

$$D = r^2\ 10^{-d} \tag{3}$$

In this example $d = 1{\cdot}52$ and the equivalent bean radius $r = 0{\cdot}40\,\text{cm}$. Hence:

$$D = 0{\cdot}40^2\ 10^{-1{\cdot}52} = 1{\cdot}34\ 10^{-6}\ \text{cm}^2\ \text{sec}^{-1} \tag{4}$$

which is about a quarter of the diffusion coefficient of caffeine in pure water and about a tenth of the diffusion coefficient of caffeine in pure methylenechloride.

We consider this figure as representative of the whole extraction process and it would be interesting to have a look at how it changes with changing experimental conditions.

Table 5 shows the whole list of the determined diffusion coefficients as a function of the coffee provenance, the solvent, the degree of swelling and the extraction temperature, respectively.

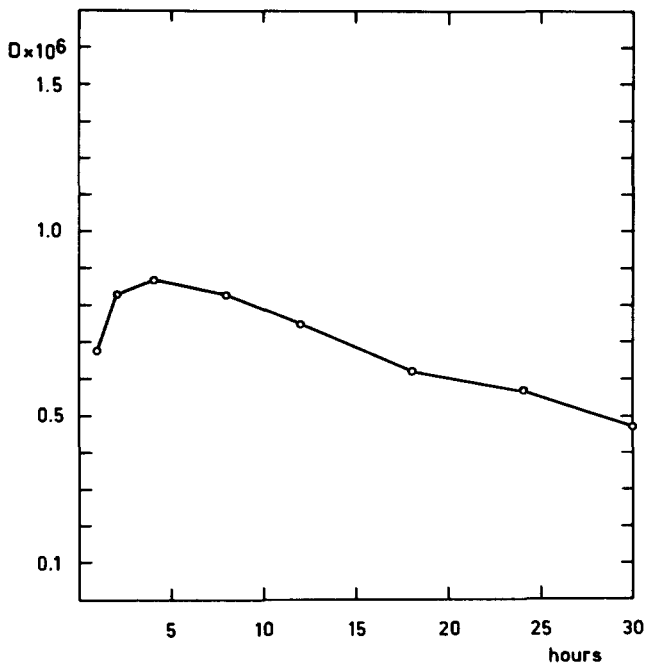

Fig. 6. Change of the diffusion coefficient with time.

These figures confirm a fact which is well known in the practice of decaffeination, namely that the diffusion coefficient increases with increasing water content of the beans and with increasing temperature.

At the same temperature and the same initial water content, the diffusion coefficient is slightly higher if chloroform instead of methylenechloride is used as a solvent.

As to the influence of the coffee provenance, we notice that, at comparable experimental conditions, the diffusion coefficient for Columbia coffee is remarkably higher than that for Robusta coffee.

There is another fact which it is worth while to look at a little closer. The listed diffusion coefficients are mean values calculated over the whole duration of the extraction experiments. A thorough examination of the sigmoidal curves of the extraction data shows that the single experimental values are not scattered at random around the hypothetical curve. Rather, there seems to be a systematic variation of the diffusion coefficient with time (Fig. 6). A slight increase during the

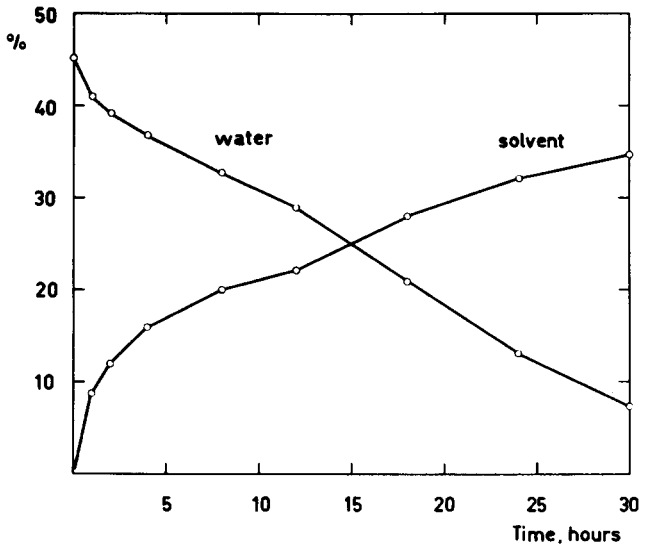

Fig. 7. Time function of water and solvent contents.

first 5 h of extraction, followed by a systematic decrease, can be noticed. This may be explained by the fact that the composition of the diffusion space varies with time. Right from the beginning of the experiment, the beans constantly lose water as a result of a considerable solvent uptake (Fig. 7). The solvent penetrating into the beans initially promotes the diffusion of the caffeine. This increase, however, is soon overcompensated by the inhibiting effect of the water loss, and the diffusion slows down again after having reached a maximum.

CHLOROGENIC ACIDS—THEIR COMPLEX NATURE AND ROUTINE DETERMINATION IN COFFEE BEANS

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(Received: 16 May, 1978)

ABSTRACT

The chlorogenic acids complex of coffee has received a vast amount of attention since the term was first applied in 1846 *to what was then thought to be a pure compound. This paper summarises the literature concerning the characterisation and measurement of the chlorogenic acids complex in coffee and coffee products. Aspects given particular attention are:* (1) *the nature of the chlorogenic acids complex and its confusing trivial nomenclature;* (2) *the behaviour of chlorogenic acids during roasting;* (3) *simple methods for accurate analysis of numerous samples on a routine basis.*

THE NATURE OF THE CHLOROGENIC ACIDS IN COFFEE

The chlorogenic acids are a complex and major component of green coffee beans and they are now known to include at least 13 isomers. These isomers may be subdivided into five groups which I shall present in order of their decreasing percentage contribution to the total chlorogenic acids (CGA) content:

(1) Caffeoylquinic acids (CQA) which are esters of caffeic acid and quinic acid.
(2) Dicaffeoylquinic acids (diCQA) which are esters involving two residues of caffeic acid attached to the same residue of quinic acid; total caffeoylquinic acids include the CQA and diCQA.
(3) Feruloylquinic acids (FQA) which are esters of ferulic acid and quinic acid and are the monomethyl ethers of the CQA.
(4) *p*-Coumaroylquinic acids (CoQA) are esters of *p*-coumaric acid (4-hydroxycinnamic acid) with quinic acid.

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(5) Caffeoylferuloylquinic acids (CFQA) are little studied esters consisting of one residue of caffeic acid and one residue of ferulic acid attached to the same residue of quinic acid—they are the monomethyl ethers of the diCQA.

This final group is a very minor component that has not been fully characterised but in coffee beans each of the preceding four groups consists of three positional isomers depending upon which of the vicinal hydroxyl(s) of the quinic acid residue are esterified.

Some of the CQA isomers can form complexes with caffeine.

THE CONFUSING TRIVIAL NOMENCLATURE OF THE CHLOROGENIC ACIDS

The term chlorogenic acid was probably first used some 130 years ago by Payen (1846) to describe phenolic material that formed a significant part of green coffee beans. Some 90 years later Fischer & Dangschat (1932) isolated 3-caffeoylquinic acid from this fraction and in the next 30 years, up to the end of the 1950s, Payen's chlorogenic acid yielded isochlorogenic acid (Barnes *et al.*, 1950), neochlorogenic acid (Corse 1953) and Band 510 (Sondheimer, 1958). Similar investigations using other plant material yielded cynarin (Panizzi & Scarpatti, 1954), pseudochlorogenic acid (Uritani & Miyano, 1955), Hauschild's substance (Badin *et al.*, 1962) and cryptochlorogenic acid (Waiss *et al.*, 1964). Fischer and Dangschat's 3-caffeoylquinic acid and these seven later fractions differed in their physical properties but all contained hydrolysable caffeic acid and quinic acid. In due course these seven substances were realised to be impure and only a little less complex than Payen's original chlorogenic acid complex. The literature contains few reports of the *thorough* characterisation of these fractions but the limited information that is available indicates that the same trivial name has been applied to fractions prepared by different methods and with notably different composition. For example, it has been reported (Lentner & Deatherage, 1958) that the isochlorogenic acids fraction of Barnes *et al.* contains 3-CQA and the three diCQA whereas the isochlorogenic acid of Sondheimer contains two isomers of FQA, one of CoQA and probably the three diCQA (Paris & Nishio, 1970). One must conclude that data reported about such variable and poorly characterised material are not amenable to comparison or precise interpretation.

A tendency during the last decade has been to use at least some of these trivial names as synonyms for individual isomers or groups of isomers that formed the major component of these ill-defined fractions. For example, the term neochlorogenic acid is often used to refer specifically to 5-CQA. Similarly, cryptochlorogenic acid and Band 510 may refer to 4-CQA and isochlorogenic acid to the group of diCQA. However, these terms are uninformative to the uninitiated and confusing if their original meaning is mistaken for their current usage. Hence my preference for a convenient and unambiguous nomenclature, with simple abbreviations as outlined at the beginning of this paper.

A CRITICAL SURVEY OF QUANTITATIVE DATA FOR THE CGA CONTENT OF GREEN COFFEE BEANS

The chlorogenic acids content of many varietes of coffee bean has been determined by many investigators using many methods which unfortunately have often differed in their specificity. I have already indicated the problems associated with making meaningful comparisons of results obtained by diverse methods. I will illustrate the situation with a few examples.

In some cases the results quoted have been inflated by the detection of compounds other than the chlorogenic acids; for example, results obtained using the original AOAC method (Weiss, 1953) which involved the measurement of the UV absorption of an alcoholic extract. Sometimes reagents have been used that are specific for the total caffeoylquinic acids (e.g. Häusserman & Brandenburger, 1961). However, results have sometimes been reported as total chlorogenic acids despite the failure (not necessarily apparent at the time) to detect the quantitatively important FQA as well as the minor CoQA. Typically, 3-CQA is used as the standard but the diCQA in particular have significantly different properties which leads to the diCQA content being overestimated by some 15% (calculating from the molar extinction coefficients reported by Rubach (1969)) if measured by UV spectrophotometry and by some 37% if measured by colorimetric reagents that react with the phenolic part of the molecule (Clifford & Staniforth, in press), a point upon which I shall elaborate below.

Having made allowances as far as one can for the foregoing points, it is clear that typically (e.g. Szilasne & Barath, 1973; Rubach, 1969; Clifford & Staniforth, in press; Rees & Theaker, in press) Robusta coffee beans contain some $7\frac{1}{2}$ to 10% of chlorogenic acids whereas Arabica coffees contain some $5\frac{1}{2}$ to $7\frac{1}{2}$% on a dry mass basis (dmb). The hybrid Arabusta has an essentially intermediate content (Clifford & Staniforth, in press; Rees & Theaker, in press) and many wild species have contents well below 2% dmb (Chassevent *et al.*, 1974). The major isomer is 3-CQA, forming some $5\frac{1}{2}$ to $6\frac{1}{2}$% dmb in the three commercially important species, i.e. some 55–65% of total CGA in Robusta coffees, some 75–80% in Arabica coffees and approximately 70% in Arabusta coffees. There are few (Rubach, 1969; Pictet & Brandenburger, 1960; Kung *et al.*, 1967) precise data available for the contents of 4-CQA and 5-CQA but together they probably account for some 10–15% of the total chlorogenic acids. The diCQA and FQA contents each appear to range between 5 and 25% of the total, and Robusta coffee beans have substantially higher contents ($\times 2$) of these two groups of compounds than Arabica coffee beans (Clifford & Wight, 1976; Häusserman & Brandenburger, 1961; Szilasne & Barath, 1973; Rubach, 1969; Clifford & Staniforth, in press; Rees & Theaker, in press). It is mainly the relatively high FQA and diCQA contents of Robusta coffees that are responsible for the higher total CGA content (Rees & Theaker, in press) of this species.

I know of only one set of quantitative data for the CoQA content (Rubach, 1969)

and it would seem that this group does not exceed some 2% of the total, but I am currently investigating a method for determining this group of compounds. The CFQA are certainly a very minor component for which I know of no quantitative data. So far this group has only been detected as a minor impurity in a diCQA preparation (Corse *et al.*, 1965).

HYPOTHESES LINKING GREEN BEAN CGA CONTENT AND BEVERAGE QUALITY

Since it is generally accepted that Robusta coffee beans yield an inferior beverage compared with Arabica coffees it is tempting to try to correlate beverage quality inversely with chlorogenic acids content. However, when looking for such correlations it must be borne in mind that there are other marked differences in composition between Arabica and Robusta coffees. Nevertheless, Amorim *et al.* (1973) have reported that the best quality soft Brazilian Arabica coffees have a lower chlorogenic acids content than the worst quality coffees which have a Rio taste. However, their data also indicated that the worst grades did not necessarily have a greater chlorogenic acids content than the intermediate grades.

The same group of workers (Amorin & Amorin, 1977) have reported a correlation between low quality, low carbonyl content and low polyphenoloxidase activity in the green bean. They postulate that the chlorogenic acids act as protective agents for some ill-defined green bean carbonyls during green bean processing and storage; presumably the chlorogenic acids operate as radical acceptors preventing radical oxidation of the carbonyls. However, more data are required before such hypotheses can be confirmed.

THE EFFECTS OF ROASTING ON CGA CONTENT

During roasting there is a progressive destruction of chlorogenic acids which may exceed 90% in severe roasts of, say, 9% or greater pyrolysis loss (Clifford & Wight, 1976; Häusserman & Brandenburger, 1961; Rees & Theaker, in press; Merrit & Proctor, 1959). If the type or blend of bean is known and the roasting equipment is constant then a measure of residual chlorogenic acids content is a fair estimate of the severity of roast to which the beans have been subjected. Beyond some 5% pyrolysis loss the chlorogenic acids are very rapidly destroyed and this loss to a large extent accounts for the rise in roast bean pH value which occurs at this stage. This may lead to a rise of as much as 0·2 pH unit in the beverage pH value (Sivetz, 1972).

Recent data indicate that while CQA, diCQA and FQA are all destroyed during roasting these subgroups are not necessarily all destroyed at the same rate even when the charge of beans and roasting procedure are standardised. For example, the results of Rees & Theaker (in press) indicate that when a Zaire Robusta coffee was

roasted to 9% pyrolysis loss the FQA content rose from 22% of the total chlorogenic acids to 33%. Similarly, the FQA content rose from 11% to 20% of the total during the roasting of a Kenyan Arabica coffee to 11·5% pyrolysis loss. I have also observed that the FQA seems to be slightly more stable than the CQA during roasting (Clifford, 1972).

The behaviour of the diCQA seems rather variable but this may reflect the use of different beans and different roasting equipment. For example, Häusserman & Brandenburger (1961), using Brazilian and Colombian Arabica coffees and a Ugandan Robusta coffee, reported that the diCQA formed an essentially constant percentage of the total chlorogenic acids during roasting. Rees & Theaker (in press) obtained similar results with the Zaire Robusta coffee but observed total destruction of the diCQA in the Kenyan Arabica coffee beyond $6\frac{1}{2}$% pyrolysis loss. These variations suggest that the residual chlorogenic acids content is of limited value in predicting the degree of roast to which an 'unknown' bean has been subjected.

Relatively little is known of the chlorogenic acids degradation products, although it would seem that a substantial amount of chlorogenic acids and/or their degradation products are incorporated into the humic acids (Clifford, 1972, 1975). The humic acids are high molecular mass components with molecular masses in the range 5000 to 50,000 and these substances form some 12–15% of roasted Arabica and Robusta coffee beans. Workers have indicated that acid, alkaline and enzymic hydrolyses release at least 12 phenolic compounds as well as amino acids and galactose and mannose (Clifford, 1972; Maier *et al.*, 1968; Maier & Buttke, 1973; Klöcking *et al.*, 1971), but it is possible that some of the phenols are artefacts arising from the effect of the severe chemical hydrolysis systems upon intact bound chlorogenic acids (Clifford, 1972).

A range of volatile and relatively non-volatile phenolic compounds has been detected in roasted Arabica, Robusta and Arabusta coffee beans (Clifford, 1972; Pypker & Brouwer, 1969; Tressl, in press). The most recent and most extensive work in this area by Tressl indicated that phenol, guaiacol, 4-ethylguaiacol and 4-vinylguaiacol are the major volatile phenols and that they are present at concentrations in excess of their threshold values and will therefore contribute to the odour and flavour of coffee and coffee brews. The content of most of these volatile phenols was higher in heavily roasted coffee than in medium roasts by a factor of between 5 and 10. The major di- and trihydroxy phenols are catechol, 4-ethylcatechol and pyrogallol; these were detected in rather greater concentrations than the more volatile monohydroxyphenols. Examination of model systems has indicated that the guaiacols are almost certainly derived from the degradation of the ferulic acid residue of the FQA (Clifford, 1972; Tressl, in press). In contrast, catechol and pyrogallol are probably mainly derived from the quinic acid residue of the chlorogenic acids.

However, it is likely that ethylcatechol, vinylcatechol and 3,4-dihydroxycinnamaldehyde and probably some of the catechol (Clifford, 1972) are produced from

the caffeic acid residue. Most of the phenols are present at the highest level in Robusta coffees, but while the Arabica coffees have the lowest content of guaiacols the Arabusta coffees have the lowest content of catechols and pyrogallols. It is not clear whether these phenols contribute desirable or undesirable notes to the roasted bean and beverage. Recent investigations on woodsmoke have attributed 'sweetish-smokey' organoleptic properties to such guaiacols and a 'heavy burnt phenolic odour with some sweetness' to such catechols (Fujimaki *et al.*, 1974; Kim *et al.*, 1974).

THE ROUTINE DETERMINATION OF THE CGA CONTENT

I mentioned earlier that many methods have been devised for the measurement of chlorogenic acids. I propose that the ideal method should have the following attributes:

(1) It should give equal emphasis on a molar basis to each of the many isomers.
(2) It should not be subject to interference from other substances.
(3) High repeatability and reproducibility.
(4) Simplicity, rapidity and economy of labour, capital and operating costs.

As I indicated earlier, some of the older methods fail to meet criteria (1) and (2).

My own search for the ideal method has been centred upon highly specific colorimetric or spectrophotometric methods and since these have been described elsewhere (Clifford & Wight, 1976; Clifford & Staniforth, in press; Clifford, 1972; Clifford & Staniforth, 1977), I shall only present here a summary of the performance of the better reagents.

The periodate reagent (0·25% aqueous sodium metaperiodate) is the most successful reagent that I have investigated to date. This reagent reacts with the CQA, FQA, diCQA and CFQA, i.e. some 98% of the total CGA, and gives an equal response to each isomer on the basis of the cinnamic acid content. A large number of non-phenolic substances have been tested and found not to interfere; the minor phenolic components of green or roasted coffee beans either do not produce colour at the wavelength employed (406 nm) or are present at such a low level that their contribution can be ignored.

The best results (Clifford & Staniforth, in press) are obtained by controlling the time and temperature of the colour producing reaction, 10 min at 27° being the optimum. Repeatability and reproducibility are very good, the standard deviations being 2·2% and 4·6% of the mean value for green beans analyses (three samples, five occasions, one laboratory). Moreover, these values were obtained using test tubes in a thermostatically controlled water bath and a stopclock—very probably better results would be obtained using an autoanalyser, a development to which the

method lends itself very easily. Using the manual method, four samples in duplicate and blanks can be determined in some 15–20 min at an approximate reagent cost of 0·1p per one sample and one blank.

Alternative good reagents include the borate reagent of Häusserman & Brandenburger (1961) that has been adopted for the Swiss (Anon., 1967) and German standard methods (Anon., 1976) and the molybdate reagent of Kendal (1949) and Goldstein & Swain (1963). Both reagents and their blanks require buffers (borate pH 7·0, molybdate pH 6·5) and therefore neither reagent is as simple nor as cheap to prepare as the periodate reagent (borate reagent 1·75p, molybdate reagent 0·6p).

Neither reagent detects the FQA, therefore giving results for total caffeoylquinic acids (CQA plus diCQA) only.

The Deutsche Institut für Normung have carried out collaborative studies (Kwasny, 1976) on the performance of the borate reagent in the determination of CGA in green, roasted and instant coffees. Very good repeatability was obtained on the three types of coffees examined. Reproducibility was also very good for instant and roasted coffee but poor for green coffee beans, due largely to variations in grinding and extraction procedures between laboratories. Inevitably the reproducibility of the periodate reagent would also suffer under such circumstances.

However, in my investigations (Clifford & Staniforth, in press) the borate reagent did not perform as well as the periodate reagent when used under conditions where differences in grinding and extraction could not be responsible. I have not yet established the cause of the discrepancy between my results and those of the Germans, but it is clear that the pH value of the borate reagent must be rigidly standardised. It is very interesting to note that Sloman & Panio (1969) reported that they could eliminate the production of occasional spurious results with the borate reagent if the procedure were automated. There is little doubt that the performance of the periodate reagent and molybdate reagent would also be improved with automation.

A limitation common to the periodate, molybdate and the borate reagents is their tendency to overestimate the content of diCQA by approximately 37%. This occurs because these reagents react with the phenolic part of the molecule and therefore each molecule of diCQA (molecular weight = 516) is determined as two molecules of CQA (molecular weight = 2 × 354 = 708),

$$\frac{708 - 516}{516} \times \frac{100}{1} = 37\%$$

and the result effectively includes a non-existent residue of quinic acid. If the diCQA content is known then an approximate correction can be applied (Clifford & Staniforth, in press).

The use of the periodate reagent with either the molybdate or borate reagent

permits the FQA content to be estimated by difference (Clifford & Wight, 1976; Clifford & Staniforth, in press; Rees & Theaker, in press; Clifford, 1972).

Recently, high pressure liquid chromatography (HPLC) has been used for the determination of chlorogenic acid isomers and there is no doubt that very good separations are possible with this type of equipment (Rees & Theaker, in press; Court, 1977). It is essential when developing HPLC methods that each peak is characterised and checked for purity and that the correct molar extinction coefficients are applied (Rubach, 1969) if quantitative results are required. However, it is debatable whether such methods can compete in terms of speed with, for example, an automated method using the periodate reagent. For example, the HPLC method reported by Court (1977) takes 1 h after preparing the extract, although the method of Rees & Theaker (in press) is probably more rapid than that reported by Court.

An alternative application of HPLC methods is to provide characteristic fingerprints for particular types of beans and/or processes and degrees of roast. For example, the method reported recently by Rees and Theaker demonstrated two unidentified components in the extract from an Angolan Robusta coffee which were not detected in Arabusta, seven other Robusta or four Arabica coffees.

CONCLUDING STATEMENT

In closing I would suggest that an automated method employing the periodate reagent comes closest to meeting the attributes required by the ideal method for routine analysis of chlorogenic acids. Some 98 % of the total chlorogenic acids are measured and interference is negligible, especially if the diCQA inflation is corrected. The procedure is simple, cheap and rapid and of high repeatability and reproducibility.

Should fingerprinting, or information on the individual isomers, be required, then a HPLC method would be a very good choice.

I would finally like to encourage everyone to stop using the confusing trivial nomenclature that currently appears in the chlorogenic acid literature.

ACKNOWLEDGEMENTS

I wish to thank Dr D. I. Rees of Lyons Central Laboratories, London, and Professor Dr R. Tressl of the Technische Universität, Berlin, for providing me with copies of the papers they presented at the Eighth International Scientific Colloquium on Coffee at Abidjan in December, 1977. I also wish to thank Herr H. Kwasny of Hag AG, Bremen for providing data from the recent D.I.N. investigations.

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COFFEE MIXTURES—SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF COFFEE CONTENT

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(Received: 16 May, 1978)

ABSTRACT

The method described in this paper gives results which are good approximations of the actual coffee contents of roasted coffee/roasted chicory and roasted coffee/roasted fig mixtures.

It has also been applied to coffee and chicory essence, cereal products and instant coffee/chicory, instant coffee/cereal/chicory mixtures.

INTRODUCTION

The Coffee and Coffee Products Regulations (SI 1967, No. 1865) specify minimum coffee contents for mixtures of roasted coffee and roasted chicory (French coffee) and for mixtures of roasted coffee and roasted fig (Viennese coffee) and these are not less than 51% and 85%, respectively.

The method used by Lyons Tetley for French coffee for a number of years is that published by Hughes and Wise (1955). This depends upon the considerable difference in water-soluble solids in roasted chicory and roasted coffee. In our experience although this method was the best available, it was unreliable because of large differences in the soluble solids present in multiple samples of chicory from the same roaster batch and also between different roasts.

The Seliwanoff test (Seliwanoff, 1887) has been used by Mitra & Roy (1953) for the detection of chicory in coffee and chicory mixtures. It has been made quantitative by Gal (1959) who has also applied it to fig. Bose *et al.* (1971) have modified the test in order to increase its sensitivity.

Our earlier work on the determination of the coffee content of French coffee and Viennese coffee involved application of the Gal method. However, the procedure

Fd. Chem. (4) (1979)—
Printed in Great Britain

was found to be too empirical and the coloured complex too unstable and very much dependent on the intensity of daylight. The colour was stabilised to some extent by making up the resorcinol solution in 95% ethanol. During the course of this work, however, some results were found to be erratic. Upon further investigation a spectral shift was observed. This phenomenon has been identified by Arsenault & Yaphe (1965) and other workers and is probably due to the presence of trace amounts of acetaldehyde in the ethanol.

The procedure of Kulka (1956), also based upon the Seliwanoff test, was next investigated and gave results which were more consistent and easily reproducible. This procedure ensured intensification of the coloured resorcinol complex by the addition of Fe^{+++} and also eliminated the spectral shift due to impurities in ethanol.

At the same time, the earlier work by Rees & Smith (1963) concerning elimination of interference by 5-hydroxy methyl furfural (HMF) in the spectrophotometric determination of caffeine in coffee mixtures was followed up by Rees (1971) who found 1·2% 5-hydroxymethyl furfural in roasted chicory and 0·1% in coffee. However, further work by the same investigator indicated that HMF could not form the basis for a method because of its loss during dry blending of coffee and chicory.

In the present method the Kulka procedure has been adapted for the determination of ketose sugars in coffee mixtures.

The method for estimating the coffee contents of such mixtures relies upon the relative preponderance of ketose sugars in chicory and fig and their virtual absence in roasted coffee.

EXPERIMENTAL

Apparatus

Absorbance measurements were made on a Unicam SP500 spectrophotometer using 1 cm glass cells. All the glassware was acid washed and the volumetric items were of Grade A accuracy.

Reagents

All reagents were of analytical reagent grade unless otherwise specified.

(1) Ethanol 95% v/v.
(2) Resorcinol.
(3) Ferric ammonium sulphate.
(4) Hydrochloric acid (SG 1·18).
(5) Fructose for biochemistry (BDH).
(6) Reagent A: Dissolve 0·050 g resorcinol in 95% ethanol and dilute to 100 ml with 95% ethanol in a calibrated flask. This solution will remain stable for up to one week if kept in an amber glass container under refrigeration.

(7) Reagent B: Dissolve 0·216 g ferric ammonium sulphate in hydrochloric acid (SG 1·18) and make up to one litre in a calibrated flask with the same acid.
(8) Alumina cream (BDH).

Calibration graph

Prepare a calibration graph by plotting absorbance readings against concentration of fructose using 0·001, 0·002, 0·003, 0·004 and 0·005 % solutions of pure fructose and applying the following procedure.

Preparation of samples for analysis

Grind not less than 10 g of well quartered sample(s) to pass through a BSS 16 mesh sieve.

Procedure

For quality control purposes it is important that the method is applied to samples of coffee mixtures and those of their ingredients.

TABLE 1
AVERAGE VALUES FOR KETOSE SUGARS CONTENTS OF INGREDIENTS OF FRENCH COFFEE AND VIENNESE COFFEE

	Ketose sugars (%)		
	Coffee	*Chicory*	*Fig*
No. of samples examined	50	100	20
Mean	0·53	48·0	23·0
Range	0·1–0·8	45·9–49·7	20·0–24·5
Standard deviation	0·23	1·18	1·1

Where no ingredients are available the sample of the mixture alone may be used and in this case the coffee content is computed from the ketose sugars found in the mixture and the average values for ketose sugars contents of the ingredients listed in Table 1.

Method

Transfer a sample of exactly 4 g to a 500 ml conical flask. Add 80 ml distilled water and boil under reflux for 10 min using a double surface coiled condenser and adjusting the heat source to prevent the slurry splashing on to the condenser. Filter the hot extract through an 18·5 cm Whatman No. 4 paper into a 200 ml calibrated flask. Wash the residue on the filter paper with approximately 80 ml distilled water. Add 10 ml alumina cream to the contents of the calibrated flask and mix. Make up to volume, again mix and filter through 18·5 cm Whatman No. 5 paper, rejecting the first 10 ml of filtrate.

Prepare the following solutions by diluting appropriate aliquots of the clear filtrates with distilled water:

(a) *Coffee and Chicory Mixtures*
Coffee 0·04%, chicory 0·01%, mixture 0·02%.
(b) *Coffee and Fig Mixtures*
Coffee 0·04%, fig 0·02%, mixture 0·04%.

Add 3 ml of Reagent A, followed by 3 ml of Reagent B, to four 8 in × 1 in glass stoppered tubes and mix. Add 1 ml coffee solution to tube 1, 1 ml chicory (or fig) solution to tube 2, 1 ml mixture solution to tube 3, 1 ml distilled water to tube 4 (reagent blank).

Close the tubes, mix and heat for 40 min in a water bath at 80 °C. Cool the tubes in an ice bath for 5 min. Bring to room temperature by immersing the tubes in a beaker containing water at 25 °C for 5 min.

Measure the absorbance of the solutions at 480 nm and deduct the reagent blank. Refer to the calibration graph to obtain the ketose sugars content (as fructose). Calculate the coffee content of the mixture from the ketose sugars content (*I*).

$$\% \text{ coffee in mixture} = \frac{\% \ I \text{ in chicory (or fig)} - \% \ I \text{ in mixture}}{\% \ I \text{ in chicory (or fig)} - \% \ I \text{ in coffee}} \times 100$$

RESULTS AND DISCUSSION

The water-soluble extractives content of roasted chicory used as a basis for the other methods is very variable. Differences of between 83% and 66% have been found between samples from the same crop year roasted to the same degree, and to a lesser extent between samples taken from the same roaster batch.

The variation is due in part to the method of roasting, product from the Barth roaster generally yielding a greater extractives content than that from the Whitmee roaster, and also to the fact that it is not possible to control the colour of the roast or its uniformity because of the absence of water quenching as a means of optimising the colour development.

Chicory from the Barth type of roaster is of a light colour outside and darker inside whereas the opposite is true for product from the Whitmee type.

The water-soluble extractives in the product are related to colour of the roast, the lightly roasted product being low in extractives and the amount increasing with the darkening of colour until an optimum value is reached. Beyond that point charring commences and extractives are destroyed. The phenomenon can be explained by the fact that the lightly roasted product contains a relatively greater amount of inulin which is very sparingly soluble in water.

Since a variation in colour exists in each roasted chicory cossett prior to it being ground, the extractives content of the powder is also variable.

It is for this reason that the methods based upon water-soluble extractives must be regarded as generally unreliable.

In the present method a calibration graph was prepared by subjecting a duplicate set of fructose solutions to the procedure. The graph was linear over the range 0–0·005% fructose (Fig. 1).

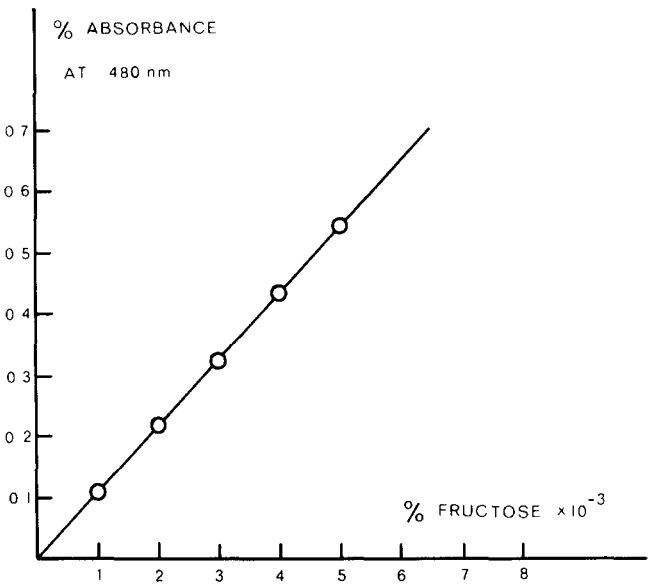

Fig. 1. Calibration graph.

The method was first applied to 25 samples of Barth roasted English chicory from the 1971 crop and representing a similar number of deliveries.

The results obtained showed the ketose sugars content of the samples to be within a restricted range. Concurrently extracts were prepared from the samples by the Hughes and Wise technique and the water-soluble extractives content calculated (Table 2).

It is evident from Table 2 that the water-soluble extractives in chicory are

TABLE 2
KETOSE SUGARS AND WATER-SOLUBLE EXTRACTIVES IN ROASTED CHICORY

	Ketose sugars (as Fructose) (%)	*Water-soluble extractives* (%)
Mean	48·2	77·3
Range	47·0–49·2	68·0–83·4
Coefficient of variation	1·14	11·38

approximately ten times more variable than the ketose sugars. Furthermore, examination of individual results did not show any sufficient relationship between the ketose sugars and the extractives.

In order to test the present method on coffee/chicory mixtures, a number of different blends were prepared and these, together with their ingredients, were analysed by the present method and by the Hughes and Wise extractives method. The results obtained are shown in Table 3.

TABLE 3
COMPARISON OF PRESENT METHOD AND HUGHES AND WISE EXTRACTIVES METHOD

Mixture % Coffee (added)	*% Coffee (found) by present method*	*% Recovery*	*% Coffee (found) by extractives method*	*% Recovery*
45	44·6	99·1	43·0	95·6
47	46·7	99·4	46·8	99·6
48	48·2	100·4	48·2	100·4
49	49·2	100·4	50·4	102·9
50	50·0	100·0	49·1	98·2
51	50·9	99·8	49·0	96·1
52	51·6	99·2	50·2	96·5
55	55·0	100·0	56·9	103·5

The method was next used to determine the ketose sugars in roasted fig. The results obtained on six samples are summarised in Table 4.

The results in Table 4 indicated that the ketose sugars content might be a suitable parameter for the calculation of the coffee content of Viennese coffee.

The results of an analysis of six samples of coffee/fig mixtures of known composition showed the method to be satisfactory for the type of product (Table 5).

TABLE 4
KETOSE SUGARS CONTENT OF ROASTED FIG

Mean	22·5
Range	21·0–23·5
Standard deviation	0·9

From the results over a three-year period average values were calculated for ketose sugars in coffee, chicory and fig (Table 1).

The average values in Table 1 were then used for the estimation of coffee content of mixtures of known composition, instead of their respective ingredients. The results of the estimation are shown in Table 6.

From the results in Table 6 it is evident that the spectrophotometric method is also suitable for analysis of coffee mixtures alone using the average ketose sugars contents of the ingredients.

TABLE 5
ANALYSIS OF COFFEE/FIG MIXTURES

% *Coffee in mixture*	% *Coffee found*	% *Recovery*
90	89·4	99·3
88	88·0	100·0
85	84·8	99·8
84	84·2	100·2
82	82·0	100·0
80	79·7	99·6

TABLE 6
COFFEE CONTENT OF MIXTURES CALCULATED FROM KETOSE SUGARS IN MIXTURES AND AVERAGE VALUES FOR THOSE IN THE INGREDIENTS

Type of mixture	% *Coffee in mixture*	% *Found*	% *Recovery*
Coffee/chicory	48	47·9	99·8
Coffee/chicory	50	49·0	98·0
Coffee/chicory	52	53·0	101·9
Coffee/chicory	52	53·5	102·8
Coffee/chicory	52	51·2	98·5
Coffee/chicory	52	52·0	100·0
Coffee/fig	85	84·6	99·5
Coffee/fig	85	85·9	101·0
Coffee/fig	85	85·3	100·3
Coffee/fig	85	86·1	101·3
Coffee/fig	85	84·2	99·1
Coffee/fig	85	83·2	97·9

TABLE 7
KETOSE SUGARS IN SPRAY DRIED CHICORY POWDER

Sample	*Origin*	*Ketose sugars* %
CHL1	France	71·0
CHL2	France	71·9
CHL3	France	71·5
CHT1	Belgium	69·9
CHT2	Belgium	70·1
CHT3	Belgium	69·6

The method has also been used for the estimation of the chicory content of coffee and chicory essence, as also for the honey content of a cereal product containing honey and sucrose.

When the method was applied to three samples of instant chicory powder of French origin and a similar number of samples of the product of Belgian manufacture the results shown in Table 7 were obtained.

From the results shown in Table 7 it was evident that the ketose sugars content of spray dried instant chicory powders was also within a restricted range.

In order to assess the suitability of the method for instant coffee/instant chicory mixtures, ketose sugars were also determined in instant coffee and the results of this determination once again showed the degree of scatter to be small (Table 8).

TABLE 8
KETOSE SUGARS IN SPRAY DRIED INSTANT COFFEE

Sample	*Ketose sugars* %
1	1·3
2	1·0
3	0·78
4	0·95
5	1·9
6	1·1

Further work on the application of the method to instant coffee/instant chicory and other soluble beverage mixtures was carried out in our Central Laboratories and subsequently the method was adopted for use within the J. Lyons group for such products.

The only modification for soluble products entails bypassing the initial extraction procedure.

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INTERNATIONAL COFFEE STANDARDISATION AND LEGISLATION

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(Received: 16 May, 1978)

ABSTRACT

The author describes the particular technical features of coffee standards used commercially and/or legislatively. Existing national standards on green coffee and their harmonisation of terminology, sampling and test method are described and discussed. Comparisons are made of the various legislative standards of different countries with respect to instant coffee and their harmonisation within the EEC member countries. Roasted coffee is considered to a lesser extent as it is not an important element in the world trade for coffee. The role of various international organisations in standardisation is described. It is emphasised that the flavour of coffee is not amenable to international standardisation.

INTRODUCTION

Coffee standards feature in both legislation, where they are statutory, and in commercial trading arrangements, where they are voluntary or contractual. Such standards are similar in purpose to those of other foodstuffs; however, raw or green coffee needs to be considered somewhat separately from its derived products (roasted and coffee extracts) for human consumption. Furthermore, we need to consider the differences in the nature of standards for legislative and for trade purposes.

Most coffee-consuming countries have national legislation for roasted coffee, coffee extracts and soluble coffee, with the main object of consumer protection against fraud and potential health risks. Similarly, every producing country of green coffee has national legislation dealing essentially with the purity of exportable coffee and each such country has its own trade grading system. The larger importing countries such as the USA and France have legislation regulating imports. The USA, through the New York Coffee and Sugar Exchange, sets commercial grading standards for green coffee from Brazil and other Latin American countries.

Fd. Chem. (4) (1979)—
Printed in Great Britain

It should be said at the outset that the use of the word 'coffee' to mean pure coffee is zealously guarded in most countries, as can be seen from the undertaking of members of the International Coffee Organisation (1962), representing both producers and users of green coffee.

Roasted and soluble coffee are perhaps more susceptible to adulteration, being less easy to detect, but national legislation in most developed countries is entirely adequate against such practices.

International standardisation has as its basic objective the removal of hindrances to trade. There are two main organisations promoting this objective: (1) the International Standards Organisation, which deals with barriers arising from differences in (and understanding of) national commercial standards (established for the convenience of those in trade) and (2) for human foodstuffs, the Codex Alimentarius Commission which tackles barriers arising from differences in national food legislation (established to protect the consumer). There are also various geographically more restricted organisations such as the EEC Commission which is concerned to harmonise legal provisions amongst its member countries. Codex and the EEC Commission are, of course, governmental bodies, whilst the ISO is non-governmental (at least amongst its non-communist members); both, however, consulting with trade and other experts.

The ISO, through its Technical Committee (TC 34), has been very active since 1963 in coffee standardisation. Sub-Committee SC15 has, since 1976, been the operating unit with delegates drawn from national standards bodies. ISO has issued a statement of policy (ISO, 1972) in respect of its activity in foodstuffs. The terms of reference show a direct involvement with terminology, sampling, methods of analysis, storage, packing and transport, and specifications for agricultural food products in their raw state not intended for direct human consumption. With finished edible product standardisation, ISO will continue to interest itself with specifications for commercial categories and to work on specifications concerning all qualities of product not on the Codex programme. It will, however, avoid duplication of effort in regard to specifications for 'minimum quality' having a regulatory aspect.

The Codex Commission, the other international body concerned with food standards, uses seven criteria to decide whether Codex Standards are warranted in a given area of foodstuffs. Codex has issued a document entitled *Coffee and the Consumer* (Secretariat, Codex, 1971).

THE NATURE OF STANDARDS

Before considering the standardisation of coffee, it is useful to summarise the basic elements of standards (Townshend, 1976). From a technical point of view, the elements are conveniently illustrated in Table 1.

TABLE 1
FOOD STANDARDS

Definition	Hygiene
Designation	Pesticide Residues
Composition	
Additions	
Quality	
Sampling and Methods of Analysis	
Marking and Labelling	

Not all of the elements shown in Table 1 are applicable, or indeed necessary, with particular foodstuffs. Such a standard for a given foodstuff is generally referred to a vertical standard; in practice, many of these elements, particularly those relating to additions (specifically additives) and pesticide residues are referred to horizontal standards for a group of foodstuffs.

Legislation is concerned with minimum standards and maximum allowable amounts of impurities whilst commercial specifications, although incorporating these standards, also provide ranges of quality (maximum and minimum) or grades.

Methods of analysis are clearly important in order to establish the value of any chemical/physical characteristics defined in the standard. Often a standard or official method is not in fact provided; for example, in Great Britain the caffeine content of decaffeinated soluble coffee is set at 0·3%—the analyst chooses the method and in a legal situation it is up to the courts to assess the validity of the analyst's choice. There is now a draft International Standard, however, for the determination of caffeine content in coffees.

Methods of sampling are equally important but also often not clearly specified. The method of sampling not only encompasses the actual method of drawing the sample but also the interpretation of the results of the sampling operation in the light of the standards.

In food legislation, enforcement enters realms which are not technical, and indeed enters areas of the wider field of law. Agreed sampling methodology, however, influences the system of enforcement which differs in different countries. Economic factors of cost also become important. The systems of sampling foodstuffs for legal purposes have been considered by Codex (Codex Committee on Methods of Analysis and Sampling, 1977) by the ISO (ISO, 1977) and by the EEC (EEC, 1976).

In Great Britain and some other European countries, official or unofficial sampling to provide samples for analysis and assessment against legal standards set takes place at retail outlets only. Official sampling is relatively infrequent, with one or two samples taken at a time. Every such item taken is expected, however, to comply with the standards set for the particular product. The fairness of this system, vis-a-vis manufacturer/consumer, is related to the type of standard in question. It is

useful to classify food standards as either 'relating to health' or not 'relating to health'. The concentration of pesticide residue could be an example of the former and allowable moisture content of the latter. A consumer might not like to see a foreign body even in one sample, though he might not object to an unobservable (to him) high moisture content in a single isolated sample.

Statistical sampling plans are frequently recommended to be applied to continuing lots at the factory (as indeed is general for net weight control), or a national border. For product characteristics (EEC, 1976) every item of the sample (say 20) might be expected to conform in order to give a tighter control than sampling at retail outlets.

STANDARDS IN RELATION TO COFFEE

It is clear that coffee as consumed is such a variable beverage, reflecting a variety of local and national tastes, that standardisation in the flavour sense is usually impractical. Coffee products generally are manufactured to meet local preferences in flavour, brewing method, serving, distribution and cost. These factors also differ widely around the world and are not amenable to international standards.

The other elements in coffee standards, relating to quality and purity, definitions and designations and therefore marking and labelling, methods of test and sampling methods are described in detail subsequently for green and soluble coffee. The total body of commercial standards and trade practices, and of national legislation, including that for roasted coffee, indicates that fraudulent practices are very rare in the developed countries with good protection to the consumer. Green coffee, like other agricultural commodities from the many tropical places, can be and is subject to conditions that generate health concerns. The potential risks from the increasing use of insecticides in coffee agriculture are being dealt with by the Codex committee on pesticides and regulations already appear in various countries by horizontal legislation. Coffee products are also now marketed as 'decaffeinated' or 'caffeine-free' after decaffeination by solvents. National coffee regulations or decrees will specify the solvents that may be used and the residual levels in decaffeinated green, and the inevitably lower levels in decaffeinated roasted and soluble, coffee. Solvents have been studied by an FAO/WHO Expert Committee on Food Additives (FAO/WHO, 1970). With good manufacturing practice, solvent residues (ppm) are negligible (Brandenburger, 1969). A problem remains with limits, which are dependent upon the method used, when GC methods are particularly sensitive.

Whilst maximum 'moisture' content values feature widely in coffee standards, high moisture content causes accelerated storage changes and deteriorating taste may be enough to protect consumers. The usual control by manufacturers is to limit moisture based on storage experience and to package to maintain the product at that level during distribution. The health risk of very stale coffee is not known.

Coffee products are fortunate in that they are not good media for the growth or survival of bacteria. Bacterial counts, diminishing on storage, can, however, be useful indicators of sanitary practices.

Minimum health requirements of packages are that they add no toxic materials to the foodstuff, that they protect from contamination and that they protect against excessive storage deterioration. Such packaging requirements are generally subject to horizontal legislation (e.g. draft EEE proposal); soluble coffee is now widely packed in glass jars with waxed glassine or metal foil seals and screw caps.

In the overall application of standards, it is important that they are carefully drafted and considered before application, since otherwise they can act as a brake on technological development and innovation, without corresponding consumer advantage.

Green coffee is a worldwide commodity in large volume, trading in which is not much impeded by legislation; terminology, resulting from numerous languages (primarily Spanish, Portugese, French and English) is, however, often confusing, and methods of tests often dissimilar. The present industry practice of avoiding 'unclean' coffee represents all the internationally decided control that seems necessary. International standardisation of terminology and test methods will, however, be a marked convenience to trade. Roasted coffee (and closely related products) is, however, a negligible element in world trade but soluble coffee, particularly so-called off-shore manufactured (i.e. from Brazil and elsewhere) is increasing in importance which has not been diminished by existing national legislation in the developed countries. Certain roasted coffee products are distinctly national in character, such as sugar-roasted coffee (e.g. *cafe tostado* in Spain), whilst roasted whole beans are given a 'shine' by enrobing materials (France and Holland). In both instances, the qualities and quantities of materials used are closely specified in these countries and appropriate designations made. This paper does not deal with additions of coffee substitutes, such as roasted malt extract, but again national legislation clearly defines labelling requirements to show these additions.

STANDARDS FOR GREEN COFFEE

Commercial standards for green coffee have been developed over a number of years by the producing organisations in the various green coffee countries and also by the New York Coffee and Sugar Exchange. Such standards (Anon., 1972) in themselves have no statutory role although, in the Latin American countries, much of the content of these standards, especially in their 'minimum quality' aspects, has been incorporated into governmental law by decrees and so on. In the former French-speaking colonies (now called OAMCAF countries) and in France itself, the link between commercial standards and legislative standards is even stronger. French law (Dehove, 1970) (primarily, the decrees of 1965) provides a comprehensive set of

regulations for controlling the import of green coffee. In the former English-speaking colonies, now independent, there are also governmental regulations on the type of green coffee that can be exported. In the UK itself, however, there are no special regulations with detailed standards on the importation of green coffee. The situation is the same with the former colonies, now independent, of Holland (e.g. Indonesia) and of Belgium (e.g. Zaire). Portugal had also a comprehensive set of regulations governing export of coffee from its formerly dependent overseas territories, presumably now adopted by these countries themselves (e.g. Angola).

The International Coffee Agreement of 1962 (International Coffee Organisation, 1962) is also an important aspect of green coffee standardisation, particularly Article 2 which provides definitions, and Article 42, which provides for, and insists on, certification of origin for exported coffee.

TABLE 2
GENERAL NATURE OF GREEN COFFEE STANDARDS

Item	*Type of description*
Definition	
Designation	
Composition	
Moisture content:	% Maximum
Quality	
Bean size:	Screen analysis
	Grade numbering/lettering
Defective beans:	Quantity per sample (count or weight)
Extraneous matter:	Commercial—type numbers
(coffee or non-coffee)	Legislative—maximum allowable

Green coffee is exported in bags, so that required bag markings are an important element of standardisation (i.e. Marking and Labelling).

The general nature (Clarke, 1972) of these green coffee standards and items (in relation to a sample which is taken) is shown in Table 2.

Definition and designation

The definition of green coffee as a natural product is fairly self-evident. Coffee is defined by the ISO as: 'General term for the fruit and seeds of plants of the genus Coffea, generally the cultivated species, as well as the products from these fruits and seeds in different stages of processing and use, intended for consumption'. Green coffee is coffee beans, with a note to say that green coffee so-called is not necessarily green in colour. A coffee bean is then a commercial term designating the dried seed of the coffee plant.

Bag markings refer to the country of origin (and usually grade/type) and bags contain green coffee of a particular species (i.e. Arabica or Robusta).

Composition

The important element is the 'moisture content' not commercially specified (often low, in fact) but under control to the point of delivery. Some national legislation (e.g. French) limits to $12\frac{1}{2}\%$. Caffeine content is a slight variable, averaging at two distinct values according to species.

Defects

Defects is a term used in commercial practice to describe the quality factor of the amount of defective beans and of extraneous matter taken together. This quality factor is more relevant, however, to dry process (e.g. Brazils and most Robustas) than to wet process green coffees. In the latter, the quantity of defective beans from such countries as Kenya and Colombia is generally negligible. The main types of defective beans are itemised in Table 3 with their French and Brazilian equivalents.

TABLE 3
DEFECTIVE COFFEE BEANS

English	*French term*	*Brazilian term*
Dried Coffee Cherry	Cerise seche	Grao em coco
(as above, small)	—	Grao marinheiro
Broken bean	Feve brisée	Grao quebrado
Bean fragment	Brisure	—
Black bean	Feve noire	Grao em preto
Semi-black bean	Feve demi-noire	—
Insect-damaged bean	Feve endommagée par insectes	Grao brocado
Sour bean	Feve sûre	Grao ardido
'Stinker'	Feve puante	—
Immature bean	Feve immature	Grao verde
White bean	Feve blonde	—
Withered bean	Feve ridée	—

In systems which involve physical counting, grades (types) are assessed according to the number of defects present per sample (300 g weight in metric countries; volume basis, 36 in^3 or approximately 1 lb US). However, not all defective beans or other defects are considered to make the same contribution. The New York Coffee and Sugar Exchange devised the 'Black-Bean-Equivalent' count basis; that is, all other defects are assessed as requiring a number to equal one black bean. Brazil also uses this system. The French system is similar except that special attention is also paid to the sour bean. The comparable ratings of some of the main types are illustrated in Table 4. The total number of equivalent defects is determined and maxima–minima established for each grade (type) e.g. NY4 means 30 maximum defects present (Brazil, 26 per 300 g sample) and NY3 means 13 (12). Mixed types are more usual, e.g. NY2/3 (Extra) means 7–12 and NY4/5 (Superior) 33–57. The French system is similar, with types known as 'Prima' (30) 'Superior' (60) and 'Courante' (120), Limite (180); Extra Prima (15 maximum). The Portuguese had a similar system. These

TABLE 4
EQUIVALENCY RATINGS OF GREEN COFFEE DEFECTS

Defect	*NYCSE System (Brazils)*	*French*	*Portugese*
Dried Coffee Cherry	1	1	1
Black	1	1	1
Insect-damaged	1/5	1/10	
Sour	$\frac{1}{2}$	1	$\frac{1}{2}$
Immature	1/5	1/5	

grades are not incorporated into legislative requirements, except for minimum importable/exportable quality. Thus green coffees with more than 120 defects cannot be imported into France, and similarly in the USA where direct legal action can be made, taken by the FDA, against importation of poorer grades than NY8. Maximum numbers of particular defects are also subject to legislation specifically (Table 5). Foreign matter (including stones) is also often separately assessed by weighing, both within the various grades (types) and also to provide a legislative limit for export/import (Table 6). The better types contain substantially less.

TABLE 5
MAXIMUM INDIVIDUAL DEFECTS IN GREEN COFFEE

France (Dehove, 1970)	*USA (Anon., undated)*
Five 'blacks' Five 'cherries' 15 brisures 15 insect-damaged (i.e. 150 actual in, say, 2000 beans (300 g usual) = 7·5 % by weight	Maximum average of 10 % or more insect-infested damaged, including mould

Bean size

Commercial standards also define bean size and range, as a result of laboratory screen analysis, using screens with round holes, numbered from No. 17 to about No. 10. These numbers refer to the diameter of the holes in 1/64 in increments. This numbering system is universally used, although the actual hole size may be specified in metric units. Insofar as screening methods (whether actual large screens or air-lifting devices) are used for the removal of small beans, foreign matter and defectives, minimum quality is also defined by a maximum amount allowable

TABLE 6
MAXIMUM EXTRANEOUS MATTER IN GREEN COFFEE

France and OAMCAF	*Portugal*	*Brazil*
0·5 % by weight	1·2 % by weight	Assessed in defect counts

through the smallest screen used. For example, screen No. 12 when the French limit the maximum through to 6% (but retained by No. 10) for the poorest grade, similarly the Portugese had a limit of 6% of 'fundos' coffee.

Traded green coffees in various specified ranges of bean size are characterised by either size designations (e.g. 'bold', 'medium', etc.) or grade numbers/letters (e.g. Ivory Coast Grade 2 (Note: 2 refers only to size grading) or Kenya A/B.

The undersize products of commercial screenings—including products of hand-picking/electronic sorting for defects—are generally not exportable/importable. The French refer to these operations as triage. Some names of these products are as follows:

Colombia	—Pasillas
Brazil	—Grinders (equivalent to > grade 8)
France	—'Residu du triage'
Mexico	—'Manchadoes'
Central America	—'Imperfectoes'

Test methods sampling and terminology

It will be clear from the foregoing that such standards covering such a large number of countries will not be uniform in respect of the test methods used, nor in terminology applied, and are primarily national. It is in this field that

TABLE 7
ISO STANDARDS FOR GREEN COFFEE

DIS 4072	'Green Coffee in Bags—Sampling'
N 263	'Coffee Tryer'
R 1447	'Green Coffee Beans—Determination of Moisture' (now under revision)
DIS 3509	'Glossary of Coffee Terms'
DIS 1445	'Test Methods Concerning Green Coffee' (now under revision)
N 422	'Screen Analysis—Manual Sieving' (now DIS 4150)
352	'Insect Damaged Beans'
423	'Olfactory and Visual Examination and Determination of Foreign Matter and Defects' (now DIS 4149)

ISO/TC34/SC15 has been particularly active; so far the documents listed in Table 7 have been prepared. DIS is a draft International Standard, and R- Recommended, of ISO.

Other quality factors

Brazilian coffee usually has specifications covering average colour of beans, characteristics of the roasted coffee (degrees of uniformity) and result of cup-testing (to assess presence or absence of hard and medicinal Rioy flavour).

Health and hygiene factors

In coffee agriculture, residues can be left from chemicals used for fertilisation, insect control, plant disease control or microbial inhibition in green coffee

processing. The health risks of such residues are well known and many countries, especially the USA, have regulations for use and maximum allowable before acceptance. Foreign matter is generally non-toxic and there are both legislative and commercial standards for maximum levels, the remainder being removed in cleaners at good coffee product manufacturing plants. Its impact is primarily economic, being material useless to the manufacturer. From both tropical sources and in subsequent shipping/warehousing, insect-damaged beans are regarded as defects, subject to legislative control. Their presence is also unaesthetic in roasted whole beans. The actual presence of insects or their remains from tropical countries is regarded as unsanitary, as are rodent droppings, etc. from warehousing; here again legislation exists to prevent importation and usage. This examination is carried out on total bags, rather than samples. An excellent survey of the legislative procedures used to control these factors in the USA is given in the booklet entitled *Health and Safety in Importation of Green Coffee* (Anon., undated). It includes the allowable pesticides and their maximum residual levels for the USA.

The numbers of mouldy beans are similarly subject to control; aflatoxin development from mould growth in coffee beans has not been found to occur, as reported in a comprehensive survey (Borker & Levi, 1975) but mouldy beans are usually undesirable in respect of flavour/odour.

STANDARDS FOR SOLUBLE COFFEE

Most of the standards that have appeared for soluble coffee are governmental. Since its general introduction after World War 2, most countries in Europe and America have national legislation setting out statutory requirements for this product when sold to the consumer by retail. These requirements differ somewhat in extent and

TABLE 8
GENERALISED LEGISLATIVE REQUIREMENTS IN SOLUBLE COFFEE

Item	*Type of Specification*
1. Definition Designation Composition	Restrictions
2. Extraction yield	Restriction
3. Dryness	
(a) Moisture content	% maximum
(b) Dried matter content	% minimum
(c) Dried soluble content	
4. Caffeine content	Allowable range
Additions	
5. Allowable additives	Type and maximum
Quality (Purity)	
6. (a) Insolubles content (b) Non-coffee substance content	Maximum amount

type over different countries. In the UK they are included in the Coffee Product Regulations of 1971 which are relatively straightforward.

The technical factors or items which are generally found in national regulations are shown in Table 8 whilst Table 9 shows the individual requirements by the countries comprising the EEC. As is now well known, an EEC Directive for Coffee Products was adopted on 12 July, 1977 and has to be introduced into national law in

TABLE 9
NATIONAL AND EEC HARMONISED REGULATIONS FOR SOLUBLE COFFEE

Item No.	*UK*	*France*	*Germany*	*Italy*	*Denmark*	*Benelux*		*EEC*
						Holland	*Belgium*	
1		Water extraction only	Water extraction only			Water extraction only, filtered	Water extraction only, filtered	Fully defined
2				2·3:1				2·3:1
3 (a)		3·5%		5·0%		—	—	—
(b)		—		—		96%	96%	96%
(c)	95%	—		—		—	—	—
4						3–6%	3–6%	Not specified
5	100 ppm of substances not specified	50% of carbohydrates (with label declaration)			Ascorbic acid, 500 ppm. Fruit acids. Flow agents, 1% maxm.	10 ppm residual polysiloxane. Flow agents (0·5%)		None (Flow agents for vending machines—national)
6	100 ppm							Traces
Date	1971	1965	1931	1976	1976	1968	1968–69	1977

Note: Blank spaces mean no reference to item.

these same countries. Table 10 shows the requirements for the same items in various non-EEC European countries, the USA and Kenya. There are additional factors for decaffeinated soluble coffees, discussed in a later section.

Definition and designation

Most European countries have made a point of insisting that soluble coffee be made from roasted coffee by extraction using water only (which includes steam) although technology is available to extract aromatic substances by means of various solvents (including liquid and gaseous carbon dioxide). This view is reflected also in the EEC Directive but this makes allowance for the commercial reality of added coffee aroma oils prepared by mechanical pressing of roasted coffee.

TABLE 10
NATIONAL STANDARDS FOR SOLUBLE COFFEE

Item No.	*US Federal Specifications (Supply agencies only)*		*Sweden (Regulatory)*	*Spain (Regulatory)*	*Kenya (Std.)*	*Soviet Union GOST/HRTU* 5.863-71
	Type 1.1	*Type* 2				
1	Defined (also coffees used)			Water extraction only	Dehydration of aqueous extract of pure freshly roasted coffee	
2						
3 (a)	3·0	2·6	5·0%	4·0%	3·5%	3·8%
(b)	—	—	—	—	—	
(c)	—	—	—	—	—	
4	3·2% min.	2·2% min.			2·8% min.	3·0% min.
5	Ascorbic acid, 15 mg per $2\frac{1}{2}$ g					
6	Sediment pad No. 2 maximum				Free of impurities	
Others					Solubility max. ash content	Solubility. Ash 10% minimum pH 4·8 minimum
Date	1974		1970		Draft 1977	1971

The EEC Directive carries a definition of soluble coffee whilst ISO has provided a similar but shorter definition in DIS 3509.

Designation

The EEC Directive only allows the terms 'soluble coffee', 'instant coffee', 'dried coffee extract' or 'dried extract of coffee' in the English language and covers the product as defined and described with two compositional requirements and allowing no additions (except nationally of flow agents in products designed for vending machines).

Compositional

The EEC Directive carries the somewhat unique element of restricting the amount of soluble coffee to be taken from the original green, that is, the soluble coffee 'is obtained by using for its manufacture a quantity of raw coffee of at least 2·3 kg per 1 kg finished product'. The reason for this was clearly stated in the motivation section submitted with the draft proposal in 1973 and the actual figures arrived at by consultation with the Eoropean soluble coffee industry. The same figures entered into national Italian law in 1976 and a lower figure of 3·0:1 featured for a time in French law between 1965 and 1967.

'Dryness' is the most obvious requirement of any soluble coffee legislation but in fact is fraught with problems in specification. Some countries define a maximum 'moisture' content for the soluble coffee product, which is difficult to measure directly as H_2O alone, even if needed. In practice, a convenient method is to determine the percentage loss of weight (mass) on drying by the application of heat under specific conditions so that an alternative means of expression is the percentage dry matter content. The present UK regulation is anomalous in that it defines a minimum soluble solids content which is only directly measurable by this method if allowance is made for any insoluble matter (in water), whether from coffee or not.

The caffeine content of soluble coffee is always specified when the product has been decaffeinated and there is general agreement with the figure of 0·3 % maximum. Decaffeinated soluble coffee is a relatively new variant of soluble coffee and this figure reflects an earlier figure of 0·08 or 0·1 % for decaffeinated roasted coffee. With normal soluble coffee that has not been decaffeinated the need for such a specification is questionable, indeed it does not appear in the EEC Directive; the previous national requirements in Holland and Benelux for a range between 3–6 % seem unrealistic.

The caffeine content of a soluble coffee will be primarily determined by the type of green coffee used.

A soluble coffee made from 100 % Brazilian coffee taken at a typical yield (say 35 %) can readily be shown to have a caffeine content of between 2·0 and 3·0 % as actual data of Brazilian coffees shows (Angelucci *et al.*, 1973). The higher limit is equally unrealistic when we compare with the caffeine content of a 100 % roasted Robusta coffee for home brewing, so frequently consumed in France and Italy, which can reach about 9 %. A recent paper (Volper & Solovyera, 1976) in an East German scientific journal by two Russian authors, making a plea for a maximum of 4·5 %, contains the specious argument.

Additions

Some soluble coffees are not especially flowable, a characteristic needed in dispensing from vending machines, so that flow agents may be needed, although manufacturing practice is in fact quite capable of making flowable instant coffee without such additives.

Soluble coffees are convenient vehicles for fortification by nutritive substances, such as iron and vitamins, as optional additives. Opinions differ on the desirability of so doing, even assuming and assuring that such additives used are fully cleared by other horizontal legislation. These allowably included substances are a feature of further Danish legislation, and in US federal specifications for supply agencies (General Services Administration, USA, 1974).

Anti-foaming agents, such as dimethyl polysiloxane, similarly cleared, are often requested for allowable use in soluble coffee. Present UK regulations allow 100 ppm maximum of substances not derived from coffee, tacitly for this purpose,

but would require ingredient declaration. Dutch coffee legislation specifically allows 10 ppm in the finished product. Sulphur dioxide as a preservative, present at not more than 150 ppm, for coffee extract, inexplicably appears in UK horizontal legislation.

Opinions differ on the desirability or otherwise of being able to add nature-identical flavour and odour substances, though the technology is available.

Quality (Purity)

Technically, the presence of some insoluble coffee (or fine particles of extracted coffee grounds) is difficult to avoid. Measurement of this actual quantity is also difficult and will include necessarily very small amounts of non-coffee insolubles that may be present. National regulations are generally silent but the EEC Directive allows 'traces' of such substances. The US federal specification (General Services Administration, USA, 1974) for instant coffee provides for assessment by the sediment pad test, used in the milk industry. Some particles of soluble coffee will be found to be of colloidal dimensions (i.e. below 100 microns) to add a further difficulty in determination.

The water used for commercial extraction will necessarily introduce mineral salts according to its hardness.

The EEC Directive sums up by saying that coffee extract may not contain any substances other than those derived from its extraction.

TABLE 11
ADDITIONAL LEGISLATIVE REQUIREMENTS FOR DECAFFEINATED SOLUBLE COFFEE

Item	*Type of Specification*
7. Caffeine content	% Maximum allowable
8. Decaffeinating solvent	Description and purity
9. Solvent residues	Maximum residual solvent (in ppm)

Decaffeinated soluble coffee

The additional requirements (actual or under consideration) for the variant of soluble coffee, in the EEC countries and in the harmonised Directive, are shown in Tables 11 and 12.

Methods of Test/Analysis

National regulations in general have not specified the exact methods of analysis to be used but for EEC harmonisation this matter is under consideration for a further Directive including the method of sampling. ISO/TC34/SC15 have interested

TABLE 12
NATIONAL AND EEC HARMONISED REGULATIONS FOR SOLUBLE COFFEE (ADDITIONAL FOR DECAFFEINATED)

Item No.	*UK*	*France*	*Germany*	*Italy*	*Benelux*		*EEC*
					Holland	*Belgium*	
7	0·3%	0·3%	0·3%	0·3%	0·3%	0·3%	0·3%
8	Now being considered in horizontal legislation	Solvents without chemical action, and not depriving product of useful constituents		Only ethyl acetate and dichlor methane of specified purity			List being determined by the Commission together with their purity
9		Removed		5 ppm EtAc, DCM			As above
Date	1971	1965	1977	1976	1968	1968–69	1977

themselves in various test methods to establish their validity and usefulness by 'round-robins' as follows:

DIS 3726 'Moisture Content of Soluble Coffee (Vacuum Oven—Mass Loss at 70 °C for 16 h) (under revision).

4052 'Determination of Caffeine Content' (Levine method).

and currently, establishing suitable methods for the sampling of soluble coffee, in bulk, solvent residues in decaffeinated coffee and insoluble matter in soluble coffee.

The US federal specification (General Services Administration, USA, 1974) sets out the required methods of analysis and of sampling for use with their standards of packaged soluble coffee in governmental supply agencies.

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THE VOLATILE CONSTITUENTS OF TEA

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(Received: 4 January, 1978)

ABSTRACT

This paper reviews the chemistry of the volatile constituents of black tea with special reference to their origin and the influence of climate, differences between cultivars and processing on their composition. This information is discussed with the object of assessing the way in which some of the volatile compounds affect the flavour of tea. Attention is drawn to gaps in present knowledge.

INTRODUCTION

The identification of more than 200 volatile constituents of black tea has been reported in the literature and many more have been detected but not identified. Comprehensive reviews of tea chemistry, including full bibliographies, have been published within the past five years by Sanderson (1972), Sanderson & Graham (1973) and Natarajan *et al.* (1975) so it is not necessary to mention all these compounds, or even all of the publications, here. This paper summarises what is known of the origin and relative importance of these compounds and those factors in growing and processing that influence the composition of tea aroma. The interpretation of these data is then discussed.

In assessing the relative importance of any volatile constituents that might be detected by gas chromatography one must apply two important principles. The first is that the concentration of a compound in the air reaching the olfactory organ must exceed a certain minimum value (the threshold value) before it can be detected. This value varies from one compound to another over a range of many orders of magnitude. Consequently, a compound that produces a large peak in a chromatogram might contribute little to the aroma while one that is not detected might be extremely important.

Fd. Chem. (**4**)(1978)—© Applied Science Publishers Ltd, England, 1978
Printed in Great Britain

Secondly, solutions containing different concentrations of the same compound cannot be distinguished unless the difference exceeds a certain value. For individual compounds this difference threshold is usually between 30% and 50%, but in complex mixtures it might be much higher.

The effect of mixing odorous chemicals is usually additive but synergism and antagonism are known to occur. In complicated mixtures several compounds with the same type of odour might be present and in such cases it is necessary to consider the concentration of the group as a whole rather than that of individual compounds.

The essential oil of tea forms only 0·2% of black tea at most and yields below 0·02% have been reported, so that the brew contains between 5 ppm and 50 ppm of essential oil.

According to Kobayashi *et al.* (1965), about half the oil consists of linalool and its oxides, while none of the remaining 45 compounds detected represents more than 7% of the total. Consequently, only compounds or groups of compounds with extremely strong odours could have much influence on the odour, which excludes the terpene hydrocarbons, the C_1 to C_3 alcohols, acetone and esters with less than five carbon atoms, all of which have relatively weak odours. Such compounds could be detected easily in air by GLC at levels well below their threshold values, where β-ionone, for example, could not be detected until its concentration was many times the threshold value of about 6×10^{-8} g/m^3 of air.

AROMA PRECURSORS

Amino acids

The concentrations of free amino acids in the green leaf increase during withering and decrease during fermentation (Roberts & Wood, 1957; Bhatia & Deb, 1965) which suggests that they may be precursors of other compounds. Co & Sanderson (1970) homogenised fresh tea leaves, added amino acids labelled with C^{14} and allowed the mixture to ferment at 25 °C for 30 min. The vapours in the headspace were then analysed by GLC using both flame ionisation and radioactivity detectors. It was found that leucine, *iso*-leucine, valine and phenylalanine were partially converted into *iso*-valeraldehyde, 2-methylbutanal, *iso*-butyraldehyde and phenylacetaldehyde, respectively, which would be expected to arise from Strecker degradations and which are known to be constituents of tea aroma. The subsequent drying of the fermented mixture caused additional quantities of aldehydes to be formed. In a similar study; Saijo & Takeo (1970*a*,*b*) showed that aldehydes were produced only if both enzymes and polyphenols were present.

The addition of other labelled amino acids to homogenised fresh leaves under the same conditions did not give rise to any detectable volatile radioactive compounds. When the enzymes were deactivated by steam treatment, no volatile compounds were formed from any of the amino acids. Identical results were obtained from a

model system consisting of crude tea-leaf enzyme extract, epigallocatechin gallate and amino acids labelled with C^{14}.

Ascorbic acid inhibited the formation of aldehydes, while dehydro-ascorbic acid produced them without the aid of enzymes, albeit with only about a quarter of the efficiency of the enzyme system.

Since the presence of tea flavanols is required for the production of aldehydes, and the process is inhibited by the reducing agent ascorbic acid, it is probable that the Strecker degradation is brought about by the oxidised flavanols which are formed by the polyphenol oxidase in tea leaves.

Linolenic acid

Linolenic acid is the major constituent of the tea leaf lipid. When Gonzalez *et al.* (1972) added this acid, labelled with C^{14}, to pulverised tea leaves and allowed the mixture to ferment for 3 h, the only volatile compound found to contain C^{14} was *trans*-2-hexenal. This compound is well known as a constituent of green leaves and has a strong grassy odour. As with the conversion of amino acids into aldehydes, this reaction did not occur when the enzymes were inactivated by steaming. Later work showed that this transformation was brought about by the action of a metalloprotein rather than a true lipoxidase (Coggon *et al.*, 1977).

Carotenes

Several studies showed that the carotenes present in fresh tea leaves decrease during fermentation, especially during the early stages. Tirimanna & Wickremasinghe (1965) suggested that this decrease might be due to the conversion of carotenes into volatile flavouring constituents. This hypothesis was substantiated by Sanderson *et al.* (1971) who demonstrated that β-carotene was converted into β-ionone and other unidentified compounds during tea fermentation. It was found that this degradation occurred readily in macerated tea leaves but not in the relatively dilute model system, unless tea flavanol oxidation had taken place and the reaction mixture was evaporated to dryness.

Sanderson & Graham (1973) listed a number of compounds that could be formed in this way. α- and β-carotenes would form β-ionone, several substituted ionones, and products of the further oxidation of ionones (such as theaspirone and 2,2,6-trimethyl*cyclo*hexanone), while other carotenoids would be expected to give linalool and several terpenoid aldehydes and ketones. More recently, Reynolds *et al.* (1974) identified β-damascenone, α-damascone and β-damascone which they considered to be derived from carotenoids.

These reactions are known to be affected by the amount of catechins present, the quantity and composition of the carotenoids, oxidase activity, the degree of mixing of cell contents and the concentration of reactants. The concentration might be partly determined by the degree of wither, and the mixing of cell contents will be largely determined by the severity of the crushing and bruising brought about by the roller, CTC machine or whatever process is used.

Other precursors

The oxidation of alcohols to aldehydes and thence to acids is an obvious possibility and the increase in acids during firing suggests that it does, in fact, occur (Yamanishi, 1967).

VARIETAL DIFFERENCES

The consideration of differences between types of tea can provide some indication of the relative importance of the constituents. Since much of the work on volatile constituents of black tea has been done on non-flavoury types it is clear that lack of the quality known to tea tasters as 'flavour' is not synonymous with lack of volatile constituents.

The work of Bondarovich *et al.* (1967) on the essential oils of six teas of different origin showed that the detectable differences were quantitative and were mainly in the less volatile constituents.

In the following year Yamanishi *et al.* (1968*a*) separated tea oils into fractions with different functional groups. The GLC analysis of the acid fractions as the methyl esters produced a different pattern from each oil but there was no relationship between it and the type of tea. Similar results were obtained from an analysis of the phenolic fraction. Although the carbonyl fraction formed a very small proportion of the oils, it had a very strong aroma. Uva tea seemed to contain more carbonyls with a light, sweet odour than a Shizuoka tea of the Benihomare type which contained more heavy flowery odours. Darjeeling tea contained unidentified carbonyls with a heavy aroma.

The bulk of the oil consisted of neutral non-carbonyl compounds, mainly alcohols, the odour of which closely resembled that of the whole oil. Quantitative analysis of the alcohols in eight black teas showed that the total content of linalool and linalool oxides was very characteristic. It is high in the flavoury teas, Dimbula, Uva, Nilgiri and Darjeeling, and low in the plain Shizuoka tea, providing at least an empirical GLC method for distinguishing between flavoury and non-flavoury teas. Since linalool and its oxides form well over half of the alcohol fraction in flavoury teas, five or six times as much as in non-flavoury teas, and linalool has a strong, agreeable odour, it is probably more than a fortuitous indicator, although it is only one of the important contributors to the aroma of flavoury tea.

Yamanishi *et al.* (1968*a*) found that the ratio of the total area of GLC peaks emerging before linalool to the total area of peaks emerging after linalool was lower in 'flavoury' teas than in 'non-flavoury' ones. In their analysis of the headspace vapours, which contain the more volatile substances and probably all the compounds that would be eluted before linalool, five peaks account for roughly 60 % of this very volatile material; there is a mixture of dimethyl sulphide (which has an extremely strong odour, reminiscent of seaweed), with propanal, *iso*-

butanal/acetone, *n*-pentanal, *iso*-pentanal and *n*-hexanal. All these aldehydes occur in rancid fat and have strong, obnoxious odours. Also present were 1-pentene-3-ol and *trans*-2-hexenal which have strong grassy or beany odours; an excess of the latter is known to be deleterious to tea aroma. Low concentrations of substances with unpleasant odours can, and often do, make a positive contribution to a perfume or flavour but larger amounts are damaging.

Yamanishi *et al.* (1968*b*) attempted to throw more light on the subject by comparing flavoury, high-grown Ceylon teas with non-flavoury, low-grown ones. Unfortunately, during 1968 their chosen high district failed to produce the expected highly flavoured teas. However, they found that high-grown teas produced more linalool and linalool oxides than the lower grown ones, and one of their high-grown samples that did exhibit slight flavour contained more geraniol and jasmone than the others. They also compared the headspace vapours and found that the low-grown teas contained far more very volatile compounds than the high-grown ones. These results are in keeping with the principles of flavour chemistry outlined above.

Wickremasinghe *et al.* (1973) compared a high-grown flavoury Ceylon tea with a high-grown non-flavoury Ceylon tea and found that, as expected, compounds with retention times longer than that of linalool were present in higher concentrations in flavoury tea than in non-flavoury tea. From their data it may be calculated that the ratio of the sums of peak areas emerging before linalool to those emerging after linalool was 1·04 for flavoury tea and 4·5 for non-flavoury tea. The flavoury tea also contained more linalool and linalool oxides than the non-flavoury one, in agreement with Yamanishi's earlier work.

Gianturco *et al.* (1974) expressed the volatile constituents of tea in terms of four parameters, one of which (parameter A) was the sum of the areas of the peaks produced by linalool and two of its oxides and represented a light, floral note. The second parameter (B) was (A) divided by the area of the *trans*-2-hexenal peak which they describe as having a coarse, vegetable aroma. The third (C) was the sum of peaks representing hexanal, 1-penten-3-ol, *cis*-2-penten-1-ol, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol (which combine to give a fresh, leafy aroma) divided by the *trans*-2-hexenal peak. The fourth parameter was more complicated but again represented a balance between fresh and coarse green odours. This system seemed to work quite well in that it classified four teas of good flavour into groups which corresponded well with the rank order given by a tea taster. It is perhaps surprising that they did not find it necessary to take into account the terpenoid compounds such as jasmone and geraniol. Nevertheless, this work demonstrates clearly that the quality of tea flavour depends upon a delicate balance between pleasant and unpleasant odours, and confirms the importance of linalool.

Over the years a number of authors have noted that certain compounds are desirable or otherwise. Saijo (1973) analysed flavoury and non-flavoury teas and identified more than 130 compounds. He concluded that linalool, linalool oxides and methyl salicylate seemed to contribute to good flavour while acetic acid,

benzaldehyde, 3-methylbutanol, benzyl alcohol and β-ocimine were undesirable. Kozhin & Treiger (1973) reported that good Georgian tea aroma was associated with linalool oxides, salicylaldehyde, methyl benzoate, α-terpinyl acetate, bornyl acetate, phenylacetaldehyde, borneol and acetophenone, while hexanal, 'leaf aldehyde', benzyl alcohol, *m*- and *p*-cresols and linalool were deleterious.

Nakabayashi (1956) found that superior black teas had a low carbonyl content and this was confirmed by the analysis of a large number of South Indian teas which showed that high quality tea contained 30 to 45 ppm of carbonyls while poor teas contained 60 to 70 ppm (Anon. 1968, 1969).

The structure of theaspirone was elucidated by Ina *et al.* (1968) who found that this compound, together with the lactone of 2-hydroxy-2,6,6-trimethyl *cyclo*hexylidene-1-acetic acid in suitable proportions in extremely dilute solutions had a fragrant, tea-like odour. Both compounds occur in tea.

A number of nitrogen compounds, mainly substituted pyridines, pyrazines, quinolines, thiazoles, aromatic amines and N-substituted amines, were discovered in tea by Vitzthum *et al.* (1975) but no estimate was made of the extent to which these compounds contribute to the aroma of black tea.

Methyl anthranilate, geranyl acetate, γ-valerolactone, γ-heptalactone, γ- and δ-decalactone and the lactone of 5-hydroxydeca-7-enoic acid were found by Cazenave *et al.* (1974) who described them as fresh, floral–fruity or slightly green and generally desirable. They probably form part of Yamanishi's desirable fraction which emerged after linalool on her PEG 6000 column.

A further 68 compounds in black tea were discovered by Reynolds *et al.* (1974). They were mainly unsaturated aliphatic aldehydes, ketones (including some aliphatic ketones that could result from lipid degradation and cyclic ketones, including β-damascenone and β-damascone, which are thought to be derived from carotenoids), some unsaturated aliphatic esters (such as hexenyl propionates), some terpene alcohols and compounds related to theaspirone.

Yamanishi *et al.* (1973) reported the identification of methyl jasmonate and several lactones, including jasmine lactone, in a flavoury Ceylon tea.

EFFECT OF CLIMATE

It is well known that very flavoury teas are produced by certain cultivars in well-defined districts at certain seasons. For example, in Sri Lanka very flavoury teas are produced in January and February in the Dimbula district and in August and September in the Uva district. In both cases the outstanding aroma is produced in cool, clear, dry windy weather when the day temperature is about 20 °C falling to between 6 and 10 °C at night. These conditions must last for at least two consecutive weeks and even slight rain sets the process back to the beginning. The famed first and second flush Darjeeling flavour is produced under similar conditions when growth is

slow and the plants are in fact under climatic stress. According to Wickremasinghe (1974) these conditions cause a kind of natural withering with increased permeability of the chloroplasts which are often further damaged at this time by mites or other parasites so that photosynthesis takes place outside the chloroplasts as well as inside. The rate of fixation of CO_2 is also very low and chlorophyll production is reduced.

Under normal conditions, terpenoids are produced from acetate, but the extra-chloroplast pathway begins with leucine and leads via the carotenes to β-ionone, theaspirone, dihydroactindiolide (i.e. the lactone of 2-hydroxy-2,6,6-trimethyl-*cyclo*hexylidene-1-acetic acid) and rhodoxanthin, all of which have floral odours. Since the carotenes are converted into volatile compounds, the carotene content of flavoury black tea is low. In contrast, flavoury teas contain relatively small amounts of *trans*-2-hexenal which is derived from acetate *via* linolenic acid. *trans*-2-Hexenal has been shown to have a deleterious influence on flavour.

PROCESSING

In some early work on the changes during manufacture, Nakabayashi (1956) found that the acid value increased during rolling and the ester value reached a maximum during fermentation. Carbonyl compounds changed little during withering but increased tenfold during rolling and even more during fermentation. Prolonging the rolling and fermentation, especially at high temperatures, increased the production of carbonyl compounds.

The most comprehensive work on the changes that take place during withering, fermentation and firing is that of Yamanishi (1967). Her results showed that during withering the proportions of hexanol, nerol, *trans*-2-hexen-oic acid, *trans*-2-hexenol, linalool oxide (*cis*-furanoid), *n*-valeraldehyde *n*-hexanal, *n*-heptanol, *trans*-2-hexenol, *trans*-2-hexenal, *trans*-2-octenal, benzaldehyde, phenylacetaldehyde, *n*-butyric acid, *iso*-valeric acid, *n*-hexenoic acid, *cis*-3-hexenoic acid, salicylic acid and *o*-cresol all increased (especially the first three) while those of *cis*-pentenol, linalool, geraniol, benzyl alcohol, phenylethanol, acetic acid and propionic acid decreased considerably. During fermentation the amounts of most of the constituents, especially 1-penten-3-ol, *cis*-2-pentenol, benzyl alcohol, *trans*-2-hexenal, benzaldehyde, *n*-hexanoic acid, *cis*-3-hexenoic acid and salicylic acid, increased but those of linalool, nerol and geraniol decreased. Saijo & Kuwabara (1967) found that the formation of volatile compounds almost ceased when the fermentation was carried out under nitrogen. This is entirely consistent with the evidence that oxidising agents play an essential part in the conversion of amino acids and carotenoids into volatile compounds.

Yamanishi also found that most alcohols, carbonyls and phenolic compounds decrease sharply on firing, but acetic, propionic and *iso*-butyric acids increase

substantially. In view of their unpleasant odour it is reasonable to assume that too high a proportion of these acids would be detrimental to the flavour of tea. It seems therefore that desirable compounds are lost during firing and some undesirable ones formed. This work was done on Benihomare tea, however, and it would be interesting to see similar work on highly flavoury teas.

Firing normally takes about 20 min, during which time the moisture content of the tea falls from about 60 % to 3 % and the temperature of the leaf rises rapidly from ambient to about 65° and then slowly to about 85°. Sanderson & Graham (1973) suggested that while the moisture remains high, it is likely that the process of fermentation will be accelerated until the enzymes are finally inactivated by heat. They also suggested that in the presence of an oxidising agent (i.e. oxidised polyphenols) the high temperature and increasing concentration of reactants will produce very high oxidation potentials which would explain why some reactions only take place during firing. The most obvious example of this is the relative increase in the volatile fatty acids which might well be formed from the alcohols and aldehydes or even from some unsaturated compounds.

Kawashima & Yamanishi (1973) have shown that ten volatile constituents, including β-ionone, were produced by heating carotenes. This reaction would supplement their formation during fermentation, which was discussed earlier. Unfortunately, a comparison of the volatile constituents of fermented leaves with those of finished tea provides little information about the chemical reactions that occur during firing because a high proportion of the volatile compounds evaporate with the water.

CONCLUSIONS

The work on precursors has revealed at least some of the constituents required for a tea leaf to produce an aroma. Yamanishi (1967) has shown that beneficial compounds are not produced at any stage of manufacture, except possibly rolling. Linalool and geraniol are in fact reduced at every other stage of the process, providing a scientific explanation of the fact that while a good tea can be ruined by bad processing, a poor tea cannot be improved by good processing.

The aroma of a flavoury tea is evidently a balance between the heavier perfume of the terpene alcohols and ketones and the harsher green and rancid odours of the aliphatic acids and carbonyls which are produced during manufacture. Hence prolonged fermentation spoils the balance by producing excessive amounts of the aliphatic carbonyls. However, no information on the optimum conditions of withering, rolling and fermentation which are required for the production of good aroma has yet been published.

Yamanishi *et al.* (1967, 1968*a*, 1968*b*), Wickremasinghe *et al.* (1973) and Gianturco *et al.* (1974) have given enough information to provide a basis for the

screening of teas for 'flavour' by gas chromatography which could be of considerable practical value in plant breeding programmes, but this information was obtained by comparing teas that differed in more than one respect. Consequently, it is uncertain whether a difference observed by gas chromatography is actually responsible for a noticeable difference in flavour or whether it is merely an indicator for important differences that have not been detected by analysis. This uncertainty would be removed by systematic, quantitative experiments in which volatile compounds, known to occur in black tea, were added to tea liquor in order to ascertain their effect on its flavour.

Sanderson & Graham (1973) appear to assume that the enzymes are inactivated by the high temperatures at the end of the drying process, but there has been much informal discussion on this point. Since the temperature rises above 80° only when the moisture content has fallen to low levels, it is equally possible that the enzymes cease to be active not because they have been denatured but because of lack of moisture. It is well known that oxidation reactions are resumed when tea becomes moist, but no studies have been published describing the mechanism of the deactivation of enzymes during firing or of the reactions that take place in moist black tea.

Although black tea does not acquire its characteristic odour until it is fired, it is nevertheless true that this part of the process causes the loss of much of the volatile material that has been shown to be necessary for the production of good flavour and at the same time produces compounds which, if not wholly undesirable, can be deleterious if present in excess. Conditions in the drier are usually adjusted to produce the most economic drying and to avoid the stewing which can result from excessive humidity at the inlet, but no quantitative work has been published on the conditions necessary to obtain the maximum retention of volatile compounds or to produce the best balance between different types of compound. Such an investigation, including a study of any changes in the composition of the vapours in the drier exhaust that occur as drying proceeds, could well lead to an improvement in the aroma of some teas.

The numerous authors who have contributed to the literature of the volatile constituents of black tea have, between them, produced results from which conclusions of scientific and practical value can be drawn but clearly much remains to be done.

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FRACTIONATION OF BOLTI FISH TISSUE LIPIDS INTO CLASSES AND WITHIN THE TRIGLYCERIDE CLASS WITH TLC

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(Received: 10 September, 1977)

ABSTRACT

By utilising TLC with a developing system of petroleum ether-diethylether-acetic acid (70:30:2 v/v) and allowing the front to travel 18 cm, the best conditions for the fractionation of Bolti tissue lipids were achieved. Four distinct classes—triglycerides, free fatty acids, sterols and phospholipids—were detected. In addition, two other faint classes—hydrocarbons and diglycerides—were identified.

Silica gel impregnated with silver nitrate has been employed for the fractionation of the triglycerides into groups. Seven groups were found when 1·5% of ethanol was added to the developing system of chloroform–acetic acid (99·5:0·5 v/v). Raising the ethanol content to 2% resulted in the detection of eight groups.

INTRODUCTION

Chemical evaluation of fish oils has changed its direction and objectives with changes in the commercial uses of these products. Formerly the main advantage of fish oils was their content of vitamins A and D and the triglyceride vehicle which contained them received little attention (Holman, 1962).

TLC, first introduced by Stahl (1956), has proved to be a very powerful and discriminating tool in the analysis of the lipid component. Mangold & Malins (1960) were the first to reveal the high potential of this technique for the separation of lipids in general. While a great deal of information exists in the literature concerning the composition of fish lipids (for example, Tsuchiya, 1961; Worthington *et al.*, 1972 and Wessels & Andrew, 1974), far less is available in this respect for Egyptian fish lipids. Bolti fish, which belongs to the family Cichlidae, constitutes

Fd. Chem. (4) (1979)—
Printed in Great Britain

about half the catch from the Egyptian inland fisheries and provides the material for the present study. Most of it is marketed as so-called fresh fish. Thus the chemical nature of the tissue lipids of fresh fish, i.e. live fish, is of paramount importance.

MATERIALS AND METHODS

Materials

Fresh Bolti fish (*Tilapia nilotica*) were obtained from Lake Mariut immediately after landing (in this study 'fresh' should be taken to be synonymous with 'live'). The fish were caught by the ordinary fish traps usually used in the area of the lake. The fish ranged between 15·5 and 17·5 cm in length and between 150 and 170 g in weight, i.e. 5–7 fish per kilogramme.

The fish were transported to the laboratory in glass jars filled with water where they were killed instantly by an electric shock to the head. The fish used throughout this work were obtained during the course of the spring season (April/June) of 1974.

Methods of analysis

Preparation of fish sample: The fish were beheaded, cut from the back and the backbone and belly were removed, thus obtaining the edible portion, i.e. the flesh. This was passed rapidly three times through a meat chopper and the comminuted flesh was then kept in a refrigerator at 4 °C in airtight jars and analysed as quickly as possible.

Extraction of tissue lipids: Lipids were extracted according to the method suggested by Bligh & Dyer (1959). In this method each 100 g of sample was homogenised with a mixture of 100 ml chloroform and 200 ml methyl alcohol. After blending 100 ml of water were added, the homogenate filtered and the alcoholic layer separated in a separatory funnel. The chloroform layer contained the purified lipids.

Fractionation of the tissue lipids into classes: Fractionation was carried out according to the method of Mangold & Malins (1960). Thin layer chromatoplates of silica gel G 10–40 μ (Merck, Germany) were prepared and 250 μg of the lipids were then separated with a mixture of petroleum ether 60–80 °C, diethylether and acetic acid with two different ratios. The first (90:10:1 v/v) has a relatively lower polarity than the second (70:30:2 v/v). Each solvent system was used with two different migrating distances (10 and 18 cm). A commercially refined cottonseed oil was used as a reference material for the different lipid classes for two reasons—lack of pure materials of the different classes and the fact that the different classes of cottonseed oil had been fractionated and identified by Osman *et al.* (1977) in the same laboratory.

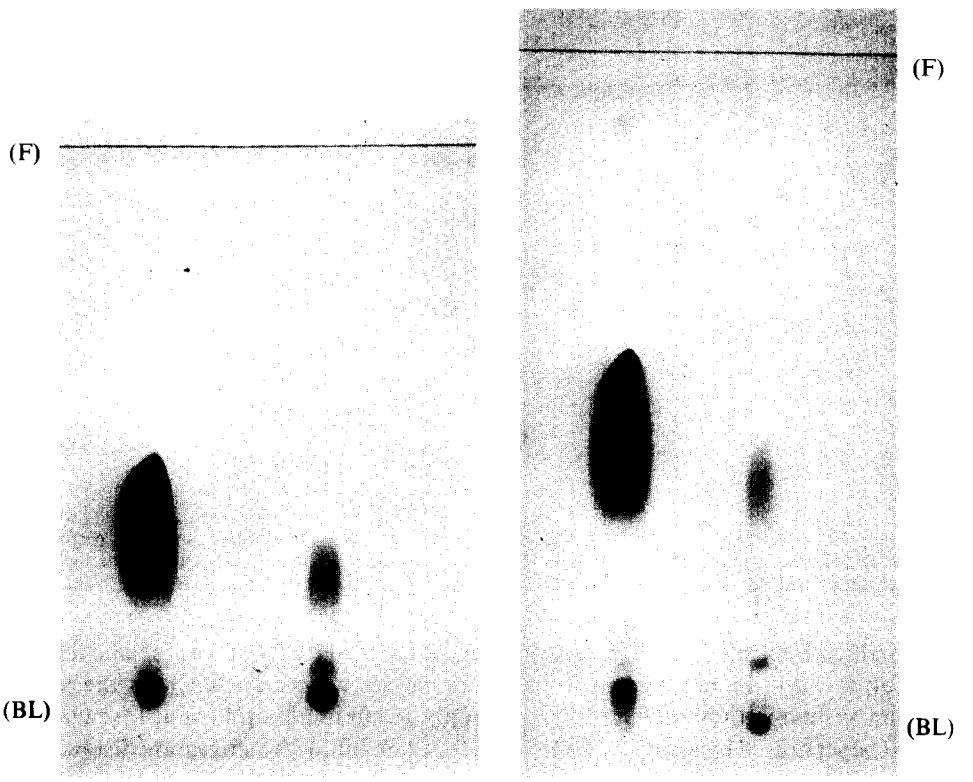
Methods of detection: Three methods of detection were used. (1) Spraying with 0·2′, 7′-dichlorofluorescein in ethanol followed by exposure to UV light; (2) exposure to iodine vapours; (3) spraying with chromic acid–sulphuric acid (5 g K dichromate in 100 ml of 40% sulphuric acid) followed by charring.

Separation of the different triglycerides: Chromatoplates of silica gel impregnated with silver nitrate were prepared. The glyceride mixtures were applied as a solution in chloroform. Development was by the ascending technique for 18 cm (65 min) with a mixture of chloroform–acetic acid (99·5:0·5 v/v) to which varying small amounts of ethanol were added. The plates were then dried and sprayed with 2′,7′-dichlorofluorescein and observed under UV illumination.

RESULTS AND DISCUSSION

Fractionation of Bolti tissue lipids into classes

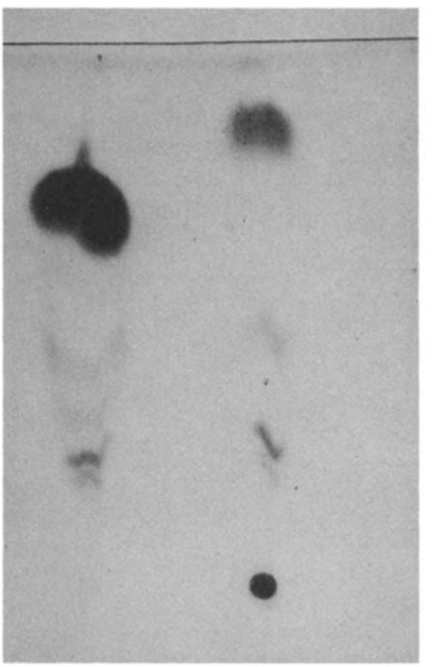
Figures 1 to 4 show that the solvent system petroleum ether–diethylether–acetic acid (70:30:2 v/v) with a migrating distance of 18 cm gave the best conditions for



Figs. 1 and 2. TLC of fish flesh oil. Coating material: silica gel G. Developing solvent: petroleum ether–diethylether–acetic acid (90:10:1 v/v). Migrating distance: Fig. 1 (left)—10 cm; Fig. 2 (right)—18 cm. Visualisation: charring with chromic acid–sulphuric acid, 250 μg amounts. A, Refined cottonseed oil; B, Bolti flesh oil. F, solvent front; BL, base line.

fractionation. Figure 4 shows four distinct classes as well as two other faint classes. These six classes would be, according to Malins & Mangold (1960), in the following sequence from the front to the origin: hydrocarbons, triglycerides, free fatty acids, diglycerides and phospholipids (which remain at the origin).

Mangold & Malins (1960) separated only three classes for fish oils—the triglycerides, the free fatty acids and the phospholipids. They used a solvent system

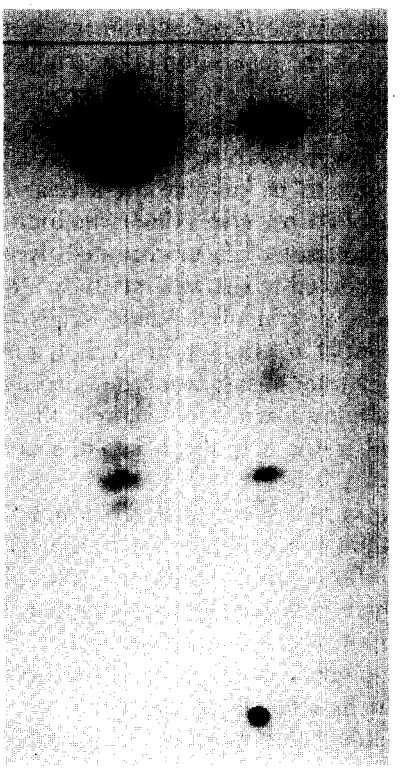


(A) (B)

Fig. 3. TLC of fish oil. Coating material: silica gel B. Developing solvent: petroleum ether–diethylether–acetic acid (70:30:2 v/v). Migrating distance 10 cm. Visualisation: charring with chromic acid–sulphuric acid, 250 μg amounts. A, Refined cottonseed oil; B, Bolti flesh oil.

consisting of petroleum, ether–diethylether–acetic acid (90:10:1 v/v) for one hour's migration only. In the present work, three classes were reported with the same solvent system letting the front travel for only 10 cm in about 40 min. Five classes could be separated by using the same solvent system but with a migrating distance of 18 cm in about 80 min.

Stansby & Olcott (1963) reported that the depot fat of fish consists primarily of triglycerides. Much of the lipid associated with the flesh cells occurred in the non-triglyceride form. Stansby and Olcott added that, in some species of fish such as haddock and cod, where the amount of total lipid content in the flesh is less than 1 %,



(A) (B)

Fig. 4. TLC of fish oil. Coating material: silica gel G. Developing solvent: petroleum ether–diethylether–acetic acid (70:30:2 v/v). Migrating distance: 18 cm. Visualisation: charring with chromic acid–sulphuric acid, 250 μg amounts. A, Refined cottonseed oil; B, Bolti flesh oil.

the vast majority of the lipids occurred as phospholipids and in other non-triglyceride forms. Olley & Lovern (1954) reported the lipids of haddock flesh to consist of lecithin, unidentified lipids, waxes and alcohols, free cholesterol, inositol lipids, cholesterol esters, triglycerides, hydrocarbons and plasmalogens.

Separation of the different triglycerides (within the triglyceride class)

The preliminary trials revealed that amounts of crude lipids between 10 and 20 μg were suitable for separation of the triglyceride fraction uncontaminated by other fractions. This is in agreement with the results of Barrett *et al.* (1963) who used a load of 1–3 μlitres at a concentration of 0·5 %. Silica gel impregnated with silver nitrate had been employed for the fractionation of the triglyceride class into groups according to the degree of unsaturation according to Barrett *et al.*, (1962) and Litchfield *et al.* (1964) who stated that the fractionation did not depend upon the presence of individual fatty acids but on the total degree of unsaturation.

Barrett *et al.* (1962) suggested the use of chloroform–acetic acid (99·5:0·5 v/v) as a developing solvent system for the separation of the triglycerides. The same authors also suggested another solvent system—carbon-tetrachloride–chloroform–acetic acid (60:40:0·5 v/v) to which varying small amounts of ethanol were added according to the type of glycerides to be separated. A level of 1·5% ethanol was found, in the present work, to be suitable for a clear fractionation. This is in agreement with the results of Barrett *et al.* (1963) who found that a level of 1 to 1·5% of ethanol improved the resolution of the unsaturated glycerides. These two types of developing system were adopted in the present study. No differences were noted between the results, whichever system was used. This agrees with the results of Osman *et al.* (1977). Barrett *et al.* (1963) found that the mobility of the glycerides was influenced not only by total unsaturation but also by the arrangement of the fatty acids within the glyceride molecule; for example, they found that the 2-oleo-di-stearin showed a higher R_f value than the 1-oleo-di-stearin.

Detection or visualisation of the separated triglycerides

When the charring method was used the spots of the triglyceride groups were faint and not suitable for photography. Spraying the plates with 0·2% 2′,7′-dichlorofluorescein in ethanol followed by exposing them to UV light (Mangold & Malins, 1960) gave clear spots with a yellow fluorescence. The intensity of the yellow fluorescence depended upon the concentration of the different groups. In addition, visualisation by UV light has the virtue that it has no destructive effect on the triglyceride groups. This is desirable if preparative separations are aimed at, in order that no significant losses may be encountered in the recovery of these compounds (Osman *et al.*, 1977).

Composition of the Bolti tissue triglycerides

Figure 5 shows the effect of adding varying amounts of ethanol on the resolution of the spots. For cottonseed oil the presence of 0·5% ethanol gave better resolution without influencing the number of fractionated groups (six in each case) which were triglycerides with 1, 2, 3, 4, 5 and 6 double bonds).

Raising the ethanol content in the mixture (from 0·5 to 1, 1·5 and 2%) made the separated spots travel more towards the front. This resulted in accumulation of the spots near the front into one spot and reduced the number of the separated spots to 5, 4 and 4 for 1, 1·5 and 2% ethanol, respectively.

Bolti lipid tissue showed a similar trend. The presence of 0·5% ethanol gave better resolution so that eight groups instead of seven could be separated. Raising the ethanol content of the mixture to 1, 1·5 and 2% also resulted in the spots travelling more towards the front, thus permitting the two groups near the front to accumulate and appear as one. In addition, in the presence of 1 or 1·5% ethanol a new group appeared near the base line and in the presence of 2% ethanol a second new group appeared near the base line. This raised the total number of the separated groups (in

the case of 2% ethanol) to eight while cottonseed oil under the same conditions showed only four groups.

These results reveal the importance of the percentage of added ethanol to the total number of the separated groups. They also indicate that the groups which constitute the triglyceride fraction of the Bolti tissue lipids differ markedly in their behaviour on chromatography (and consequently in their degree of unsaturation) from those

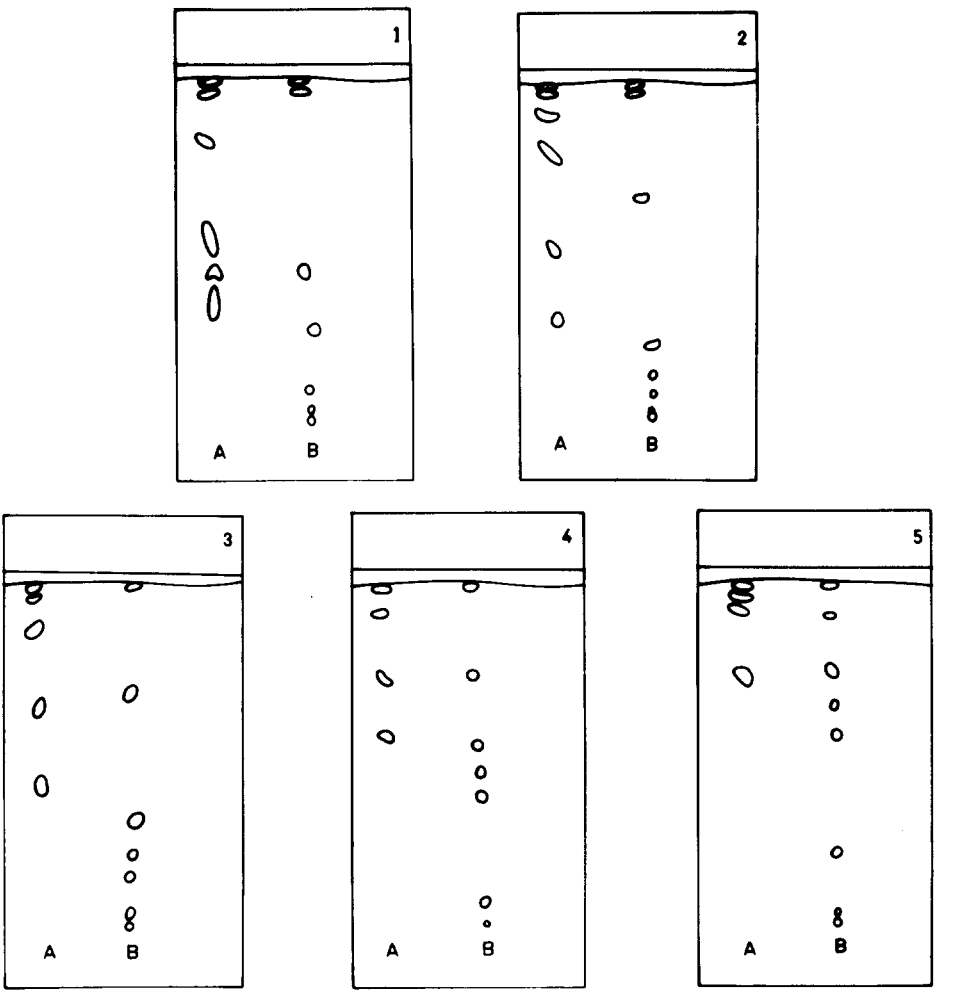


Fig. 5. $AgNO_3$-TLC of the triglycerides of Bolti flesh oil. Coating material: silica gel G–$AgNO_3$. Solvent system: chloroform (99·5%); acetic acid (0·5%). Indicator: 0·2% 2′-7′-dichlorofluorescein–ultraviolet light. A, Triglycerides of refined cottonseed oil; B, triglycerides of Bolti fish. 1, Without ethanol; 2, with 0·5% ethanol; 3, with 1% ethanol; 4, with 1·5% ethanol; 5, with 2% ethanol.

of cottonseed oil. This means that Bolti tissue triglycerides contain groups of higher degrees of unsaturation (of more than six double bonds each) than does cottonseed oil. In addition, the more ethanol that is added to the solvent mixture the more such groups will appear, as may be seen from Fig. 5. Further study of the internal structure of each group is recommended in order to obtain more information.

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BIOTIN AND CHOLINE IN FOODS—NUTRITIONAL IMPORTANCE AND METHODS OF ANALYSIS: A REVIEW

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(Received: 21 February, 1978)

ABSTRACT

Biotin and choline are essential nutrients for many animals and man. Biotin has been shown to be an active component of several enzymes. The nutrient is involved in regulating fatty acid synthesis and also influences carbohydrate metabolism. Generally, the need for biotin in the human body is easily met through the dietary supply combined with endogenous synthesis by the gut microflora.

Choline plays a crucial role in maintaining cellular membrane structure and permeability and in transporting fat in the bloodstream. The body can synthesise choline provided adequate amounts of precursors, folic acid and vitamin B_{12} *are available. A choline deficiency is only possible when both the amount of choline in the diet and the supply of nutrients required for* de novo *synthesis are limiting.*

This review discusses the nutritional importance of biotin and choline and methods of analysis for these nutrients.

INTRODUCTION

The nutritional significance of biotin and choline was realised in the 1930s and it became of interest to determine the distribution of these two compounds in food materials. The amounts of biotin and choline found in various food products are given in Table 1.

BIOTIN: HISTORICAL ASPECTS

Many diverse lines of research led to the discovery and isolation of biotin. Kögl & Tönnis (1936) isolated a minute amount of a crystalline substance from egg yolks

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TABLE 1
CHOLINE AND BIOTIN IN FOODS*

A. Meat, Poultry and Fish Products

Product	*Choline (mg/100 g)*	*Biotin (mg/100 g)*
Beef		
heart	170 (McIntire *et al.*, 1944)	6·8–7·8 (Schweigert *et al.*, 1943)
kidney	240–333 (Engel, 1943; McIntire *et al.*, 1944)	92·3–250 (Schweigert *et al.*, 1943; Lampen *et al.*, 1942)
liver	470–630 (Engel, 1943; McIntire *et al.*, 1944)	88·0–118·0 (Schweigert *et al.*, 1943)
round	65–95 (Engel, 1943; McIntire *et al.*, 1944)	2·9–6·3 (Schweigert *et al.*, 1943)
tongue	108 (McIntire *et al.*, 1944)	3·3 (Schweigert *et al.*, 1943)
Chicken		
dark meat	—	10·1 (Schweigert *et al.*, 1943)
light meat	—	11·3 (Schweigert *et al.*, 1943)
Lamb		
chop sirloin	75–77 (McIntire *et al.*, 1944)	—
leg	75–92 (McIntire *et al.*, 1944)	—
liver	—	127·0 (Schweigert *et al.*, 1943)
roast	122–124 (McIntire *et al.*, 1944)	—
Pork		
ham	101–129 (McIntire *et al.*, 1944)	4·4–6·9 (Schweigert *et al.*, 1943)
ham, cured	98–129 (McIntire *et al.*, 1944)	4·6–6·1 (Schweigert *et al.*, 1943)
loin	—	4·7–6·1 (Schweigert *et al.*, 1943)
bacon	80 (McIntire *et al.*, 1944)	—
Veal	83–149 (Engel, 1943; McIntire *et al.*, 1944)	3·8–7·7 (Schweigert *et al.*, 1943)
Fish		
cod, Atlantic	—	3–4 (Neilands *et al.*, 1947)
cod, Pacific	—	12·23 (Neilands *et al.*, 1947)
salmon	—	5·9–19·0 (Ives *et al.*, 1946; Schweigert *et al.*, 1943; Neilands *et al.*, 1947)
tuna	—	3·6–6·0 (Neilands *et al.*, 1947)
trout	87 (Engel, 1943)	—

B. Cereals Products

Barley	139 (Engel, 1943)	—
Corn		
whole	—	8·3–10·0 (Schiener & De Ritter, 1975)
germ	160 (Engel, 1943)	—
cornmeal, yellow	37 (Engel, 1943)	—
cornmeal, white	10–42 (Engel, 1943)	6·5 (Hardinge & Crooks, 1961)
Oats	94 (Engel, 1943)	—
Rice		
whole	112·4 (Kik, 1957)	12·0 (Kik, 1957)
parboiled	98·2 (Kik, 1957)	10 (Kik, 1957)
bran	170·0 (Kik, 1957)	60 (Kik, 1957)
germ	300 (Kik, 1957)	58 (Kik, 1957)
polishings	102–126 (Engel, 1943; Kik, 1957)	57 (Kik, 1957)

TABLE 1—*contd.*

B. Cereals Products—*contd.*

Product	*Choline (mg/100 g)*	*Biotin (mg/100 g)*
Wheat		
bran	143–269 (Engel, 1943; Calhoun *et al.*, 1960; Glick, 1944; Waggle *et al.*, 1967)	44–49 (Schiener & De Ritter, 1975; Calhoun *et al.*, 1960)
germ	135–423 (Engel, 1943; Calhoun *et al.*, 1960; Glick, 1944; Waggle *et al.*, 1967)	17·4 (Calhoun *et al.*, 1960)
shorts	176–328 (Calhoun *et al.*, 1960; Waggle *et al.*, 1967)	35–37 (Schiener & De Ritter, 1975; Calhoun *et al.*, 1960)
white flour	52–211 (Engel, 1943; Calhoun *et al.*, 1960; Glick, 1944; Waggle *et al.*, 1967; Calhoun *et al.*, 1958)	0·7–8·4 (Schiener & De Ritter, 1975; Calhoun *et al.*, 1960; Chedelin & Williams, 1942)
whole meal	86·7–244 (Engel, 1943; Calhoun *et al.*, 1960; Glick, 1944; Waggle *et al.*, 1967; Calhoun *et al.*, 1958)	5·2–11·4 (Neilands *et al.*, 1947; Calhoun *et al.*, 1960)
white bread	188–209 (Calhoun *et al.*, 1958)	1·1 (Chedelin & Williams, 1942)
whole wheat bread	—	1·9 (Chedelin & Williams, 1942)
C. Vegetables and Fruits		
Vegetables		
asparagus	128 (Engel, 1943)	1·7 (Hardinge & Crooks, 1961)
beans, green	340 (Engel, 1943)	—
beets	—	1·9 (Hardinge & Crooks, 1961)
cabbage	251 (Engel, 1943)	2·4 (Hardinge & Crooks, 1961)
carrots	95 (Engel, 1943)	2·5 (Hardinge & Crooks, 1961)
cauliflower	—	17·0 (Hardinge & Crooks, 1961)
onions	—	3·5 (Hardinge & Crooks, 1961)
peas, green	263 (Engel, 1943)	1·4–3·5 (Ives *et al.*, 1946)
potatoes, white	106 (Engel, 1943)	—
potatoes, sweet	35 (Engel, 1943)	4·3 (Hardinge & Crooks, 1961)
spinach	238 (Engel, 1943)	2·3 (Hardinge & Crooks, 1961)
tomatoes	95 (Engel, 1943)	0·8–2·4 (Ives *et al.*, 1946)
turnip greens	245 (Engel, 1943)	—
Fruits and Juices		
apples	—	0·92 (Hardinge & Crooks, 1961)
apple juice	0·58 (Hardinge & Crooks, 1961)	0·42 (Hardinge & Crooks, 1961)
bananas	—	4·4 (Hardinge & Crooks, 1961)
grapefruit	—	0·81–3·0 (Krehl & Cowgill, 1950; Hardinge & Crooks, 1961)
grapefruit juice	—	0·3–0·97 (Ives *et al.*, 1946; Krehl & Cowgill, 1950)
grapes	—	1·6 (Hardinge & Crooks, 1961)
oranges	—	1·5–1·9 (Krehl & Cowgill, 1950; Hardinge & Crooks, 1961)

TABLE 1—*contd.*

C. Vegetables and Fruits—*contd.*

Product	*Choline (mg/100 g)*	*Biotin (mg/100 g)*
Fruits and Juices—*contd.*		
orange juice	7–11 (Rakieten *et al.*, 1952; Krehl & Cowgill, 1950)	0·3–1·5 (Rakieten *et al.*, 1952; Krehl & Cowgill, 1950; Hardinge & Crooks, 1961)
peaches, canned	—	0·05–0·3 (Ives *et al.*, 1946)
strawberries	—	4·0 (Hardinge & Crooks, 1961)
tangerine juice	—	0·45–0·46 (Krehl & Cowgill, 1950)
watermelon	—	3·6 (Hardinge & Crooks, 1961)
D. Dairy Products, Fats, Oils, Nuts and Legumes		
Dairy		
cheese	45 (Hardinge & Crooks, 1961)	2·6 (Lampen *et al.*, 1942)
egg, whole	504 (Evans & Davidson, 1951)	22·5 (Evans *et al.*, 1953)
egg yolk	1490 (Evans & Davidson, 1951)	52·0 (Evans *et al.*, 1953)
egg, white	1·9 (Evans & Davidson, 1951)	7·0 (Evans *et al.*, 1953)
milk, whole	14·7 (Engel, 1943)	3·0–4·0 (Lampen *et al.*, 1942)
milk, skim	35–47 (Rhian *et al.*, 1943)	—
milk, dried skim milk powder	159 (Engel, 1943)	—
milk, dried whole milk powder	107 (Engel, 1943)	—
Fats and Oils		
butter	<5·0 (Engel, 1943)	—
cod liver oil	<5·0 (Engel, 1943)	—
corn oil, refined	<5·0 (Engel, 1943)	—
oleomargarine	<5·0 (Engel, 1943)	—
soybean oils, refined	<5·0 (Engel, 1943)	—
Nuts		
almonds	—	18–21 (Hall *et al.*, 1958)
peanuts	162 (Hardinge & Crooks, 1961)	34 (Hoffpauir, 1953)
peanut butter	145 (Hardinge & Crooks, 1961)	39 (Hardinge & Crooks, 1961)
pecan halves	50 (Hardinge & Crooks, 1961)	26·7 (Hardinge & Crooks, 1961)
walnut halves	—	36·7 (Hardinge & Crooks, 1961)
Legumes		
blackeyed peas	197 (Chattopadhyay & Banerjee, 1951)	—
cowpeas	257 (Hardinge & Crooks, 1961)	21 (Hardinge & Crooks, 1961)
garbonzos	245 (Hardinge & Crooks, 1961)	10 (Hardinge & Crooks, 1961)
lentils	223 (Hardinge & Crooks, 1961)	13 (Hardinge & Crooks, 1961)
mung beans	209 (Hardinge & Crooks, 1961)	7·5 (Hardinge & Crooks, 1961)
soy beans	345 (Engel, 1943)	61 (Hardinge & Crooks, 1961)

which was shown to have great potency as a yeast growth factor. At about the same time, Allison *et al.* (1933) reported that a factor, extractable from various foodstuffs, stimulated the growth and respiration of a strain of nitrogen-fixing bacteria. They called the factor coenzyme R. In 1927, still another new 'factor' was reported in the literature. Bateman (1916) observed that laboratory animals which were fed huge quantities of raw egg whites developed dermatitis, diarrhoea and haemorrhages of the skin that could be prevented by feeding a variety of foods. The protective action was thought to be due to a substance present in the foodstuffs. Eventually, it was shown that the substance isolated by Kögl & Tönnis (1936), Allison's coenzyme R and the protective factor against egg white injury were identical (György *et al.*, 1940). The compound was given the name biotin.

Determination of the chemical structure and the laboratory synthesis of biotin had been accomplished by 1944, but the function of biotin in the body largely remained a mystery (Harris *et al.*, 1945). The first real breakthrough in the elucidation of biotin's function came from the laboratory of Lynen *et al.* (1959) who demonstrated that biotin played a role in carboxylation reactions. Since Lynen and his co-workers provided this first piece of evidence, biotin has been shown to be an active component of several enzymes (Wood, 1976). One of the first enzymes shown to contain biotin as its prosthetic group was acetyl coenzyme A carboxylase, which catalyses the addition of a carboxyl group to acetyl coenzyme A (Bridges, 1967). This is the first and rate-limiting enzyme of fatty acid synthesis and is under allosteric control by intermediates of the Krebs cycle. Biotin is thus intimately involved in regulating the levels at which fatty acid synthesis takes place in the body.

Eight other enzymes have been shown to require biotin (Wood, 1976). These are: pyruvate carboxylase, a regulatory enzyme of gluconeogenesis; propionyl CoA carboxylase, also a gluconeogenic enzyme; β-methyl crotonyl CoA carboxylase, an enzyme required for the catabolism of leucine; geranyl CoA carboxylase and urea carboxylase. In all of the above enzymes, biotin is covalently linked via its valeric side chain to the ε-amino group of a lysine residue. The catalytic group of biotin is the ureido-1'-nitrogen which acts as an HCO_3^- acceptor and a carboxyl group carrier (Wood, 1976). Transcarboxylase is a biotin enzyme which differs from those already mentioned in that it does not utilise free CO_2 or HCO_3^-. Transcarboxylase is a key enzyme for propionic acid bacteria. Two biotin enzymes catalyse decarboxylation reactions. These are methyl malonyl CoA decarboxylase and oxaloacetate decarboxylase.

BIOTIN: DEFICIENCY SYMPTOMS

Biotin is considered to be a necessary constituent of all living cells, both plant and animal, but its high potency makes the requirements for it quite low (Woodward, 1961). Generally, the need for biotin in the human body is easily met through the

dietary supply combined with endogenous synthesis by gut microflora (McCormick, 1975). Thus, biotin deficiency results only when a biotin-free diet is fed, coupled with consumption of raw egg white or antibiotics. Raw egg white contains the glycoprotein avidin which forms an unabsorbable complex with biotin, whereas antibiotics destroy the intestinal flora which normally manufacture biotin (McCormick, 1975; Hamm & Scott, 1953).

The clinical manifestations of biotin deficiency are many and diverse and are often not readily linked to a known biochemical function of the vitamin. The most extensively studied of the deficiency conditions has been the so-called 'egg white injury' as first described in the rat (Bateman, 1916; Sebrell & Harris, 1968). This condition is characterised by dermatitis of the skin, severely affecting the area around the mouth. There is often a denuding around the eyes which explains the name 'spectacle eye condition'. Biotin deficiency in black mice is characterised not so much by dermatitis, as in the rat, but by depigmentation of the fur and alopecia (Wilson *et al.*, 1949).

Recent work by Petrelli *et al.* (1974) with female rats has shown that the relative amounts of the different types of circulating leukocytes change during biotin deficiency. The primary change was an increase in the number of circulating neutrophils. The significance of such an alteration has not been fully explored.

Biotin deficiency has been associated with a spasticity and paralysis of the hind legs in dogs (Smith & Lester, 1945; Smith, 1946), rats (Servigne & Terroine, 1954) and cattle (Wiese *et al.*, 1946). Pigs have been shown to develop seborrheic changes, spasticity of the hind legs and cracks in the feet when fed a diet devoid of biotin and high in raw egg white (Cunha *et al.*, 1946). In poultry, the primary signs of biotin deficiency are perosis and dermatitis which may be produced by feeding a low biotin diet, without the addition of raw egg white (Patrick *et al.*, 1943; Jensen & Martinson, 1969). As will be discussed later, the chick is apparently more dependent on a dietary supply of biotin than are other species such as the rat (Hegsted *et al.*, 1940).

A lack of biotin has been shown to lead to an impairment of protein synthesis and to diminished activity of certain enzymes (Dakshinamurti & Mistry, 1963). Poznansksys (1957) reported a sharp decrease in the synthesis of albumin and in the activity of amylase in biotin deficient chicks. The defect in protein synthesis may be the result of an abnormal production of amino acid transfer RNA (Woodward, 1961; Robinson, 1972). The impaired protein synthesis may also be due, in part, to an inadequate supply of energy caused by the reduction in synthesis of dicarboxylic acids which accompanies biotin deficiency (Woodward, 1961; Dakshinamurti & Mistry, 1963; Dakshinamurti & Litvak, 1970).

The availability of biotin also influences carbohydrate metabolism. An inadequate supply of biotin is associated with decreased incorporation of glucose into glycogen (Bhagavan *et al.*, 1965). As previously mentioned, biotin is an essential component of two gluconeogenic enzymes. Thus, during biotin deficiency a decreased activity of these two enzymes may lead to a decreased substrate

availability for glycogenesis. Biotin has also been observed to stimulate the activity of hepatic glucokinase, an enzyme which regulates the uptake of glucose by the liver (Anon, 1970).

Much attention has been given to the interrelationships between biotin, vitamin B_{12} and folic acid. Evidence suggests that there is an interference in the utilisation of vitamin B_{12} and in the conversion of folate to its coenzyme active derivatives when inadequate levels of biotin are present (Bridges, 1967; Marchetti *et al.*, 1966).

It is obvious from the discussion above that a deficiency of biotin has profound effects in the animal body. However, a spontaneous biotin deficiency is extremely rare, due to the widespread occurrence of this substance in food and its synthesis by intestinal bacteria. In man, only two reports of biotin deficiency outside of the laboratory environment have ever been published and these two were associated with an excessive consumption of raw egg white (Williams, 1943; Baugh, 1968). There is at present no RDA for biotin, although it has been estimated that man requires 150 μg per day (Williams, 1942).

Some recent studies suggest that domestic fowl may be exceptional in their susceptibility to a deficiency of biotin. Biotin deficiency may spontaneously arise in young fowl raised on wheat-based diets (Payne *et al.*, 1974). Fatty liver and kidney syndrome have frequently been observed in immature domestic fowl fed diets composed mainly of wheat, its by-products and small amounts of bone meal, and these abnormalities have been attributed to a lack of biotin. Biotin supplementation has been shown to prevent the development of this syndrome in broilers (Payne *et al.*, 1974; Whitehead & Blair, 1964) but Balnave (1975) has questioned the idea that biotin deficiency is the sole factor responsible for the development of the condition in immature fowl.

Apparently, there are species differences in the requirement for biotin. In many species, such as the rat, the intestinal synthesis of the vitamin is able to satisfy the animal's need (György, 1968) but in other animals, notably the chick, the intestinal synthesis of biotin is too limited to meet the demand (Wagstaff *et al.*, 1961). As stated previously, biotin deficiency can be produced in the chick by the feeding of a low biotin diet without the addition of avidin. The chick is thus dependent on a dietary supply of biotin and so is more vulnerable to a deficiency condition than is an animal with an adequate endogenous supply. This vulnerability may explain the development of biotin deficiency in chicks fed a diet based on wheat, which may contain less biotin than the suggested dietary allowance for chicks (Wagstaff *et al.*, 1961) particularly when incomplete bioavailability of biotin in wheat is taken into account.

CHOLINE: HISTORICAL ASPECTS AND PRESENT KNOWLEDGE

Choline is unique in its status as a vitamin since its synthesis in the animal body appears to be universal (Sebrell & Harris, 1968). However, the demand for choline

can surpass the body's capacity for *de novo* synthesis which explains why it is considered to be a dietary essential.

Choline was isolated from biological tissue as early as 1849, but the nutritional significance of this compound was not realised until 1932, when Best *et al.* (1932) demonstrated its lipogenic activity in the animal liver. Since that time, three functions for choline in the body have been firmly established: choline is a substrate for the synthesis of the neurotransmitter acetylcholine, it is a source of labile methyl groups and it is an essential component of phospholipids (Pike & Brown, 1975). As a component of phospholipids, choline plays a crucial role in maintaining cellular membrane structure and permeability and in transporting fat in the bloodstream.

After the initial discovery by Best *et al.* (1932) that choline was able to prevent fatty infiltration of the liver, the relationship of other dietary factors to choline's lipotropic activity became of interest. It was noted that the addition of methionine to the diet produced a further protective effect against the occurrence of fatty liver (Tucker & Eckstein, 1937). It was soon demonstrated that methionine's protective effect was due to its ability to donate the labile methyl groups required for the synthesis of choline (duVigneaud *et al.*, 1941). The concept of transmethylation was

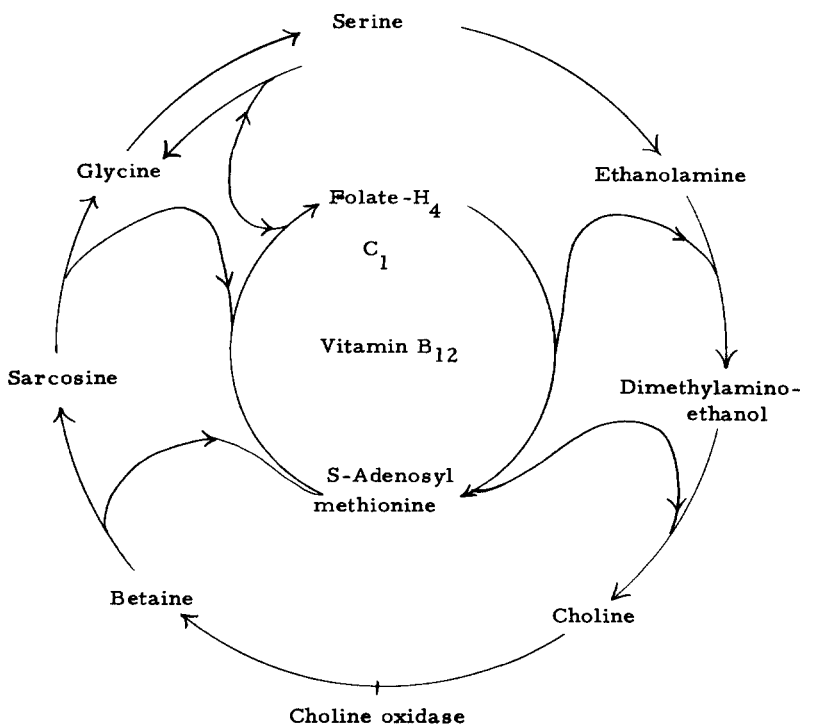

Fig. 1. Labile methyl metabolism (Pike & Brown, 1975).

proposed and amply substantiated by the work of Keller *et al.* (1949) and duVigneaud (1941). The transmethylation scheme as presented in Fig. 1 is now universally accepted.

CHOLINE: DEFICIENCY SYMPTOMS

Figure 1 indicates that the body can synthesise choline provided that adequate amounts of precursors, folic acid and vitamin B_{12} are available. Therefore, in order to induce a choline deficiency it is necessary not only to limit the amount of choline in the diet but also to limit the supply of nutrients which are required for its *de novo* synthesis (Newberne *et al.*, 1970).

Perhaps the most extensively studied manifestation of choline deficiency has been the fatty infiltration of the liver. Development of a fatty liver is the first indication of a choline deficiency in the rat and other species (Lombardi, 1971). It has been detected as early as twenty-four hours after the start of the choline-free diet (Olson, 1971). In the early stages, the liver condition may be rapidly reversed if choline is administered to the afflicted animal; however, if the animal is maintained on the deficient diet for one to two months, atrophy and regression of liver cells occurs, followed by the growth of fibrous tissue (MacLean & Best, 1934; Hartroft, 1950).

It is well established that choline is an integral component of hepatically-synthesised phospholipids which serve to transport fat out of the liver. The cause of the fatty liver during choline deficiency appears to be an impaired secretion of lipoproteins out of the liver and into the plasma (Lombardi & Oler, 1967). This impaired secretion is evidenced by a decreased level of circulating lipoproteins in the plasma (Lombardi, 1971; Lombardi & Oler, 1967). Using radio-isotope labelling techniques, Lombardi *et al.* (1968) have shown that as early as eight hours after a choline-deficient diet is given to rats there is interference in the movement of newly synthesised very low density lipoproteins out of the liver.

Renal degeneration is another of the classic symptoms which occurs during choline deficiency. The condition was first described by Griffith & Wade (1939) in rats fed on choline-free diets. Wells (1971) has hypothesised that the haemorrhagic changes in the kidney are due to both a shortage of acetylcholine, which leads to tubular necrosis, and to a deficiency of one of the blood clotting factors. An unusual feature of the renal degeneration accompanying choline deficiency is the spontaneous recovery observed within a few days among the survivors while still on the deficient diet. The recovery has been attributed to lysis of accumulated red blood cells, which liberates enough choline for the production of acetylcholine in the kidneys (Wells, 1971). In addition to the haemorrhagic condition seen in the kidney, haemorrhagic damage has also been observed in the eyes, heart, liver, adrenals and brain of weanling rats fed on choline deficient diets (Sebrell & Harris, 1968).

Considerable serum changes are also associated with choline deficiency. Increases

in α and β globulins and decreases in serum albumin and in all classes of serum lipids have been documented (Fischer & Garrity, 1945; Tinoco *et al.*, 1964). In addition, choline deficiency is observed to lead to hypertension in young rats (Kratzig & Wetzig, 1970) and may also lead to irreparable damage of the developing immune system in prenatally deprived rats (Newberne *et al.*, 1970).

Species differences exist both in the requirement for choline and in the response to its deficiency. The primary pathology observed in chicks and turkey poults which are choline deficient is a decreased growth rate and the development of perosis or 'slipped tendon disease' (Sebrell & Harris, 1968). The choline deficient guinea pig primarily shows signs of fatty infiltration of the liver but, unlike the rat, the condition takes about three weeks to appear and is accompanied by the disappearance of adipose fat (Anon, 1957). Apparently, the chick and guinea pig differ from the rat in their capacity to synthesise choline. The former two animals are unable to methylate ethanolamine directly and so, unlike the rat, cannot use betaine as a substitute for choline in the diet (Anon, 1957).

A specific deficiency disease in man has never been attributed to lack of choline. Its widespread distribution in foods, coupled with the body's capacity to manufacture choline, makes a shortage situation for the vitamin unlikely. There have been some attempts to link the liver damage which accompanies chronic alcoholism in man to a deficiency of choline.

BIOTIN: ASSAY PROCEDURES

(a) *Microbiological assays*

Until recently the levels of biotin naturally occurring in foods were far beyond the sensitivity of chemical assays. The problem of how to estimate the microquantities of biotin present in food materials was solved by the development of microbiological techniques. Snell & Strong (1939) performed the first microbiological assay for a B-vitamin and thereby established the principles of the microbiological technique which are still in use today.

Microbiological methods for biotin estimation are used extensively but, according to György (1968), these methods have certain limitations. First, most of the organisms used for assay are unable to use bound forms of biotin. Therefore, food samples under investigation must first be hydrolysed to convert the bound biotin to its free form. Biotin is among the most stable of vitamins and thus is well suited to acid hydrolysis (Bell, 1971) but there may be some destruction of the vitamin during this process. A second limitation of microbiological methods, described by György and Langer, is the tendency for many microbes to respond non-specifically to compounds with biotin-like activity. Limitations such as these sometimes render results of microbiological assay difficult to interpret; however, the method is technically simple and demands considerably less time and expense than do animal assays (Sebrell & Harris, 1968; Snell & Wright, 1941).

To be used as a test organism for biotin assays, a microbe must be unable to manufacture its own supply of biotin and thereby depend upon its medium to furnish the vitamin. Many microorganisms fit this criterion but the most extensively used has been *Lactobacillus plantarum* (ATCC 8014). Snell & Wright (1941) reported that biotin was an essential growth factor for *L. plantarum* and it was later established that this *Lactobacillus* is highly specific in its response to biotin (Wright & Skeggs, 1944).

Lactobacillus casei (ATCC 7469) has also been recommended as a test organism for biotin assays (Lampen *et al.*, 1940). However, this *Lactobacillus* is reportedly less specific for biotin and more complex in its medium requirements than is *L. plantarum* (Wright & Skeggs, 1944). The yeast, *Saccharomyces cerevisiae*, was the original test organism for biotin assay (György, 1968). Its advantages are its high degree of sensitivity and wide working range. However, the yeast displays a certain lack of specificity for biotin (Wright & Skeggs, 1944). Other organisms which have been tried in biotin assays but which have received little acceptance are *Clostridium butylicum* (Lampen *et al.*, 1940) and *Ochromonas danica* (Aaronson, 1959; Baker *et al.*, 1962).

(b) *Animal assays*

Animal assays for biotin have been described (Sebrell & Harris, 1968). They offer the advantages of being highly specific and of measuring the vitamin's bioavailability, in contrast to microbiological assays which measure the amount of biotin freed in chemical hydrolysis. This is an important distinction since incomplete availability of biotin to animals has been demonstrated (Patrick & Knandel, 1942).

When using the rat as a test animal, a diet free of biotin and high in avidin is fed (Sebrell & Harris, 1968). Traditionally, the curative effect of biotin on the growth rate of the rat is used as the basis for the bioassay because this parameter is easily assessed quantitatively. The chick may also be used as a test animal for the bioassay of biotin.

(c) *Chemical assays*

Viswanathan *et al.* (1970) described a gas–liquid chromatographic method for the quantitative analysis of biotin in premixes and parenteral preparations. The method involved the conversion of biotin to its silyl ester with bis-(trimethyl silyl) acetamide, followed by analysis using an H-flame ionisation detector. This method has not been used extensively. Other chemical methods which have been described include a colorimetric determination, involving thin layer chromatography (McCormick & Roth, 1970) and spectrophotometric techniques for the determination of both biotin and avidin (Green, 1970). The advantages of the chemical methods are their convenience, wide working range and precision; they are, however, much less sensitive than microbiological methods.

CHOLINE: ASSAY PROCEDURES

(a) *Chemical assays*

One of the earliest methods proposed for estimating choline was devised by Kapfhammer & Bischoff (1930). They employed a chemical method based on the precipitation of choline as a Reineckate complex, followed by gravimetric determination. Precipitation of choline as a Reineckate has since gained the most widespread use of the various methods available for choline assay.

In 1936, greater sensitivity was gained with the Reineckate method by Beattie (1936) who dissolved the Reineckate precipitate in acetone and analysed the solution by colorimetry. The use of photoelectric colorimetry improved the method still further such that choline concentrations of the order of 0·003 % could be measured with an error of no more than 3 % (Engel, 1942; Glick, 1944).

Engel (1942) proposed a more efficient and rigorous extraction process using methanol as the solvent. Further modifications were made by Glick (1944) who advised the use of an alkaline medium for the precipitation of the choline–Reineckate complex to avoid contamination by other compounds which also have the ability to form insoluble Reineckates. Glick also demonstrated that *n*-propanol was the most effective solvent for rinsing the precipitate. These modifications added by Glick resulted in the form of the procedure which is still in use today.

Precipitation of choline as the periodide has also been suggested for the estimation of choline. The method was first studied by Staněk (1905) and has since been improved upon by other researchers (Sebrell & Harris, 1968).

Hanin (1971) has devoted a book to the compilation of methods available for assaying choline and acetylcholine in biological tissue. Methods discussed include fluorometric and radioassay techniques and are particularly well suited to the measurement of the small amounts of choline and related compounds present in animal tissue. Other chemical methods for the estimation of choline have been described. Extensive reviews of the available methods have been presented by Harris & Thimann (1943) and by György (1968).

(b) *Microbiological assay*

Microbiological assays for choline have also been described (Difco Laboratories, 1953; Horowitz & Beadle, 1943). The most recommended organism is a mutant strain of *Neurospora crassa* (ATCC 34486) but other organisms have been used (Sebrell & Harris, 1968). As was previously discussed with respect to biotin, the microbiological method suffers from the limitation of requiring hydrolysis of the material to be analysed in order to release the choline from its bound forms. This practice may destroy a certain amount of the vitamin and also fails to indicate the vitamin's bioavailability. Gravimetric analysis, in which the mycelia are filtered,

dried and weighed, completes the microbiological procedure for the estimation of choline.

Lueke & Pearson (1944) have used the microbiological method to analyse for choline in blood and urine samples. The results obtained were in excellent agreement with those obtained by chemical methods. Kent-Jones (1950), however, has expressed dissatisfaction with the microbiological method.

(c) *Animal assays*

Biological assay is a third type of method available for the quantitative determination of choline. The degree of prevention of renal pathology has been used as a parameter to estimate choline; however, determination of choline by such a method is complicated by the *de novo* synthesis of choline in the presence of adequate folic acid and cobalamine (Sebrell & Harris, 1968). Although this type of procedure is seldom used today, it has yielded satisfactory results in the past. Engel (1943) reported fair agreement between values for choline content of peanut meal and dried brewer's yeast obtained with a bioassay and with the Reineckate chemical method.

More recently, Roberts and Ritz (1968) used a bioassay for choline in chick feeds. The researchers also used the Reineckate chemical method and a microbiological procedure to assay for choline. Ritz and Roberts reported that the values obtained with the Reineckate method tended to be low, those from the microbiological method tended to be high, with the values from the chick bioassay falling in between. Where large discrepancies were noted, the bioassay tended to agree more closely with the microbiological method.

The pharmacological action of acetylated choline on tissues isolated from the animal body has been used successfully as an assay for choline. In 1935, Fletcher *et al.* (1935) assayed for acetylated choline using the isolated intestine of the rabbit. Jacobi *et al.* (1941) assayed for acetylated choline using contraction experiments with frog muscle and reported satisfactory agreement with results obtained by the Reineckate chemical method. The accuracy of such a method has been questioned because many other physiologically active substances, such as histamine and potassium, may interfere with the determination.

SUMMARY

Biotin and choline are essential nutrients for man. However, once the widespread occurrence of biotin and choline in foods was demonstrated, coupled with the growing realisation that a deficiency of biotin or choline was not a problem for man or most animals, the interest in estimating their distribution diminished considerably. Yet biotin and choline have retained their status as essential nutrients and so assays to determine their concentration in foods are still performed.

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A RADIATION-CHEMICAL APPROACH TO THE EVALUATION OF THE POSSIBLE TOXICITY OF IRRADIATED FRUITS: PART 1—THE EFFECT OF PROTECTION BY CARBOHYDRATES

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(Received: 14 March, 1978)

ABSTRACT

Radiation-chemical principles are applied in an attempt to identify only those components in a foodstuff likely to undergo chemical change during ionising radiation.

The stabilities of some components of a representative fruit (mango) with respect to irradiation were determined. The rate constants of the reactions of the constituents with hydroxyl radicals were then calculated. These constants and the concentrations of the components in the fruit were used to calculate the stabilities of the constituents in the actual fruit.

The only compounds to undergo significant modifications are the sugars which account for nearly 99% of the reactions. The other components which are slightly reactive are starch (0·2%), protein (0·2%), phenol (0·4%) and ascorbic acid (0·2%). Therefore only carbohydrate degradation need be considered. Furthermore, carbohydrate reactivity tends to protect the other components from degradative changes.

INTRODUCTION

One of the main reasons for the slow progress of irradiation as an acceptable method of food processing has been the failure of health authorities to recognise the process as universally safe. Justification for this is the known formation of radiolysis products in treated foods, although the same argument is not applied to processes

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Fd. Chem. (4) (1979)—
Printed in Great Britain

such as heating or freezing where greater chemical changes can take place. The problem is that the nature and concentration of the radiolysis products are generally not known *a priori* and the task of preparing a complete inventory is virtually impossible. This is due to the complexity of the chemical composition of foodstuffs and the large number of possible radiolysis products formed in exceedingly low yields.

If all the radiolysis products were known, their toxicity could be established either from the literature or from limited animal feeding studies designed to determine the effect level for a toxic reaction. This level is generally extrapolated to man on a body-mass basis; a safety factor of 100 is introduced in order to determine an acceptable level in the diet. As the radiolysis products are not known, however, it becomes necessary to feed the integral foodstuff.

The feeding studies required usually involve large numbers of rodents and chronic and reproduction data take from two to three years to accumulate. In terms of existing legislation in many countries, such studies must be repeated for every foodstuff to be treated.

It is generally recognised that such feeding studies cannot be continued *ad infinitum*. With this in mind there has been a resurgence of interest in attempting to identify the chemical changes produced by irradiation. In the present approach, radiation-chemical principles are applied in an attempt to identify only those components in a foodstuff which are likely to undergo chemical change. In this way it is hoped to be able to pinpoint a limited number of possible reaction pathways and to indicate the reaction products which can be expected to be formed.

At this stage it is proposed to concentrate on fruits in view of the fact that they consist predominantly of water and carbohydrates—a situation which can be expected to simplify the radiolysis reaction mechanism. As a further simplification, a 'model fruit' may be defined as an aqueous solution of typical components of fruits in the concentrations in which they are normally found in nature. The effect of irradiation on the model fruit may now be considered.

When the model fruit is exposed to ionising radiation, most of the energy will be absorbed by the major component, water. The consequence of this absorption of radiant energy may be written as follows (Draganić & Draganić, 1971):

$$H_2O \longrightarrow\!\!\!\!\!\sim\!\!\!\!\!\rightarrow H_3O^+,\ e^-_{aq},\ H,\ OH,\ H_2 \text{ and } H_2O_2$$

Although H_3O^+, H_2 and H_2O_2 may enter into further reactions under certain specific conditions, the major part of radiolytic decomposition will be due to the attack on the solutes by the radicals e^-_{aq}, H and OH (Swallow, 1977). In the case of a complex mixture, such as the model fruit, competitive reactions will take place.

In a multicomponent system the probability of component X reacting with OH is:

$$\phi(X) = \frac{k_x[X]}{\sum k_s\ [\text{solute}]}$$

where $\sum k_s$ [solute] represents the sum of the products of rate constant and concentration for all the components.

The yield of the reaction of OH with X may be written as a G-value (molecules converted or formed per 100 eV energy absorbed) in the following form:

$$G(X) = G_{OH} \Big/ \frac{\sum_{k_s} [\text{solute}]}{k_x[X]} \quad \text{(Draganić \& Draganić, 1971)} \qquad (1)$$

Similar expressions may be derived for reactions involving e_{aq}^- and H but they react almost exclusively with dissolved oxygen and it is essentially only the hydroxyl radical which is involved in competitive reactions with components.

More accurate competition kinetics could be obtained in N_2O where hydroxyl radicals are the only reactive species present. Since the fruit contains oxygen, it was felt inappropriate to investigate the competitive reactions of the model fruit in another atmosphere. The secondary radical processes with oxygen (Von Sonntag & Dizdaroglu, 1977) are not being considered in this first approximation of the model fruit.

It is clear from eqn. (1) that in the model fruit only those components for which $k_x[X]$ is comparable with $\sum k_s$ [solute] will be significantly changed as the result of irradiation. Thus, if the rate constants of all the possible reactions between intermediates and components are known, it will be possible to pinpoint those pathways which are of importance in the model fruit. Unfortunately, many of the rate constants of reactions involving food components are not known. In the present work, rate constants and concentrations which have been determined for all the components in the model fruit are presented. These data allow the determination of the probability of the various reaction pathways from which certain meaningful conclusions may be derived.

MATERIALS AND METHODS

Materials

All chemicals were of analytical quality, used without further purification.

Composition determinations

The details of determinations and the results of the chemical composition of mangoes, papayas, strawberries and litchis appear elsewhere (Beyers *et al.*, 1979; Blakesley *et al.*, 1979).

Rate constant determination

Relative rate constants were determined by comparing the sensitivities of the components of the model fruit with that of glucose. In these experiments solutions containing 10^{-3} M glucose and 10^{-3} M of the component being tested were irradiated

in a Gammacell 220 (AECL) to a total dose of 1 kGy (average dose rate 6 kGy/h, temperature 22 °C). Following irradiation, the glucose concentration was determined by employing the enzymatic hexokinase kit as supplied commercially by Boehringer Mannheim, GmbH, Germany.

RESULTS AND DISCUSSION

Composition of model fruit

A number of factors, including the following, were taken into account in deciding on the exact composition of the model fruit:

(i) This study is part of a project aimed at establishing the wholesomeness of irradiated mangoes, papayas and strawberries in order to obtain a clearance for unlimited sale from the South African Department of Health. The composition of the model fruit should therefore be similar to these South African fruits.

(ii) Detailed analytical data are not available for all fruits; the use of locally obtained detailed data was thus favoured.

(iii) Considerable variation in values quoted in the literature is encountered. Reasons for this may be differences in variety, soil conditions, climate or maturity.

In view of these factors a detailed analysis of four varieties of mangoes (Kent, Haden, Zill and Peach), two varieties of papayas (Papino (Solo) and Hortus Gold) and two varieties of strawberries (Selecta and Parfait) was undertaken. A summary of these results appears in Table 1.

Values (Fox, 1966) for unspecified South African papaya and strawberry cultivars agree well with the figures reported here but the literature values for mangoes (unspecified) are higher in water and lower in carbohydrate content than indicated in the present results. However, when the observations for the cultivars are pooled and averaged, the average values agree well with average values determined from the literature (Coetzee & Burger, 1953; Watt & Merrill, 1963; Wenkam & Miller, 1965). The free amino acid concentrations (Blakesley *et al.*, 1979) are reported in Table 1. Six (Jain, 1961) and eleven (Shanta, 1969) free amino acids have been reported for mangoes. Relative values of amino acids in strawberries have been reported previously (Burroughs, 1960) and indicate aspartic acid, asparagine, glutamic acid and glutamine as the principal components.

The overall similarities in chemical composition between the three fruits are evident, but from the point of view of this investigation it is important to note that the percentage carbohydrate in mango is double that found in strawberry. The distribution of the major sugars in the carbohydrate fraction of the model fruit is the same as those found in Kent mangoes. The experimentally determined ratios of

TABLE 1
CHEMICAL COMPOSITION OF RIPE EDIBLE PORTIONS OF MANGOES, PAPAYAS AND STRAWBERRIES

	Fruit							
	Mango			*Papaya*			*Strawberry*	
	Varieties							
Component	*Kent*	*Zill*	*Haden*	*Peach*	*Papino*	*Hortus Gold*	*Selekta*	*Parfait*
g/100 g								
Water	81·85	78·52	82·26	80·50	86·10	87·85	89·25	91·70
Fat	0·08	0·09	0·07	0·05	0·11	0·07	0·15	0·20
Ash	0·32	0·32	0·33	0·48	0·44	0·46	0·50	0·40
Titratable acidity[a]	0·24	0·25	0·29	0·41	0·08	0·12	0·75	0·60
Protein	0·46	0·47	0·34	0·50	0·68	0·52	0·84	0·70
Starch	0·74	1·60	1·45	0·49	0·079	0·124	—	—
Total sugar	12·36	10·53	5·27	8·13	7·58	5·52	6·05	4·90
IU/100 g Carotene	5169	11021	4693	6024	4285	2819	382	326
mg/100 g								
Ascorbic acid	20·05	10·17	4·43	14·28	89·61	68·96	59·80	57·80
Riboflavin	0·06	0·09	--	0·06	0·031	0·02	0·03	0·03
Nicotinamide	0·42	1·65	--	0·09	0·77	0·33	0·47	0·63
Thiamine	0·06	0·09	--	0·05	0·04	0·03	0·02	0·02
Calcium	8·73	10·35	10·90	22·45	34·75	27·05	13·27	4·25
Phosphorus	10·18	14·58	9·90	14·00	10·05	7·50	17·85	12·50
Iron	0·16	0·34	0·19	0·45	0·18	0·43	0·54	0·84
Sodium	0·84	0·29	0·30	1·20	2·31	5·81	0·60	0·66
Potassium	115·00	66·45	64·75	103·95	40·52	105·10	84·73	116·65
Histidine[b]	0				0		1·00	
Lysine	0·30				2·00		0	
Leucine	0·10				2·00		0	
Isoleucine	0·10				1·00		1·00	
Valine	0·30				1·00		2·00	
Arginine	1·70				2·00		0	
Tyrosine	Trace				0		0	
Glycine	0·20				7·00		1·00	
Serine	2·80				23·00		84·00	
Glutamic acid	2·90				0		14·00	
Aspartic acid	2·50				3·00		10·00	
Alanine	5·40				3·00		17·00	

[a] = calculated as g citric acid.
[b] = free amino acids.

sugar concentrations in these fruits were glucose:fructose:sucrose:maltose = 1:1·67:1·29:0·017. The model fruit contains the components in the concentrations found in the actual mango. Most of these values were obtained experimentally (Table 1). Concentrations cited in the literature were used for glycollic acid, oxalic acid, malic acid, citric acid, tartaric acid (Fang, 1965), fibre, represented by cellulose (Wenkam & Miller, 1965), tannin, added in the form of phenol (Soule & Harding, 1956) and magnesium (Fox, 1966). The pectin concentration of Kent mangoes was determined according to the method of the AOAC (1975). The consistency and appearance of the model fruit closely resemble those of mango juice.

TABLE 2

KINETIC PARAMETERS FOR MODEL FRUIT

Component	*Concentration*	*Rate constant*	k_s [*solute*]
Glucose	$1{\cdot}90 \times 10^{-1}$ M	$2{\cdot}0 \times 10^{9}$	$3{\cdot}80 \times 10^{8}$
Fructose	$3{\cdot}10 \times 10^{-1}$ M	$1{\cdot}0 \times 10^{9}$	$3{\cdot}10 \times 10^{8}$
Sucrose	$2{\cdot}40 \times 10^{-1}$ M	$2{\cdot}6 \times 10^{9}$	$6{\cdot}20 \times 10^{8}$
Maltose	$3{\cdot}20 \times 10^{-2}$ M	$5{\cdot}8 \times 10^{9}$	$1{\cdot}90 \times 10^{8}$
Starch	$7{\cdot}50 \times 10^{-4}$ M	$4{\cdot}2 \times 10^{9}$	$3{\cdot}20 \times 10^{6}$
Pectin (Apple)	$6{\cdot}70 \times 10^{-7}$ M	$4{\cdot}3 \times 10^{10}$	$2{\cdot}90 \times 10^{4}$
Cellulose	$6{\cdot}30 \times 10^{-4}$ M	$9{\cdot}1 \times 10^{9}$	$5{\cdot}70 \times 10^{6}$
Fat (Olive oil)	$4{\cdot}50 \times 10^{-4}$ M	$1{\cdot}2 \times 10^{10}$	$5{\cdot}30 \times 10^{5}$
Protein (Catalase)	$2{\cdot}80 \times 10^{-5}$ M	$1{\cdot}7 \times 10^{11}$	$4{\cdot}80 \times 10^{6}$
Carotene[a]	$5{\cdot}60 \times 10^{-5}$ M	$1{\cdot}0 \times 10^{10}$	$5{\cdot}00 \times 10^{5}$
Thiamine	$1{\cdot}60 \times 10^{-6}$ M	$8{\cdot}3 \times 10^{9}$	$1{\cdot}30 \times 10^{4}$
Riboflavin	$1{\cdot}40 \times 10^{-6}$ M	$1{\cdot}4 \times 10^{10}$	$2{\cdot}20 \times 10^{4}$
Nicotinamide	$6{\cdot}20 \times 10^{-5}$ M	$3{\cdot}0 \times 10^{9}$	$1{\cdot}90 \times 10^{5}$
Ascorbic acid	$2{\cdot}30 \times 10^{-3}$ M	$1{\cdot}1 \times 10^{10}$	$2{\cdot}50 \times 10^{7}$
Adipic acid	—	$4{\cdot}2 \times 10^{9}$	—
Citric acid	$1{\cdot}70 \times 10^{-2}$ M	$8{\cdot}2 \times 10^{7}$	$1{\cdot}40 \times 10^{6}$
Galacturonic acid	—	$3{\cdot}6 \times 10^{9}$	—
Gallic acid	—	$7{\cdot}8 \times 10^{8}$	—
Glucuronic acid	—	$1{\cdot}7 \times 10^{9}$	—
Glycollic acid	$8{\cdot}00 \times 10^{-3}$ M	$1{\cdot}8 \times 10^{9}$	$1{\cdot}40 \times 10^{7}$
Malic acid	$5{\cdot}50 \times 10^{-3}$ M	$7{\cdot}4 \times 10^{8}$	$4{\cdot}10 \times 10^{6}$
Malonic acid	—	$2{\cdot}8 \times 10^{7}$	—
Mucic acid	—	$1{\cdot}2 \times 10^{9}$	—
Oxalic acid	$4{\cdot}00 \times 10^{-3}$ M	$4{\cdot}6 \times 10^{7}$	$1{\cdot}80 \times 10^{5}$
Pyruvic acid	—	$1{\cdot}4 \times 10^{9}$	—
Succinic acid	—	$1{\cdot}9 \times 10^{8}$	—
Tartaric acid	$5{\cdot}40 \times 10^{-3}$ M	$5{\cdot}8 \times 10^{8}$	$3{\cdot}10 \times 10^{6}$
Phenol	$5{\cdot}10 \times 10^{-3}$ M	$1{\cdot}3 \times 10^{10}$	$6{\cdot}60 \times 10^{7}$
Glycine	$2{\cdot}70 \times 10^{-5}$ M	—	—
DL-Alanine	—	$5{\cdot}2 \times 10^{8}$	—
L-Alanine	$6{\cdot}10 \times 10^{-4}$ M	$7{\cdot}2 \times 10^{8}$	$4{\cdot}40 \times 10^{5}$
L-Valine	$2{\cdot}60 \times 10^{-5}$ M	$1{\cdot}2 \times 10^{9}$	$3{\cdot}20 \times 10^{4}$
L-Leucine	$7{\cdot}60 \times 10^{-6}$ M	$3{\cdot}7 \times 10^{9}$	$2{\cdot}80 \times 10^{4}$
L-Isoleucine	$7{\cdot}60 \times 10^{-6}$ M	$4{\cdot}0 \times 10^{9}$	$3{\cdot}10 \times 10^{4}$
L-Serine	$2{\cdot}70 \times 10^{-4}$ M	$6{\cdot}8 \times 10^{8}$	$1{\cdot}80 \times 10^{5}$
L-Methionine	—	$1{\cdot}4 \times 10^{10}$	—
L-Aspartic acid	$1{\cdot}90 \times 10^{-4}$ M	$6{\cdot}0 \times 10^{7}$	$1{\cdot}10 \times 10^{4}$
L-Asparagine	—	$8{\cdot}0 \times 10^{7}$	—
L-Glutamic acid	$2{\cdot}00 \times 10^{-4}$ M	$1{\cdot}4 \times 10^{8}$	$2{\cdot}80 \times 10^{4}$
L-Glutamine	—	$5{\cdot}4 \times 10^{8}$	—
L-Arginine	$9{\cdot}80 \times 10^{-5}$ M	$3{\cdot}7 \times 10^{9}$	$3{\cdot}60 \times 10^{5}$
L-Ornithine	—	$1{\cdot}6 \times 10^{8}$	—
L-Citrulline	—	$3{\cdot}1 \times 10^{9}$	—
L-Lysine	$2{\cdot}10 \times 10^{-5}$ M	$1{\cdot}1 \times 10^{9}$	$2{\cdot}30 \times 10^{4}$
Sodium	$3{\cdot}30 \times 10^{-3}$ M	$4{\cdot}0 \times 10^{7}$	—
Potassium	$5{\cdot}10 \times 10^{-2}$ M	—	—
Calcium	$3{\cdot}40 \times 10^{-3}$ M	—	—
Iron	$6{\cdot}70 \times 10^{-5}$ M	—	—
Magnesium	$3{\cdot}30 \times 10^{-3}$ M	$4{\cdot}0 \times 10^{7}$	—
Phosphorus (as $(HPO_4)^{2-}$)	$4{\cdot}50 \times 10^{-4}$ M	—	—
			$\sum k_s$ [solute] $= 1{\cdot}63 \times 10^{9}$

[a] The carotene rate constant could not be determined because of low solubility in water. An attempt was made to adsorb it on glucose but the maximum concentration ratio obtained was 1:330. Estimated value taken from OH and cyclohexadiene.

The composition of the model fruit is given in Table 2 which also includes the kinetic parameters discussed in another section of this paper. In addition to the components of the model fruit, certain other compounds of possible importance in future experiments have been included in Table 2. The values are expressed in terms of molar concentrations, rather than the more conventional units, as these are required for the kinetic calculations.

Radiation stability of components

The stability of the various components in the model fruit with respect to radiation were determined by measuring the extent to which they modify the radiation-induced degradation of a 10^{-3} M aqueous solution of glucose. The results are shown in Table 3 which gives the decrease in glucose concentration after a radiation dose of 1 kGy in the absence ($\Delta[\text{glucose}]_o$) and presence ($\Delta[\text{glucose}]_c$) of an added component. Δ' is simply the difference between these two values.

The ratio $\Delta G/(G_{OH} - \Delta G)$ represents the reactivity of the added component relative to glucose when the hydroxyl radical is the reacting species. With a knowledge of this ratio and of the absolute rate constant of the reaction of OH with glucose, it is possible to calculate absolute rate constants for the reactions of the other components with OH. Table 4 lists absolute rate constants calculated from measured values of $\Delta G/(G_{OH} - \Delta G)$ and k (OH + glucose) $= 2 \times 10^9$ M^{-1} sec^{-1} (Anbar & Neta, 1967; Dorfman & Adams, 1973). Also included are values of rate constants which have appeared in the literature (Anbar & Neta, 1967; Dorfman & Adams, 1973).

Of twenty-one cases where comparison is possible between experimental and literature values, the agreement is regarded as good in eleven cases (within a factor of 2) and reasonable in six cases (within a factor of 3). The remaining four cases are the rate constants determined for oxalic acid, DL-alanine, L-alanine and glutamine and of these only the oxalic acid and L-alanine values are in serious error. It should also be noted that, in the case of oxalic acid, the pH employed in the determination of the literature values was 9, while in the present investigation it was 3. (Rate constants for organic acids are known to be pH dependent.) The pH of the actual fruit is 4·6 while that of the model is 3·6.

No reaction is reported for glycine. This simply means that under the conditions of the experiment no competition between glucose and the added component was observed, indicating a rate constant $< 10^7$ M^{-1} sec^{-1}. In the case of added Fe^{2+}, the degradation of glucose is enhanced.

Using the results for composition and rate constants in Table 2, we may now apply eqn. (1) to determine the stabilities of the components in the model fruit.

It is clear from the last column of Table 2 that the only components which undergo significant modification are the four sugars which account for nearly 99% of the reactions of OH radicals. Other components which are slightly reactive are starch (0·2% of OH radicals), protein (0·2%), phenol (0·4%) and ascorbic acid (0·2%).

TABLE 3

COMPETITION BETWEEN GLUCOSE AND OTHER COMPONENTS FOR OH RADICALS

(Initial glucose = 10^{-3} M, D = 1 kGy)

Conc. (M)	*Component*	Δ [*Glucose*]$_o$ (*mg*/100 *ml*)	Δ [*Glucose*]$_c$ (*mg*/100 *ml*)	Δ'	ΔG	$\Delta G/(G_{OH} - \Delta G)$
10^{-4}	Fructose	5·87	5·63	0·24	0·13	0·05
10^{-4}	Sucrose	6·06	5·50	0·56	0·31	0·13
10^{-4}	Maltose	5·39	4·30	1·09	0·61	0·29
10^{-3}	Starch	6·07	2·77	3·30	1·83	2·12
$1·3 \times 10^{-5}$	Pectin	5·39	4·32	1·07	0·59	0·28
$4·4 \times 10^{-6}$	Cellulose	5·39	5·31	0·08	0·04	0·02
10^{-3}	Fat	6·00	4·20	1·80	1·00	0·59
10^{-5}	Protein	5·87	3·62	2·25	1·25	0·87
10^{-3}	Thiamine	6·20	2·25	3·95	2·17	4·14
10^{-5}	Riboflavin	6·20	4·20	2·00	1·11	7·00
10^{-3}	Nicotinamide	5·80	2·90	2·90	1·62	1·51
10^{-3}	Ascorbic acid	6·00	1·90	4·10	2·28	5·50
10^{-3}	Adipic acid	7·20	3·92	3·28	1·82	2·09
10^{-2}	Citric acid	6·00	4·60	1·40	0·78	0·41
10^{-3}	Galacturonic acid	7·20	4·07	3·13	1·74	1·82
10^{-3}	Gallic acid	7·20	1·35	5·90	0·75	0·39
10^{-3}	Glucuronic acid	6·10	3·90	2·20	1·22	0·83
10^{-3}	Glycollic acid	6·00	4·44	2·26	1·26	0·88
10^{-3}	Malic acid	5·80	4·50	1·30	0·72	0·37
10^{-2}	Malonic acid	6·10	5·50	0·60	0·33	0·14
10^{-3}	Mucic acid	7·20	5·38	1·82	1·01	0·60
10^{-2}	Oxalic acid	6·10	5·20	0·90	0·50	0·23
10^{-2}	Pyruvic acid	6·10	4·10	2·00	1·11	7·00
10^{-2}	Succinic acid	6·10	3·70	2·40	1·33	0·97
10^{-3}	Tartaric acid	5·80	4·70	1·10	0·61	0·29
10^{-4}	Phenol	6·10	4·20	1·90	1·06	0·65
10^{-3}	Glycine	7·06	7·02	0·04	—	—
10^{-3}	DL-Alanine	7·66	6·67	0·99	0·55	0·26
10^{-3}	L-Alanine	7·66	6·37	1·29	0·72	0·36
10^{-3}	L-Valine	7·02	5·16	1·86	1·03	0·62
10^{-3}	L-Leucine	7·34	4·19	3·15	1·75	1·85
10^{-3}	L-Isoleucine	6·06	2·82	3·24	1·80	2·01
10^{-3}	L-Serine	7·02	5·80	1·22	0·68	0·34
10^{-5}	L-Methionine	6·06	5·73	0·33	0·18	0·07
10^{-3}	L-Aspartic acid	7·34	7·21	0·13	0·07	0·03
10^{-3}	L-Asparagine	7·02	6·83	0·19	0·11	0·04
10^{-3}	L-Glutamic acid	7·34	6·90	0·33	0·18	0·07
10^{-3}	L-Glutamine	7·02	5·98	1·04	0·58	0·27
10^{-3}	L-Arginine	7·02	3·87	3·15	1·75	1·85
10^{-3}	L-Ornithine	7·02	6·68	0·34	0·19	0·08
10^{-3}	L-Citrulline	7·02	4·42	2·60	1·44	1·55
10^{-3}	L-Lysine	6·14	4·45	1·69	0·94	0·54
10^{-3}	Na^+	6·10	6·00	0·10	0·06	0·02
10^{-3}	K^+	6·10	6·30	—	—	—
10^{-3}	Ca^{2+}	6·10	6·20	—	—	—
10^{-4}	Fe^{2+}	6·10	7·30	—	—	—
10^{-3}	Mg^{2+}	6·10	6·00	0·10	0·06	0·02
10^{-3}	$(HPO_4)^{2-}$	6·10	6·35	—	—	—

TABLE 4
RATE CONSTANTS OF MODEL FRUIT COMPONENTS

Component	$k_{(OH+x)}/k_{(OH+Gluc.)}$	$k_{(OH+x)}$	$k_{(OH+x)}(litt.)$
Glucose	1·00	$2·0 \times 10^9$	$2·0 \times 10^9$
Fructose	0·50	$1·0 \times 10^9$	—
Sucrose	1·30	$2·6 \times 10^9$	$2·5 \times 10^9$
Maltose	2·90	$5·8 \times 10^9$	—
Starch	2·10	$4·2 \times 10^9$	—
Pectin	21·55	$4·3 \times 10^{10}$	—
Cellulose	4·55	$9·1 \times 10^9$	—
Fat	0·60	$1·2 \times 10^9$	—
Protein	85·00	$1·7 \times 10^{11}$	—
Carotene[a]	—	$1·0 \times 10^{10}$	—
Thiamine	4·20	$8·3 \times 10^9$	—
Riboflavin	70·00	$1·4 \times 10^{11}$	—
Nicotinamide	1·50	$3·0 \times 10^9$	—
Ascorbic acid	5·50	$1·1 \times 10^{10}$	$1·2 \times 10^{10}$
Adipic acid	2·10	$4·2 \times 10^9$	—
Citric acid	0·04	$8·2 \times 10^7$	3×10^7
Galacturonic acid	1·80	$3·6 \times 10^9$	—
Gallic acid	0·39	$7·8 \times 10^8$	—
Glucuronic acid	0·85	$1·7 \times 10^9$	3×10^9
Glycollic acid	0·90	$1·8 \times 10^9$	4×10^8
Malic acid	0·37	$7·4 \times 10^8$	—
Malonic acid	0·01	$2·8 \times 10^7$	$3·3 \times 10^7$
Mucic acid	0·60	$1·2 \times 10^9$	—
Oxalic acid	0·02	$4·6 \times 10^7$	5×10^6
Pyruvic acid	0·70	$1·4 \times 10^9$	—
Succinic acid	0·10	$1·9 \times 10^8$	—
Tartaric acid	0·29	$5·8 \times 10^8$	—
Phenol	6·50	$1·3 \times 10^{10}$	$1·2 \times 10^{10}$
Glycine	No reaction	—	$2·7 \times 10^2$
DL-Alanine	0·26	$5·2 \times 10^8$	$1·2 \times 10^8$
L-Alanine	0·36	$7·2 \times 10^8$	$1·2 \times 10^8$
L-Valine	0·60	$1·2 \times 10^9$	$6·6 \times 10^8$
L-Leucine	1·85	$3·7 \times 10^9$	$1·6 \times 10^9$
L-Isoleucine	2·00	$4·0 \times 10^9$	$1·7 \times 10^9$
L-Serine	0·34	$6·8 \times 10^8$	$3·2 \times 10^8$
L-Methionine	7·00	$1·4 \times 10^{10}$	5×10^9
L-Aspartic acid	0·03	$6·0 \times 10^7$	$7·5 \times 10^7$
L-Asparagine	0·04	$8·0 \times 10^7$	$3·1 \times 10^7$
L-Glutamic acid	0·07	$1·4 \times 10^8$	$1·5 \times 10^8$
L-Glutamine	0·27	$5·4 \times 10^8$	$1·5 \times 10^8$
L-Arginine	1·85	$3·7 \times 10^9$	$3·5 \times 10^9$
L-Ornithine	0·08	$1·6 \times 10^8$	$1·6 \times 10^8$
L-Citrulline	1·15	$2·3 \times 10^9$	—
L-Lysine	0·55	$1·1 \times 10^9$	—
Na^+	0·02	$4·2 \times 10^7$	—
K^+	No reaction	—	—
Ca^{2+}	No reaction	—	—
Fe^{2+}	Sensitisation	—	3×10^8
Mg^{2+}	0·02	$4·2 \times 10^7$	—
$(HPO_4)^{2-}$	No reaction	—	—

[a] See Table 2.

Since the effect of radiation on starch is to degrade it to monosaccharides and disaccharides and simple polysaccharides (Scherz, 1974), a change in concentration of 0·08 % is irrelevant. The change in protein concentration in the model fruit is significant but it must be remembered that the situation in a real fruit is very different, with a large number of enzymes constituting the protein fraction. Thus the change in any one protein due to irradiation will be much smaller. In the case of phenol and ascorbic acid, the concentrations will be decreased by about $1{\cdot}7 \times 10^{-6}$ M or 0·2 ppm.

Toxicological implications of sugar degradation

The products found in the radiolysis of aqueous solutions of sugars have been studied by a number of workers (Dizdaroglu *et al.*, 1975; Kawakishi *et al.*, 1975; Phillips, 1972; Von Sonntag *et al.*, 1976). Of these, only hydrogen peroxide is known to have any direct toxicological action. It was shown that hydrogen peroxide does not produce cytotoxic agents in the presence of high concentrations of sugars (Schubert & Sanders, 1971). In addition, the deoxy sugars produced during irradiation of oxygen-free sugar solutions may be dehydrated under certain conditions, leading to the formation of α–β unsaturated carbonyls (Schubert & Sanders, 1971). The cytotoxic effects observed in plant cells grown in irradiated sucrose solutions could be due to the radiolytic production of these α–β unsaturated compounds (Schubert & Sanders, 1971). They are cytotoxic *in vitro*, but practically non-cytotoxic *in vivo*; furthermore, their acute toxicity in mammals is fairly low (Schubert, 1974). When a real fruit, such as strawberry, is irradiated, little or no α–β unsaturated carbonyls are produced (Schubert *et al.*, 1973) despite the high concentration of sugars present. This is also true when the juice is irradiated (Schubert *et al.*, 1973).

Recent work (Von Sonntag & Dizdaroglu, 1977) demonstrated that oxygen suppressed the formation of deoxy sugars during the irradiation of D-ribose. Sugars irradiated in oxygen are nevertheless mutagenic towards *Salmonella typhimurium* but failed to show a mutagenic response in a host-mediated assay (Aiyar & SubbaRao, 1977).

Therefore we may conclude that the possible cytotoxins of irradiated sugar solutions have not been positively identified.

Applicability of the model to a real fruit

It has been shown, using the concept of an aqueous solution model, that the number of significant reaction pathways due to radiolysis is small and that the products of these reactions are generally non-toxic. If this is also the situation in a real fruit, the implications are extremely important and could lead to the clearance of many, if not all, irradiated fruits for human consumption. Thus the relevance of the aqueous model to the structure and nature of a real fruit should be considered:

(a) A unique feature of a fruit parenchyma cell is that a major part (up to 90 %) of the interior of the cell is occupied by one or more vacuoles or sac-like spaces

(Charley, 1970). The bulk of the water content of fruits is found in these vacuoles, together with soluble substances like sugars, salts, organic acids and water-soluble pigments. Substances (such as starch) unable to dissolve in water are colloidally dispersed in it. Components which are not found in the aqueous phase are cell wall constituents (such as cellulose, haemicellulose and pectic substances) and fat droplets together with the fat-soluble pigments which are contained in plastids embedded in the cytoplasm. Most enzymes are located in the mitochondria which are distributed throughout the cytoplasm. It would thus appear that the aqueous model does, in fact, represent a considerable part of the fruit structure rather well. In a more refined model attention should be paid to effects in the cell wall, the plastids and in the mitochondria where direct effects of radiation could be important.

(b) The aqueous model does not take into account the fact that there are ongoing biochemical processes in a fruit which might be affected by irradiation. It is well known, for example, that enzymes are deactivated (or occasionally activated) and that this affects certain metabolic processes. Similarly, changes in the cell wall affecting permeability can have important biochemical consequences. It is proposed to investigate such effects in future work but for the present it is sufficient to state that metabolic changes seem to be quantitative rather than qualitative and are probably not related to the production of new compounds with toxicological significance.

CONCLUSIONS

On the basis of the aqueous model concept of a fruit we may conclude that the components which are most sensitive to radiation damage are the carbohydrates. Furthermore, because of their reactivity, they tend to protect all other components from degradative changes. The products of the radiolysis of aqueous solutions of sugars are known and are generally non-toxic. One exception is the α–β unsaturated carbonyl fraction which has been shown to be cytotoxic. However, because they react rapidly with many other components, they do not represent a hazard to health when ingested.

Future experiments will be aimed at determining whether any complications occur when all the components are present at the same time. As part of this study it is intended to investigate the possibility of post-irradiation thermal reactions involving radiolysis products and other components. At a later stage the model fruit will be modified by adding starch and cellulose in the quantities found in mature green fruit (for example, mango) and comparing the radiolysis behaviour with that of irradiated fruit puree.

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RELATIONSHIP BETWEEN PECTIN METHYLESTERASE ACTIVITY AND THE FORMATION OF METHANOL IN CONCORD GRAPE JUICE AND WINE

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(Received: 12 May, 1978)

ABSTRACT

Pectin methylesterase (PME) activity in grapes was studied. Concord grape PME has optimum activity at pH 7·5 and the activity was the highest among several grape varieties tested. PME activity in Concord grapes increased continuously throughout the maturation period and reached a maximum at harvest. Consequently, this reflected the large amount of methanol formation during wine fermentation.

INTRODUCTION

Pectin methylesterase (PME) specifically catalyses hydrolysis of the methyl ester of the galacturonic acid units of pectin and releases methanol. Pectic substances present in most fruits and vegetables play an important role in their textural quality. PME is found in many plants and is also produced by some microorganisms. It is believed to be relatively inactive in most intact plant tissues but when the tissues are macerated, the enzyme rapidly converts the pectin to pectic acid (Lineweaver & Jensen, 1951; Kertesz, 1951). The presence of PME has been studied in cherries, apples, pears (Davignon, 1961), tomatoes (Kertesz, 1938), strawberries, raspberries, apricots, radish, carrots (Polacsek-Racz & Pozsar-Hajnal, 1976), orange juice (Guyer *et al.*, 1956), cauliflower (Hoogzand & Doesburg, 1961), snap beans (Van Buren *et al.*, 1962) and Carignan grapes (Marteau *et al.*, 1961).

PME in grapes is very important because of its relationship to methanol formation during wine fermentation. It has been previously shown that Concord and Ives grapes produce significant amounts of methanol during fermentation compared with several varieties of *Vitis vinifera* and other *V. labruscana* grapes (Lee *et al.*, 1975). Therefore, an experiment was designed to determine whether the

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activity of Concord PME differs from that of other varieties of grapes and to monitor the changes in the activity of the enzyme over the period of maturation.

MATERIALS AND METHODS

Grapes and wine fermentation

All grapes used in this study were grown in the New York State Agricultural Experiment Station vineyards, Geneva, New York, during the 1976 season. Concord and Niagara grape wine was produced by fermenting the juice with and without skins at the pilot-plant scale (Nelson *et al.*, 1977).

Methanol analysis by gas chromatography

Methanol was analysed by a gas chromatographic 'direct-injection' method (Lee *et al.*, 1975). In this study, a Hewlett-Packard instrument (5830A) equipped with a 18850A GC terminal was used. A coiled stainless steel column (2 m long × 0·2 cm id) packed with Porapak QS (silylated ethylvinylbenzene polymer), nitrogen carrier gas at a flow rate of 30 ml/min, an injector temperature of 240 °C and a detector temperature of 260 °C were used. The column was operated isothermally at 115 °C. Standard solutions of methanol at various concentrations (10–500 μl/litre) were analysed regularly by injecting 1 μl of sample and a calibration curve was prepared by plotting the concentration versus peak area.

Preparation of crude enzyme solution

Representative samples of grapes picked by hand during the course of maturation were transferred to the laboratory and tested immediately. Randomly selected grape berries (50 g) were homogenised for 3 min in a pre-cooled blender with an equal amount of cold (0 °–2 °C) 1M K_2HPO_4 solution and used immediately as a source of PME.

Assay of PME

PME activity was assayed in a 0·5 % solution of pectin (Sigma Chem. Co.) in 0·2M K_2HPO_4 at pH 7·5. A 5 ml aliquot of the pectin solution was pipetted into a 25 ml test tube and placed in a 30 °C water bath. The pectin solution was allowed to equilibrate for 5 min and then 0·5 ml of crude enzyme solution was added to the tube and mixed well. Immediately sampling was carried out for methanol analysis for the 0 time and then samples were taken at convenient intervals with a 10 μl Hamilton syringe. One microlitre of sample was injected directly into the gas chromatograph and the methanol content of the solution was obtained by reference to the standard curve. One unit of enzyme activity is defined as the amount of crude enzyme that produced 1 ppm (μl/litre) methanol in 20 min from 5 ml of a 0·5 % pectin solution at 30 °C, pH 7·5. All results presented are the average of duplicate analyses.

RESULTS AND DISCUSSION

The activity of crude PME in Concord grapes is influenced by pH. Figure 1 shows that the activity increased rapidly from pH 5 to a maximum at pH 7·5 and then decreased sharply with further increase in the pH. Thus, the behaviour of Concord grape PME is similar to that found in other fruits, such as citrus and tomatoes, in regard to the effect of pH on its activity (Kertesz, 1938; Guyer *et al.*, 1956). Since the pH of Concord grape juice or must is generally less than 4, the conditions are not optimal for PME activity. Cation concentration was not taken into account in the determination of the effect of pH on enzyme activity in this study.

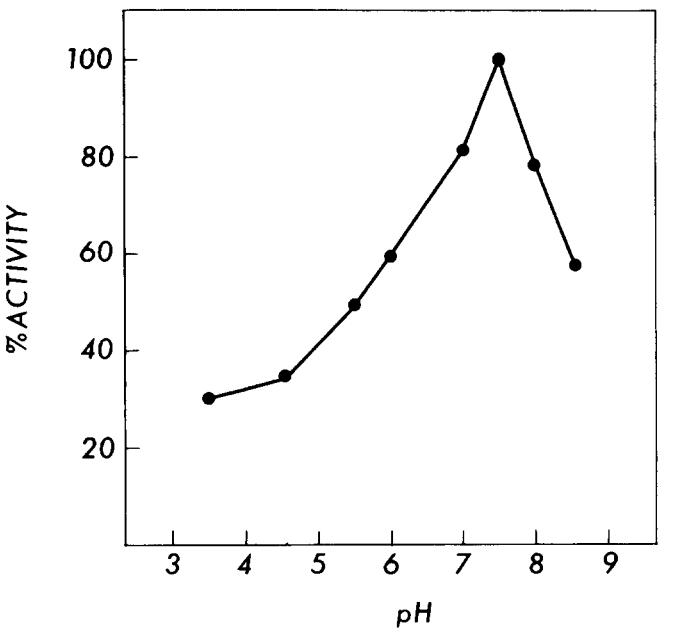

Fig. 1. Activity of crude PME in Concord grapes at different pHs.

A comparison of PME activity among different varieties of grapes is shown in Table 1 with their maturity as expressed in degrees Brix. Concord grape PME shows the highest activity among the several *V. vinifera* and *V. labruscana* grapes tested. In general, *vinifera* grapes contain lower levels of PME than *labruscana* grapes and red grapes show higher PME activity than white grapes. Since pectic substances in grapes were not measured in this study, it may be possible that additional methanol, even in small quantities, may be derived from pectic substances in the enzyme solution.

TABLE 1
PME ACTIVITY IN GRAPES

Variety		Date of harvest	Activity (unit/ml)	°Brix
Concord	*V. labruscana*	20 Oct.	106	15·0
Ives	*V. labruscana*	19 Oct.	81	16·5
Leon Millot	Interspecific hybrid	27 Sept.	78	20·4
Niagara	*V. labruscana*	28 Sept.	74	12·3
Chardonnay	*V. vinifera*	7 Oct.	64	18·1
Cabernet Sauvignon	*V. vinifera*	7 Oct.	62	19·5
Riesling	*V. vinifera*	12 Oct.	44	16·7
Vincent	Interspecific hybrid	15 Oct.	42	14·6

Figure 2 shows PME activity change in Concord and Riesling grapes during the course of maturation in the 1976 season. PME activity in Riesling grapes increased slowly during maturation and reached a maximum 1–2 weeks before harvest. This maximum was maintained. Over the period of maturation, PME activity of Riesling grapes was only about half that of Concord grapes. PME activity in Concord grapes increased continuously throughout the maturation period and reached a maximum

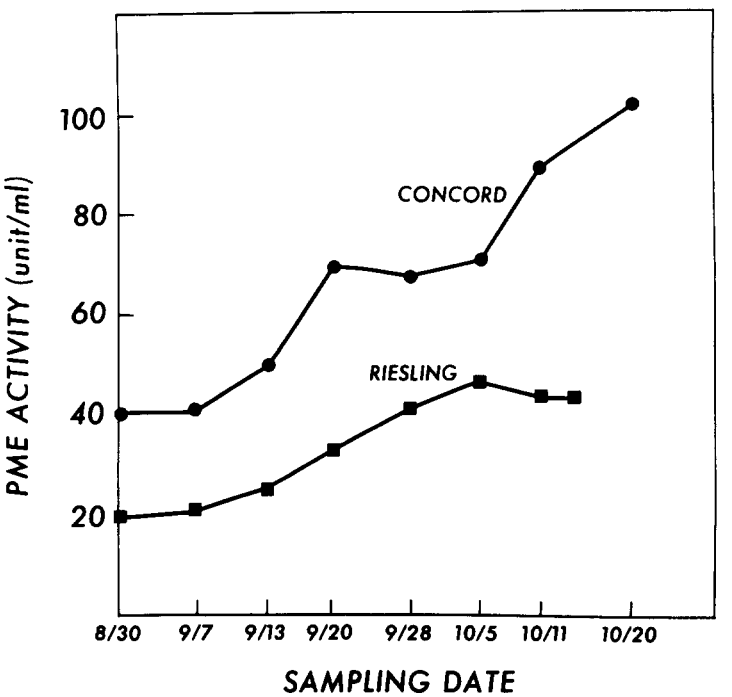

Fig. 2. Changes in PME activity in Concord and Riesling grapes during maturation.

at harvest time. Similar changes in PME activity during maturation were observed in cherries (Davignon, 1961). Since the total pectic substances in Concord grapes decreases considerably during maturation (Carter, 1968) while PME activity increases, any additional methanol derived from pectic substances in the enzyme solution would seem to be negligible.

Figure 3 shows the change in methanol content during the fermentation of Concord (red) and Niagara (white) grapes with and without skins.

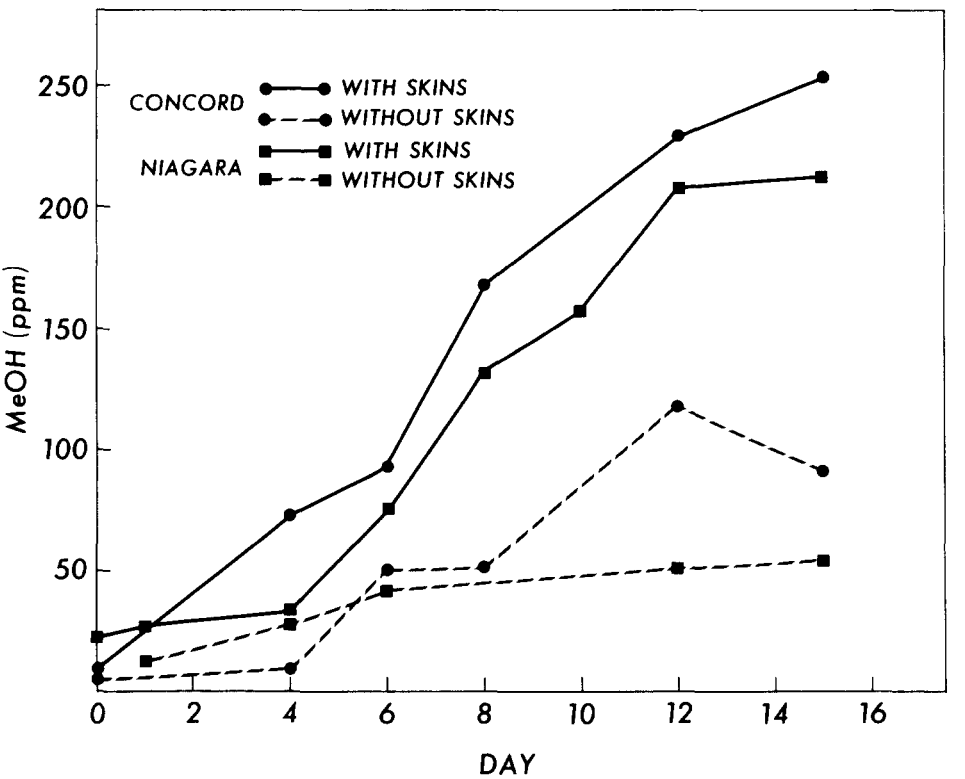

Fig. 3. Formation of methanol in Concord and Niagara grapes during fermentation with and without skins.

The methanol concentrations of Concord and Niagara wines fermented without skins were very low compared with those of the same grapes fermented with the skins. All Concord grape samples were higher in methanol content than the corresponding Niagara samples. It has been shown in previous work that white wines generally contain much less methanol than red wines (Lee *et al.*, 1975). This now seems to be due to the fact that the PME activity in red grapes is much higher than that in white grapes (Table 1). The major reason for the large amount of

methanol formation in the must with skins is that large portions of PME and pectin substances are located in the skin. Therefore, to minimise methanol formation in Concord grapes during the preparation of juice or wine, it is recommended that PME should be deactivated as soon as possible.

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VOLATILE COMPOSITION OF CERTAIN AMAZONIAN FRUITS

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(Received: 12 June, 1978)

ABSTRACT

The volatile constituents of several Amazonian fruits—bacuri, cupuacu, maruci and taperebá—were isolated by steam distillation–extraction of pulp or juice from the canned fruits. Essences were subjected to gas chromatographic analysis in high resolution wall-coated open tubular glass capillary columns; identification of the volatile constituents was based on mass spectral analysis, supported by gas chromatographic retentions under linearly temperature-programmed conditions. Considered individually, none of the compounds identified duplicates the aroma of any of these fruits. Many of them do, however, contribute fruity notes. This indicates that the typical aroma for each individual fruit is due not to one compound, but is probably the result of an integrated response to the contribution of a wide spectrum of compounds.

INTRODUCTION

A great deal of attention has been directed towards the volatile compositions of a wide variety of fruits over the past several decades. In a few cases, individual 'character-impact compounds' (Jennings & Sevenants, 1964) have been pinpointed as being responsible for a characteristic flavour, but most fruit flavours seem to be due to the integrated response to a large number of contributing compounds.

The Amazon region abounds in a rich variety of plant life which includes at least 124 edible varieties of fruit (Cavalcante, 1972, 1974); almost all of these have commercial significance and can be found in the marketplace. Horticultural

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research has been pursued on some of these fruits, but there is a paucity of information relative to their volatile compositions. For this preliminary study of the aromatic compounds in fruits native to the Amazon region, four popular dessert fruits that are also used in the industrial production of ice-cream and juice were selected: bacuri, capuacu, muruci and taperebá.

METHODS AND MATERIALS

Fruit samples

All of the fruit analysed in these studies was received from EMBRAPA (Empresa Brasileira de Pesquisa Agro-Pecuaria), Para, Brazil in the form of purée, canned juice or canned fruit. To facilitate importation under the rules of the US and California Departments of Agriculture, all fruits were subjected to 85 °C for 12 to 15 min and canned.

Platonia insignis, commonly known as bacuri, is a thick-skinned fruit, approximately the size of an orange, and contains a large quantity of resins. The pulp enclosing the seeds is white, bittersweet, with a pleasant smell and taste. The fruit can be consumed raw or in the form of juice, ice-cream or jam. The pulp had been diluted 1:1 with water and pasteurised for 12 min at 85 °C prior to canning.

Theobroma grandiflora, commonly known as cupuacu, contains a white–yellow pulp with an acid taste and a strong smell. It is used to make juice, ice-cream, liqueurs and jam. The seeds contain caffein and theobromine, an alkaloid with stimulant properties (Cuatrecasas, 1964). The seeds also contain about 48 % of a white fat very similar to cocoa butter. The undiluted pulp had been heated 15 min at 85 °C prior to canning.

Byrsonima crassifolia—common name muruci—is a yellow fruit, smaller than a cherry. It grows cherry-like on a tree, and has a strong odour. Both the pulp and the skin are used in producing juice, ice-cream and candies. Four parts of pulp had been diluted with one part of water prior to heating and canning.

Spondias lutea, commonly known as taperebá, is a small, thin-skinned fruit, approximately 3/4 cm long and yellow–orange in colour. It contains only a small amount of pulp but produces a considerable amount of bittersweet juice with a pleasant taste and smell. The pulp and skin are used in the preparation of juices, ice-creams and liqueurs.

Volatile extracts

Each of the fruit samples was subjected to simultaneous steam distillation–extraction in a modified Nickerson-Likens apparatus (Maarse & Kepner, 1970). A total of 200 g of pulp or juice of the canned sample was placed in a 1000 ml round-bottomed flask. An equal volume of boiling water was added in the preparation of essences from taperebá and bacuri. For cupuacu and muruci, 400 ml

of boiling water was added because they were much more viscous than the other fruits. Following 90 min of distillation–extraction with 5 ml of pentane, the essence was dried over magnesium sulphate and concentrated under a stream of purified nitrogen.

Gas chromatography

Gas chromatographic separations utilised a Hewlett-Packard 5720A gas chromatograph, adapted to a highly linear all-glass inlet splitter (Jennings, 1975) and a glass capillary column containing SE 30 admixed with 7 % Igepal CO 990. The inlet was maintained at 250 °C, the split ratio was *ca.* 1:100 and the column was operated at a linear velocity of *ca.* 16 cm/sec of nitrogen carrier gas. The temperature of the detector was maintained at 250 °C and supplied with 30 ml/min of nitrogen as a make-up gas, 30 ml/min hydrogen and 240 ml/min air as a combustion mixture. The oven temperature was programmed from 70 to 170 °C at a rate of 1 °C/min and held until the end of the analysis.

Gas chromatography–mass spectrometry

For the GC–MS analyses a Finnigan Model 3200 quadrapole mass spectrometer equipped with a Model 6000 computerised data system was coupled to a Model 9500 gas chromatograph, fitted with a glass capillary column coated with methyl silicone SE 30 that terminated in the EI source. The inlet was maintained at 250 °C and the split ratio was *ca.* 1:100. Helium was used as a carrier gas. The oven temperature of the gas chromatograph was programmed from 70 to 170 °C at a rate of 2 °C/min. Mass spectra were taken every 3 sec over the range from *m/e* 35 to *m/e* 250, utilising an ionisation voltage of 70 eV. After completion of the chromatographic run, all mass spectra data for significant GC peaks were recalled from the disc memory and drawn by computer plotter.

RESULTS AND DISCUSSION

Table 1 lists the compounds identified in these studies. In most cases, clear spectra were obtained from the GC/MS runs in spite of the complex composition of the essences, due to the very high column efficiencies; a few compounds were not completely resolved. Identifications were based on agreement of the mass spectral data with published spectra (Index of Mass Spectral Data, 1969; Silverstein *et al.*, 1974; Stenhagen *et al.*, 1974; Kennett *et al.*, 1977). Assignments were supported by gas chromatographic retention indices on the high resolution SE 30 column as confirmation.

Retention indices can be reproducible (Ettre, 1964) and under closely controlled isothermal conditions in high resolution columns they can be useful as a criterion of identification (Rijks, 1973). Provided the temperature coefficient of the retention

TABLE I

CONSTITUENTS IDENTIFIED IN AMAZONIAN FRUITS[a]

	Retention index[b]	*Bacuri*	*Cupuacu*	*Muruci*	*Taperebá*
HYDROCARBONS					
Heptane	700	xxx			
2-Heptene	704	xx			xx
Ocimene	1040				xx
Gamma terpinene	1046	x		x	
ALCOHOLS					
2-Butanol	628			xx	
Hexanol	912		x		
Linalool	1118	xxx			
CARBONYLS					
2-Butanone	580			xxxx	
2-Pentanone	677	xx			
n-Hexanal	790			xx	
Furfural	880	x	x	xx	
2-Heptanone	883	xx		xx	
Hept-3-ene-2-one	940			xx	
2-Nonanone	1090	xx			
Nona-2-ene-4-one	1140			x	
ESTERS					
Ethyl acetate	615		xx	xx	
Ethyl butyrate	794		xxxx	xx	
Butyl acetate	808		x		
Ethyl 2-methylbutanoate	845		xx		
Methyl hexanoate	921			xx	
Butyl *iso*butyrate	945		x		
Butyl butyrate	987		xx		
Ethyl hexanoate	991		xxx	xxxx	
Cis-3-hexenyl acetate	991	x			
Butyl-2-methylbutanoate	1038		xx		
*Iso*amylbutyrate	1040			x	
Methyl benzoate	1075				xx
2-Methylbutyl-2-methylbutanoate	1096		x		
Methyl octanoate	1110			x	
Ethyl benzoate	1184				x
Butyl hexanoate	1177			xx	
Ethyl octanoate	1184			xx	x
Hexyl hexanoate	1379			xx	
Ethyl decanoate	1393			xx	
Ethyl cinnamate	1455			xx	x
Methyl dodecanoate	1510	x			
Ethyl dodecanoate	1570			xx	
Ethyl tetradecanoate	1772			xx	
Methyl hexadecanoate	1905			*	
Ethyl hexadecanoate	1975			*	
Ethyl-9-octadecenoate	2150			*	
MISCELLANEOUS					
1-Methoxy 1-ethoxy ethane	641			xx	
1,1-Diethoxy ethane	727			xxx	
Cis-linalool oxide	1086	xxx			
Trans-linalool oxide	1100	xxx			

[a] Relative concentrations are indicated by xxxx = large, xxx = medium, xx = small, x = trace.

[b] On SE 30 as determined under programmed conditions.

* These compounds were detected in the GC/MS analysis but were not apparent on the GC runs.

index is not large, reasonably accurate retention indices can be determined under conditions of temperature programming (Van den Dool & Kratz, 1973). Yabumoto *et al.* (1977) studied these coefficients for a number of compounds and found they were small for esters, ketones and hydrocarbons of small to moderate molecular weight and larger for alcohols. Comparison of the retention data from Table 1 with literature values would support that observation, but there were some exceptions where a compound as identified from the mass spectral data exhibited a programmed retention index significantly different from the isothermal value in our files or in the literature. In these cases it was established that under these same separation conditions, a sample of the authentic compound exhibited a programmed retention index as shown in Table 1. Compounds for which this step was required included 2-butanol, hexanol, furfural, methyl and ethyl benzoate, ethyl octanoate and other larger molecular weight esters. Chromatograms typical of bacuri, cupuacu, muruci and taperebá on the SE 30 column are shown in Figs. 1 to 4, respectively.

It is widely recognised that the contribution of an individual component to the aroma or flavour of a complex mixture may bear little relationship to its concentration in that mixture. The threshold of the compound in question, and synergistic or antagonistic interactions with other compounds of the mixture, may complicate the issue, but some generalities can usually be drawn.

We are unable to assess the aroma impact of the unidentified peaks, some of which are major components. If we make the admittedly risky assumption that none of the unidentified peaks are major contributors to the aroma, at first glance the compounds responsible for the flavour of bacuri would appear to be linalool (peak No. 37), 2-heptanone (peak No. 20) and *cis*-3-hexenyl acetate (peak No. 22). Linalool was present in fairly large amounts while the amounts of 2-heptanone and *cis*-3-hexenyl acetate were relatively small. Nevertheless, the latter two are fairly powerful odorants and even though the amounts are small their contribution to the aroma is probably significant.

For some of the mass spectra from the volatiles of cupuacu we were unable to locate similar spectra in the literature. Several appear to be aldehydic in nature. The compounds possessing pleasant aromas which were identified in cupuacu are mainly esters and included relatively large amounts of ethyl butyrate (peak No. 21) and smaller amounts of ethyl acetate (peak No. 1), butyl acetate (peak No. 9), butyl *iso*butyrate (peak No. 18) and butyl butyrate (peak No. 20). While the concentration of these latter was relatively low, all of these esters undoubtedly contribute to the aroma of cupuacu.

The compounds that occurred in larger amounts but resisted identification in the analysis of muruci are represented by peaks 17, 34 and 50. The chromatograms show that peak 17 was not completely resolved and the mass spectrum of peak 17 is therefore that of a mixture. Peak 34 is the dominant volatile of muruci and shows a base peak at *m/e* 281; we were unable to find matching spectra in the literature. The

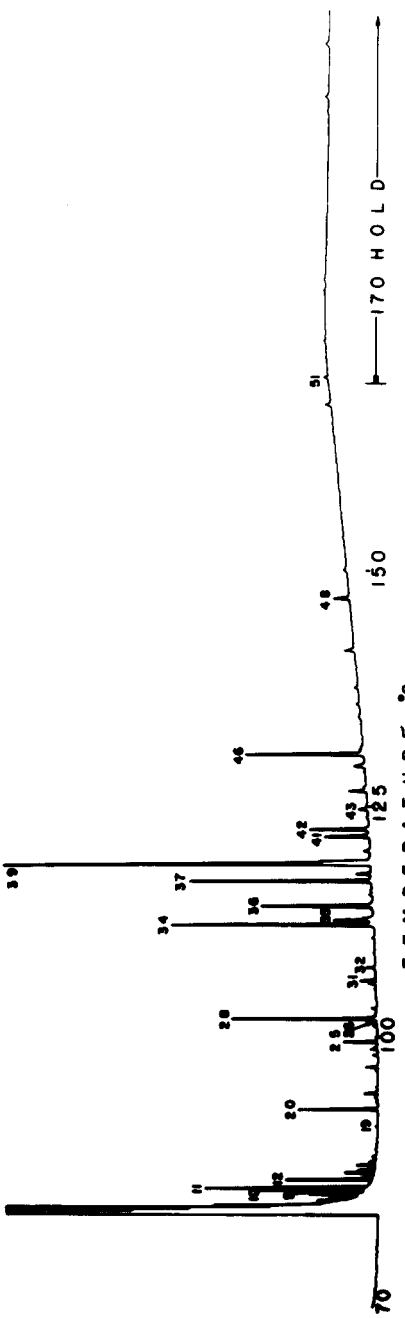

Fig. 1. Chromatogram of 1 μlitre bacuri essence, split 1:100 and programmed from 70 to 170 °C at 1 °/min on a WCOT glass capillary column coated with SE 30. Individual peaks were identified as (see Table 1): 2,2-pentanone; 11, heptane; 12,2-heptene; 19, furfural; 20,2-heptanone; 26, *cis*-3-hexenyl acetate; 32, γ-terpinene; 34, *cis*-linalool oxide; 35, 2-nonanone; 36, *trans*-linalool oxide; 37, linalool; 43 and 46, monoterpene alcohols; 51, methyl dodecanoate.

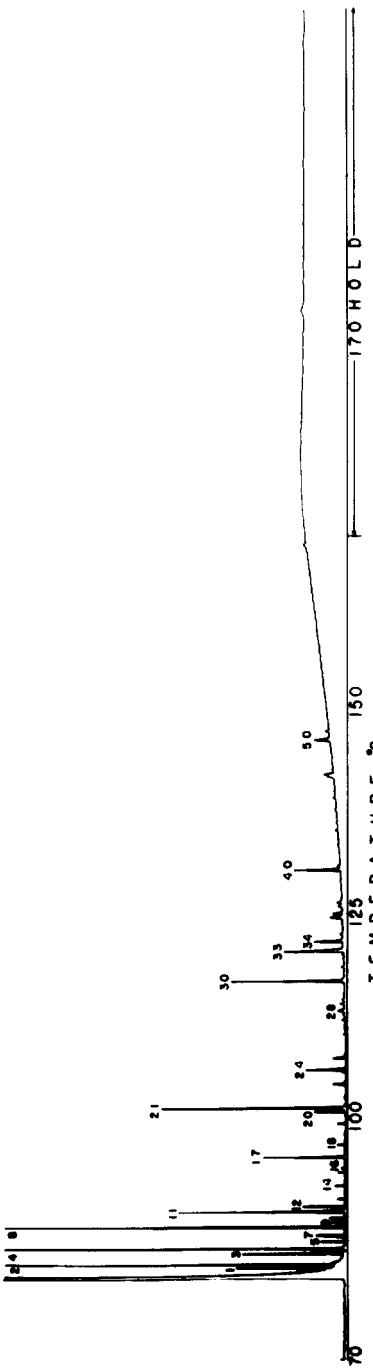

Fig. 2. Chromatogram of 1 μlitre cupuacu essence, split 1:100 and programmed from 70 to 170 °C at 1 °/min on a WCOT glass capillary column coated with SE 30. Individual peaks were identified as (see Table 1): 1, ethyl acetate; 8, ethyl butyrate; 9, butyl acetate; 12, ethyl 2-methyl butanoate; 14, furfural; 16, hexanol; 18, butyl *iso*butyrate; 20, butyl butyrate; 21, ethyl hexanoate; 24, butyl 2-methyl butanoate; 28, 2-methyl butyl 2-methyl butanoate; 40, monoterpene alcohol.

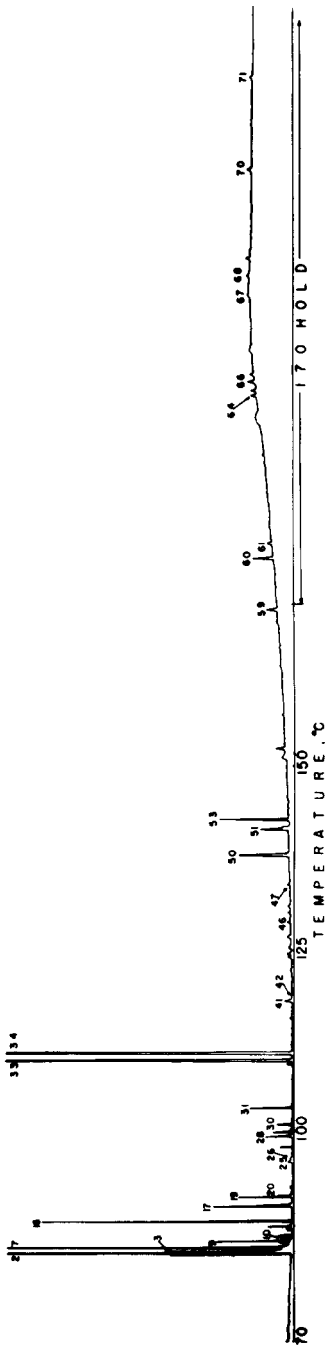

Fig. 3. Chromatogram of 1 μlitre of muruci essence, split 1:100 and programmed from 70 to 170°C at 1°/min on a WCOT glass capillary column coated with SE 30. Individual peaks were identified as (see Table 1): 7, 2-butanone; 8, ethyl acetate; 9, 2-butanol; 10, 1-methoxy-1-ethoxy ethane; 16, 1,1-diethoxyethane; 19, *n*-hexanal; 20, ethyl butyrate; 25, furfural; 26, 2-heptanone; 30, methyl hexanoate; 31, hept-3-ene-2-one; 33, ethyl hexanoate; 41, γ-terpinene; 42, *iso*-amyl butyrate; 46, methyl octanoate; 47, nona-2-ene-4-one; 51, butyl hexanoate; 53, ethyl octanoate; 60, hexyl hexanoate; 61, ethyl decanoate; 64, ethyl cinnamate; 68, ethyl dodecanoate; 70, ethyl tetradecanoate.

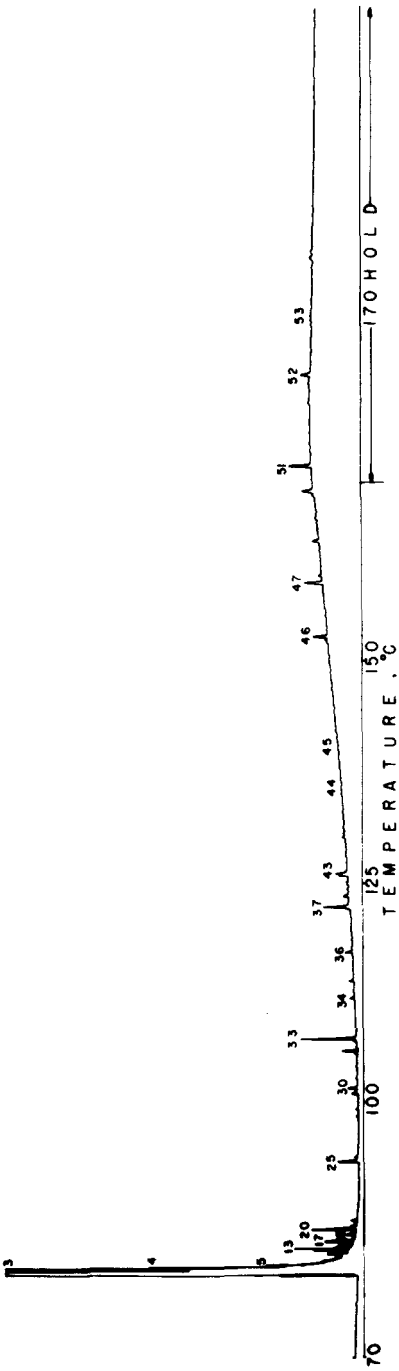

Fig. 4. Chromatogram of 1 μlitre of taperebá, split 1:100 and programmed from 70 to 170 °C at 1 °/min on a WCOT glass capillary column coated with SE 30. Individual peaks were identified as (see Table 1): 17, 2-heptene; 34, ocimene; 36, methyl benzoate; 44, ethyl benzoate; 45, ethyl octanoate; 53, ethyl cinnamate.

major fragments in the mass spectrum of peak 50 occur at *m/e* 73 and *m/e* 267 with smaller ions scattered through the spectrum. These fragments are typical of silicone-type compounds and may indicate that these are artifact peaks. As shown in Table 1, the major volatile constituents isolated from muruci were esters, dominated by ethyl esters, and included ethyl butyrate (peak No. 20), ethyl hexanoate (peak No. 33) and ethyl octanoate (peak No. 53). These occurred in fairly large amounts while smaller amounts of ethyl acetate (peak No. 6), ethyl decanoate (peak No. 61) and ethyl cinnamate (peak No. 64) were present. Other compounds of aroma significance included a large amount of butyl hexanoate (peak No. 51) and lesser quantities of methyl hexanoate (peak No. 30), hexanal (peak No. 19) and methyl octanoate (peak No. 46). None of these compounds alone represents the aroma of muruci; it is probable that the typical muruci aroma results from the interaction of a number of these compounds.

Only a few usable spectra were obtained from the GC–MS analysis of taperebá, but it was possible to identify a few of the volatiles. These included traces of methyl benzoate (peak No. 19) and ethyl benzoate (peak No. 36), each of which possesses strong fruity odours; a small amount of ethyl octanoate (peak No. 41) was present while ethyl cinnamate (peak No. 76) was present in moderate concentrations.

Considered individually, none of these compounds duplicates the aroma of any of these fruits, but many do possess aromaticity and contribute fruity notes. It seems likely that the typical aroma for each individual fruit is due not to one compound but is more probably an integrated response to a wide spectrum of compounds.

ACKNOWLEDGEMENTS

The authors are grateful to EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria, Brazil) for financial support, in the form of a Fellowship for the senior author, that made these studies possible. This paper is from a thesis submitted by the senior author in partial fulfilment of the requirements of the Group in Agricultural Chemistry for a Master of Science degree.

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Technical Note

Drawbacks in the use of the Biuret Method for Determination of the Same Protein in Differently Treated Fish Samples

There was a considerable difference in absorption (due to proteins) at 540 nm between fresh fish and frozen, thawed fish flesh using the Biuret method on the same raw material on the same day. This difference was increased after a day, giving lower results.

INTRODUCTION

It has been observed that proteins are able, under specific conditions, to complex with certain organic dye molecules. Some enlightening work has been done (Franenkel & Cooper, 1944) on the determination of total acidic and basic groups of proteins. Many workers have investigated the applicability of the dye-binding method for the quantitative protein determination of food substances (Udy, 1954, 1956; Mackenzie & Perrier, 1969; Mossberg, 1970).

The Biuret reaction involves the reaction of alkaline cupric ions with the peptide linkage. The method has an advantage over most others in that the colour formed is nearly independent of the nature of the protein. It is used routinely in most biochemical laboratories for the estimation of small amounts of cod muscle (Dyer *et al.*, 1950), for protein on rabbit livers (Hirayama, 1954), on fish muscle (Matsumoto & Perrier, 1955) and on beef, pork, chicken breast and cod (Torten & Whitker, 1964).

EXPERIMENTAL

The Biuret reagent was prepared as described by Layne (1957). The method itself is five times more rapid than the Kjeldahl method and with a little care an unskilled

Fd. Chem. (4) (1978)—
Printed in Great Britain

operator can obtain good results for a particular type of treated product. During the present study, however, protein estimation was carried out at different times on differently treated fish flesh.

Every reading was repeated four times during the investigation.

RESULTS AND DISCUSSION

The results are shown in Tables 1–3 and Figs. 1–3. There was a considerable difference in absorption at 540 nm between fresh fish flesh and frozen, thawed fish flesh. (Table 1, (1) and (2) and Fig. 1, curves 1 and 2). This could be due to some denaturation during freezing and thawing of the fish flesh. Torten & Whitaker (1964) suggested that the method is independent of the nature of the protein. In this case the nature of the protein was the same but treatments were different which could therefore affect the absorbance produced. This could mean that during freezing and thawing some of the peptide bonds were broken down, giving lower results.

The difference between fresh fish flesh and frozen, thawed fish flesh can be seen from Fig. 1, curves 1 and 2. The equation and slope for the fresh fish flesh is $y = 0{\cdot}0678x - 0{\cdot}027$ and the correlation factor is $r = 0{\cdot}98$ while for frozen, thawed fish flesh they are $y = 0{\cdot}0463x + 0{\cdot}0202$ and $r = 0{\cdot}99$.

There was a promising result between frozen, thawed fish flesh and frozen, thawed washed fish flesh (Table 1, (2) and (3) and Fig. 1, curves 2 and 3). The equation is $y = 0{\cdot}00463x + 0{\cdot}0202$ and the correlation factor is $r = 0{\cdot}99$ for the frozen, thawed fish flesh and $y = 0{\cdot}0435x + 0{\cdot}0291$ and $r = 0{\cdot}95$ for the frozen thawed washed fish flesh. From these results we can say that the difference is almost negligible and it is appropriate to draw a standard curve.

The above estimates were carried out on the same day. However, after storage at 3 °C for one day, followed by the application of the Biuret method, the rate of colour production was dramatically decreased. (Table 2, (4) and (5) and Fig. 2, curves 4 and 5.) This could be due to enzyme action on the proteins after thawing and during storage for 24 h. These are more precisely shown by the equations of curves 4 and 5. The equations and correlation factors are $y = 0{\cdot}0503x - 0{\cdot}0075$ and $r = 0{\cdot}99$ for the frozen, thawed fish flesh and $y = 0{\cdot}0589x - 0{\cdot}0686$ and $r = 0{\cdot}99$ for the frozen, thawed fish flesh kept at 3 °C for one day.

There was also a difference in absorbance between frozen, thawed, washed fish flesh and frozen, thawed, washed fish flesh kept at 3 °C for one day. (Table 3, (6) and (7) and Fig. 3, curves 6 and 7.)

This also could be due to enzyme action affecting the absorbance obtained. The equations and correlation factors are $y = 0{\cdot}084x - 0{\cdot}009$ and $r = 0{\cdot}99$ for the frozen, thawed washed fish flesh and $y = 0{\cdot}0759x - 0{\cdot}0646$ and $r = 0{\cdot}98$ for the frozen, thawed, washed fish flesh kept at 3 °C for one day.

TABLE 1

ABSORBANCES AT 540 NM OF SAMPLES (1), (2) AND (3)

	Millilitre sample	*% protein in sample*	*Quadruplet readings at 540 nm*		
			Minimum	*Maximum*	*Mean*
Fresh	0·4	1·39	0·073	0·081	0·077
fish flesh (1)	0·6	2·10	0·099	0·103	0·101
	0·8	2·80	0·154	0·171	0·162
	1·0	3·49	0·207	0·224	0·215
Frozen thawed	0·4	2·30	0·130	0·130	0·130
fish flesh (2)	0·6	3·45	0·175	0·190	0·182
	0·8	4·60	0·210	0·230	0·220
	1·0	5·75	0·280	0·310	0·295
Frozen thawed	0·4	2·05	0·135	0·135	0·135
washed fish	0·6	3·08	0·141	0·143	0·142
flesh (3)	0·8	4·11	0·199	0·201	0·200
	1·0	5·14	0·265	0·265	0·265

TABLE 2

ABSORBANCES AT 540 NM OF SAMPLES (4) AND (5)

	Millilitre sample	*% protein in sample*	*Quadruplet readings at 540 nm*		
			Minimum	*Maximum*	*Mean*
Frozen thawed	0·4	1·84	0·080	0·090	0·086
fish flesh (4)	0·6	2·77	0·127	0·134	0·130
	0·8	3·60	0·173	0·185	0·179
	1·0	4·62	0·222	0·230	0·225
Frozen thawed fish	0·4	1·83	0·038	0·039	0·038
flesh kept at 3°C	0·6	2·75	0·085	0·095	0·090
for one day (5)	0·8	3·67	0·152	0·168	0·158
	1·0	4·59	0·186	0·200	0·196

TABLE 3

ABSORBANCES AT 540 NM OF SAMPLES (6) AND (7)

	Millilitre sample	*% protein in sample*	*Quadruplet readings at 540 nm*		
			Minimum	*Maximum*	*Mean*
Frozen, thawed washed	0·4	1·77	0·136	0·145	0·139
fish flesh (6)	0·6	2·66	0·210	0·215	0·213
	0·8	3·55	0·280	0·309	0·293
	1·0	4·44	0·352	0·370	0·362
Frozen, thawed washed	0·4	1·37	0·045	0·050	0·047
(kept at 3°C for	0·6	2·06	0·077	0·105	0·086
one day) fish flesh (7)	0·8	2·75	0·120	0·157	0·133
	1·0	3·44	0·156	0·240	0·206

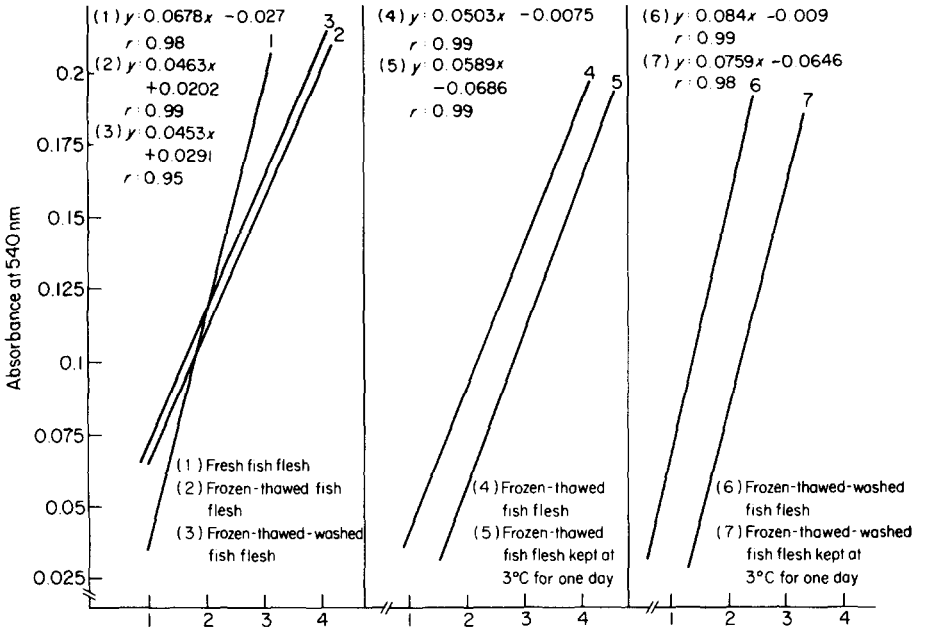

Fig. 1. Fig. 2. Fig. 3.

Fig. 1. Absorbances at 540 nm of samples (1) to (3) (% protein in sample).

Fig. 2. Absorbances at 540 nm of samples (4) and (5) (% protein in sample).

Fig. 3. Absorbances at 540 nm of samples (6) and (7) (% protein in sample).

From the equations obtained using the Biuret reaction on the above seven differently treated fish samples, we can conclude that the results are considerably different. Therefore it was difficult to use the results obtained from any one of them to draw a standard curve. For this reason it appears that the Biuret method is not a good method for determining the protein contents of the same substance which has undergone different treatments.

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BOOK REVIEWS

Protein Crosslinking. Edited by Mendel Friedman. (In two volumes, Nos. 86A and 86B in the series 'Advances in Experimental Medicine and Biology'.) Plenum Press: New York and London. 1977. Vol. 86A, xix + 760 pp., Vol. 86B, xx + 740 pp. Price: US$95·00.

This book contains a collection of papers presented at a symposium on the nutritional and biochemical consequences of protein cross-linking held in the Autumn of 1976 in San Francisco and organised by the American Chemical Society as part of its Centennial celebrations. The papers have not been set in type but are reproductions of the authors' typescripts. It is disappointing that the use of this short-cut method of publishing does not seem to have led to any noticeable decrease in the time interval between the meeting and publication; nor are printers' errors entirely eliminated—for example, Figs. 4 and 5 on page 191, Vol. 86A, are transposed. Each volume has its own index.

The usefulness of such collections depends not only on the speed of publication but also on the range and calibre of the contributions. Judged by these criteria, this book reaches a very high standard. The range of papers is particularly impressive and it is hard to think of any aspect of protein cross-linking that is not discussed somewhere in these pages. Nor are the contributors—essentially all active workers in their fields—drawn only from the USA; of the total of 82 papers, 30 are from other countries.

Volume 86A deals with chemical and biochemical aspects of cross-linking and starts with five papers on that classical cross-linking reaction, the thiol–disulphide interchange. These are followed by papers on new cross-linking reagents and their application to various systems. Most of the other papers are less easily categorised, however; they include one on the thermodynamics of cross-links by Rupley *et al.*, in which the authors found close agreement between experimental thermodynamic

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values and calculations based on Flory's statistical theory of polymers, and also one on an NMR study of the reaction between formaldehyde and amines. The overall impression of this volume is of the great ingenuity displayed in studies of this difficult subject which requires application of a very wide range of chemical techniques.

Volume 86B is sub-titled 'Nutritional and Medical Consequences'. Emphasis in the early papers is on lysinoalanine (LAL)—its formation during alkaline treatment of food proteins, its metabolism and its role in the pathogenesis of nephrocytomegalia in the rat. The question as to whether LAL and other unnatural amino acid derivatives formed during heat treatment of protein at high pH could significantly affect human health is discussed. The evidence seems equivocal and there is a clear need for further study in this area. Later papers extend the discussion to the formation of isopeptide and other cross-linkages during severe heat processing and to protein–carbohydrate interactions and their nutritional consequences. Three papers deal with protein utilisation in ruminants and with the 'protection' of feed proteins against degradation by the ruminal micro-organisms. Several papers discuss cross-linking in collagen and other structural and tissue proteins and the interesting concept that an underlying process in ageing, and in the development of disease in connective and vascular tissue, is the accretion with time of cross-linkages between protein molecules that cannot be broken by the cell enzymes.

In spite of some reservations, as noted above, this collection can be highly recommended as a stimulating and wide-ranging contribution to the literature of a difficult field; it is the sort of book that it will be a pleasure to dip into on many occasions. Unfortunately, its high price will inhibit its purchase by many organisations.

J. E. FORD and
R. L. J. LYSTER

Developments in Food Analysis Techniques. Edited by R. D. King. Applied Science Publishers Ltd, London. 1978. 223 pp. Price: £25.

Dr King's book is the first of a series planned by Applied Science which is intended to provide a 'state of the art' compilation on a range of topics. The individual chapters have been written by authors who are all actively involved in their subjects and one therefore expects—and in most cases finds—an authoritative and up-to-date review. In a compilation of this kind, the quality is bound to be a little uneven and this book is no exception. Several chapters discuss the same techniques applied to different constituents of foods but the overlapping sections have been carefully edited and do not detract from the usefulness of the book.

The first chapter, by A. A. Christie and R. A. Wiggins, on vitamin analysis is very comprehensive and gives a very balanced account of the current state of vitamin

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analyses. The determination of nitrogen and protein in foods is discussed by A. L. Lakin and the chapter is a useful introduction to the techniques in current use. The author also raises the problems arising when one attempts to calculate protein values from total nitrogen—he suggests that calculations based on amino acid content would be more accurate but a more detailed discussion of this point seems to be called for, and I personally do not agree that the measurement of protein-N is an intractable problem.

The third chapter, on the role of water, is by T. M. Hardman and is very valuable but seems out of place in the volume as a whole.

The techniques of HPLC, GLC enzymic methods and ion selection electrodes are reviewed in the next four chapters and these could with advantage have been more detailed—the chapter on enzymic methods really only summarises the position, this topic could readily be expanded into a later volume on its own in view of its potential importance.

The chapter on automated methods by P. B. Stockwell covers the many different facets of food analysis and demonstrates the many advantages that the large laboratory, in this case that of the Government Chemist, has in the development of automated systems. The introduction to the economics of automation would have been even more valuable if it included some specific examples of the savings introduced by automation. The determination of carbohydrates is covered by C. K. Lee who has restricted his discussion to free sugars and presumably will discuss polysaccharides later. This again is a competent introduction to the field.

The final chapter by K. M. Cowley deals with atomic absorption spectroscopy and this again is a useful, but rather short, introduction to the technique. The section on sample preparation is valuable and the short paragraphs on some inorganic constituents of interest provide an introduction to the problems.

In all, this collection of chapters provides a useful introduction to some important food analysis techniques; it will be especially useful to the analyst entering this field—the practising analyst may have his attention drawn to some facet of a technique that he has missed in his reading but may be disappointed with the depth of treatment. This volume is a good start to the series and gives one hope that this series will eventually provide a selection of texts for the food scientists, food analysts and nutritional workers.

D. A. T. SOUTHGATE

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Chapter 1 presents an up-to-date account of glucose syrup research, dealing with most of the recent developments in this field, and underlines how little we know about these very versatile carbohydrates which have been used for so many years. Other ACS papers deal in turn with fructose production, conformation of polysaccharides, occurrence of heptuloses and lactose recovery from various milk sources.

Fructose production from maize starch is a very topical subject at the present time and the conversion of starch to glucose to fructose, with its subsequent separation, is described. The enzymes used in the process and the separation techniques involved are discussed and the author foresees cheap fructose as a reality in the future.

X-ray diffraction studies are used to determine the conformations of gel-forming polysaccharides, more especially carageenan and agar, and the rheological and textural properties of these carbohydrates are thought to be associated with the helical secondary structures these compounds appear to possess.

Two later chapters describe physiological effects of carbohydrates and are of more general interest to the reader. Both deal broadly with sucrose ingestion, first, with its effect in the body and, secondly, with its cariogenic effect when eaten in the form of biscuits.

The eating properties of rice, browning of orange juice and carbohydrate dehydration reactions form the basis of three chapters relating the reactions of carbohydrate components of foods with the food's quality and illustrate the complexity of trying to relate chemical changes in foods to the quality.

The book follows the pattern of many other similar texts in that it is a collection of papers relating to one subject. Whilst there is an obvious need for these papers to be published it may be that the book is not specific enough in its choice of material for research use and is too detailed in certain areas for more general reading. It does, however, provide many useful references which increase its value to a certain extent.

MALCOLM W. KEARSLEY

ANNOUNCEMENT

The Fourth International IUPAC Symposium on Mycotoxins and Phycotoxins, co-sponsored by the World Health Organization and the Swiss Society for Analytical and Applied Chemistry, will be held in Lausanne, Switzerland, from 29 to 31 August, 1979.

The programme on the fungal and algal toxins includes plenary lectures and poster sessions on the following topics: Biogenesis, chemistry and analysis; Occurrence in foodstuffs and feedstuffs; Fate of mycotoxins/phycotoxins during food processing and storage; Decontamination procedures; Toxicology and implication of mycotoxins/phycotoxins in human and animal health.

A round-table discussion will review recent problems on peanut and corn crop contamination and shell-fish poisoning.

Further details may be obtained from: Professor P. Krogh, Department of Veterinary Microbiology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907, USA or Professor D. Reymond, IUPAC, Case postale 88, 1814 La Tour de Peilz, Switzerland.

Fd. Chem. (4) (1979)—
Printed in Great Britain

STUDIES ON α-AMYLASE INHIBITORS IN FOODS

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(Received: 14 June, 1978)

ABSTRACT

Different foods have been tested for α-amylase inhibitor activity on human salivary α-amylase. High activity was found in wheat flour (590 *units/g*), *whole wheat flour* (351 *units/g*) *and whole rye flour* (186 *units/g*) *but oat flour and barley flour had no activity. Bread baking reduced the α-amylase inhibitor activity in white bread and rye bread about* 80–90 % *and no activity was left in whole wheat bread. Spaghetti had high activity* (248 *units/g*) *before boiling but less than* 2 % *of the activity remained after* 15 *min of boiling. Red beans had some activity before boiling* (41 *units/g*) *but no activity was left after boiling for* $1\frac{1}{2}$ *h. Split peas, brown rice, potato, carrot and swede did not contain any α-amylase inhibitor activity.*

INTRODUCTION

Many plants are known to have naturally occurring enzyme inhibitors of a protein nature. The most interesting inhibitors from a nutritional point of view are the protease- and the α-amylase inhibitors. Compared with protease inhibitors, little is known about the structure and function of the α-amylase inhibitors (Liener *et al.*, 1969; Ryan, 1973). However, in recent years several papers have been published on the subject. α-Amylase inhibitor was first reported from buckwheat (Chrzaszcz & Janicki, 1933, 1934) and later from wheat (Kneen & Sandstedt, 1943, 1946; Shainkin & Birk, 1970; Saunders & Lang, 1973; Silano *et al.*, 1973; Petrucci *et al.*, 1974; O'Donnell & McGeeney, 1976; Granum & Whitaker, 1977), rye (Kneen & Sandstedt, 1946; Strumeyer, 1972; Marshall, 1977) and beans (Bowman, 1945; Hernandez & Jaffé, 1963; Jaffé *et al.*, 1973; Marshall & Lauda, 1975; Powers & Whitaker, 1977). No α-amylase inhibitors were found in barley, corn, oats, millet or

Fd. Chem. 0308-8146/79/0004-0173/$02·25

rice (Kneen & Sandstedt, 1946) but later an α-amylase inhibitor capable of inhibiting *Bacillus subtilis* α-amylase was reported from oat seeds (Elliott & Leopold, 1953). α-Amylase inhibitors have also been found in mangoes (Mattoo & Modi, 1970), taro root (Narayana Rao *et al.*, 1967, 1970) and acorns (Stankovic & Markovic, 1960–61).

Bessho & Kurosawa (1967) tested the heat inactivation of the amylase inhibitors in flour, by using pancreatic amylase. They found that the inhibitor activity decreased with length of baking time of bread and cakes but that more than half of the activity remained in cakes and about 13% of the activity remained in bread after baking.

In this paper experiments are reported in which some of the most commonly used flours, breads, spaghetti and vegetables employed in Norway were tested for α-amylase inhibitor activity on human salivary α-amylase.

MATERIALS AND METHODS

Materials

All food samples tested were bought in a local grocery store. The samples tested were whole wheat flour, wheat flour, oat flour, barley flour and whole rye flour, all obtained from Møllesentralen A/S. Whole wheat bread, white bread and rye bread came from Bakers A/S. Split peas came from H. Løken & Co. A/S, red beans from Pelmo, brown rice from Møllesentralen A/S and spaghetti from Den Norske Naeringsmiddelfabrikk A/S. Potato, carrot and swede (*Brassica napus* var. napobrassica) were local varieties.

Methods

The peas, the beans, the rice and the spaghetti were finely ground in a Wiley mill equipped with a No. 20 screen. The resulting flour was extracted by mechanical stirring in 0·02M phosphate buffer, pH 6·9 (1:10, w/v) for two hours at room temperature. The vegetables were mashed and extracted in the same manner. Boiled spaghetti was also tested. The spaghetti was boiled for 15 min in 0·02M phosphate buffer, pH 6·9, (1:10, w/v), cooled to room temperature and then mechanically stirred for two hours.

The bread samples were separated into two parts; the central part (3 cm diameter in the centre) and the peripheral part starting just inside the crust and going into the central part. The bread samples were crumbled and extracted in the same manner as the flour samples.

After stirring, all samples were centrifuged at 10,000 × *g* for 30 min at 4 °C. The clear supernatants were then heat treated for 10 min at 70 °C and centrifuged again under the same conditions. This last supernatant was then tested for α-amylase inhibitor activity.

Activity determination

α-Amylase inhibitor activity was tested on human salivary α-amylase as described by Granum & Whitaker (1977). One amylase unit was defined as an increase of 1·0 in $A^{1\,cm}_{540\,nm}$ per minute at 30 °C and pH 6·9. One amylase inhibiting unit was defined as a decrease of one amylase unit.

RESULTS AND DISCUSSION

Flour

α-Amylase inhibitor activity in different types of flour was tested on human salivary α-amylase. The results are shown in Table 1. The highest specific activity was

TABLE 1
AMYLASE INHIBITOR ACTIVITY IN DIFFERENT TYPES OF FLOUR, TESTED ON HUMAN SALIVARY α-AMYLASE AT pH 6·9, 30 °C, 30 MIN PRE-INCUBATION

Sample	*Units/g*
Whole wheat flour	351
Wheat flour	590
Whole rye flour	186
Barley flour	0
Oat flour	0

found in wheat flour, while whole wheat flour had about 60 % of that activity. These findings are in agreement with those of Kneen & Sandstedt (1946) who compared α-amylase inhibitor activity in ground whole wheat flour with the activity in wheat bran. They found a much higher specific activity in the flour than in the bran. It was concluded that the majority of the inhibitor is present in the wheat endosperm. As a result of removing the bran from wheat flour, the specific activity of the α-amylase inhibitor increased. The ground whole rye flour had about 53 % of the specific activity found in ground whole wheat flour, which is close to the 60 % found by Kneen & Sandstedt (1946). No amylase inhibitor activity was found in either barley or oat flour. The same results were obtained by Kneen & Sandstedt (1946) but Elliot & Leopold (1953) reported a protein from oat seeds able to inhibit α-amylase from *Bacillus subtilis*. This inhibition, however, was reversed by sulphydryl compounds and was believed to be an interaction between the inhibitor and the thiol groups in the enzyme, rather than a specific inhibition reaction.

Bread

Different types of bread were tested for amylase inhibitor activity on human salivary α-amylase. These results are shown in Table 2. White bread, commercially produced, is made of white wheat flour only and is baked for 36 min at 210 °C. In

TABLE 2
AMYLASE INHIBITOR ACTIVITY IN DIFFERENT TYPES OF BREAD, TESTED ON HUMAN SALIVARY α-AMYLASE AT pH 6·9, 30 °C, 30 MIN PRE-INCUBATION

Sample	*Units/g*
White bread, central part	54
White bread, peripheral part	30
Whole wheat bread, central part	0
Rye bread, central part	60
Rye bread, peripheral part	30

spite of the baking the central part of the bread had about 10% of the amylase inhibitor activity found in white wheat flour.

The peripheral part of the bread had lower activity due to a higher baking temperature. Bessho & Kurosawa (1967) reported the activity in white bread to decrease to about 13% of the activity found in the dough, after 40 min baking time (baking temperature not given). They also found about the same activity ratio between the central and the peripheral parts of the bread.

No α-amylase inhibitor activity was found in whole wheat bread even in the centre. The same result was found for the same kind of bread from three different bakeries. This must partly be due to the lower inhibitor activity in the flour used, partly to the baking temperature (220 °C, 36 min) and probably to better heat transfer.

Rye bread is made of 50% white rye flour (containing 15% wheat) and 50% white wheat flour and is baked for 36 min at 220 °C. This bread would be expected to have lower α-amylase inhibitor activity than white bread, since rye flour has lower activity than wheat. Rye bread, however, usually has a larger diameter than white bread and has a higher specific weight. This may explain the relatively high α-amylase inhibitor activity remaining in rye bread.

No α-amylase inhibitory activity was found in brown rice, split peas, potato, carrot or swede (Table 3). Kneen & Sandstedt (1946) found no inhibitor activity in

TABLE 3
AMYLASE INHIBITOR ACTIVITY IN DIFFERENT TYPES OF FOODS, TESTED ON HUMAN SALIVARY α-AMYLASE AT pH 6·9, 30 °C, 30 MIN PRE-INCUBATION

Sample	*Units/g*
Spaghetti	248
Boiled spaghetti (15 min)	3
Brown rice	0
Red beans (dry)	41
Boiled beans (1½ h)	0
Split peas (dry)	0
Potato	0
Carrot	0
Swede	0

rice, either. Non-proteinaceous inhibitors have been found in potato tubers by Hemberg & Larsson (1961). Beans have long been known to have α-amylase inhibitor activity (Bowman, 1945) but this activity is not present after $1\frac{1}{2}$ hours' of boiling. Spaghetti is made of durum wheat which is known to have several α-amylase inhibitors (Bedetti *et al.*, 1974). The activity in spaghetti, however, is reduced to about 1 % after boiling for 15 min—the normal boiling time given for this type of spaghetti.

Some reports on the nutritional significance of α-amylase inhibitors have been published. An α-amylase inhibitor from red kidney beans did not affect the rate of growth of weanling rats (Savaiano *et al.*, 1977). These authors concluded that the inhibitor was not toxic and did not affect the availability of energy from dietary starch. Lang *et al.* (1974), however, demonstrated that the inclusion of wheat α-amylase inhibitors (2, 4 and 8 % of the diet) in a starch-enriched diet caused a large decrease in starch availability. Continuous feeding of α-amylase inhibitors from wheat albumins to chickens (Macri *et al.*, 1977) did not change the growth rate and it was concluded that the gastric digestion in chickens was very effective in inactivating albumin amylase inhibitors. Macri *et al.* also demonstrated that intake of the same inhibitors in gastro-resistant microgranules depressed chicken growth rate, but this was overcome after four weeks of treatment. At that time the chicken pancreases showed hypertrophy and a number of histological changes indicating degenerative processes were in progress. This casts doubt upon the desirability of using oral administration of amylase inhibitors to control post-prandial hyperglycaemia in Man, suggested by Puls & Keup (1973). These findings cannot be directly related to Man, but it would seem that naturally occurring amounts of α-amylase inhibitors only have marginal negative effects on human nutrition. Protein α-amylase inhibitors may represent as much as 1 % of wheat flour (Petrucci *et al.*, 1974) and a large intake of wheat protein which has not been heat treated sufficiently may be a nutritional problem. This has, however, not been demonstrated so far. Most of the food containing α-amylase inhibitors will be heat treated (boiled, baked) before being eaten and only small amounts of the inhibitor activity will be left.

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EATING QUALITY INDICATORS FOR WAXY RICES

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(Received: 26 June, 1978)

ABSTRACT

A study of the properties of raw and cooked waxy milled rices from the Republic of Korea, the Philippines and Thailand verified that the hardness and stickiness values of cooked rices, as measured by an Instron food tester, tended to be related to final gelatinisation or birefringence end-point temperature (BEPT), neutral gel consistency or both properties of raw rices. Actual texture determination of cooked rice is required among the low final BEPT rices that have similar gel consistency and alkali test values. Intermediate final BEPT waxy rices as cooked rice had hardness and stickiness properties similar to those of high final BEPT rices.

INTRODUCTION

Previous studies have indicated that waxy or glutinous rices differ in their eating quality, particularly in processed rice products not usually consumed immediately after processing, e.g. Japanese rice cakes (Palmiano & Juliano, 1972) and Filipino puffed parboiled rice (Antonio & Juliano, 1973, 1974) and rice cakes (Antonio *et al.*, 1975). Such differences have been correlated with gel consistency and final gelatinisation or birefringence end-point temperature (BEPT) of the waxy rice starch (Antonio & Juliano, 1974; Antonio *et al.*, 1975; Perdon & Juliano, 1975). The consistency of neutral or alkaline gels of waxy rice was positively correlated with molecular size of amylopectin (Perdon & Juliano, 1975). Final BEPT is classified as low $<70\,^{\circ}C$; intermediate 70–74 °C and high $>74\,^{\circ}C$ (Juliano, 1972).

With the breeding programmes for waxy rices in Thailand, South Korea, the Philippines and the IRRI and the development of more objective means of instrumental measurement of the stickiness and hardness of cooked rice, the properties of waxy samples differing in final BEPT were studied. Intermediate BEPT

Fd. Chem. 0308-8146/79/0004-0179/$02·25

waxy rices were included for the first time. Among non-waxy rices of similar amylose content, we recently found that differences in hardness of cooked rice were related to differences in gel consistency or final BEPT (Perez & Juliano, 1979).

MATERIALS AND METHODS

Materials

Rice samples were obtained from breeding programmes in the Philippines, Thailand and South Korea and from Niigata, Japan. The South Korean samples were derived from crosses of Tongil (indica × japonica rice) with various waxy rices. The Japanese rices from Niigata were those previously studied by Palmiano & Juliano (1972). Samples other than milled rice were dehulled with a Satake THU test husker and milled with a Satake TM-05 grain testing mill. Rice flour for amylography and protein analysis was prepared with a Udy Cyclone mill with a 40-mesh sieve.

Methods

Alkali spreading values were determined by incubating six whole milled rice grains in 10 ml 1·7% and 1·3% KOH for 23 h at 30 °C and scoring the degree of spreading according to Little *et al.* (1958). Final birefringence end-point temperature (BEPT) was measured photometrically using a modified Spectronic 20 colorimeter at 525 nm with heated test tube holder (Ignacio & Juliano, 1968). Protein content of rice was determined from micro-Kjeldahl N multiplied by the factor 5·95. An amylogramme was obtained with 40 g flour in 360 ml water in a Brabender Visco/amylograph with 700 g/cm sensitivity cartridge (Juliano *et al.*, 1969). Amylograph consistency is the difference between viscosity on cooling to 50 °C and final viscosity at 94 °C.

Milled rice (20 g) was cooked in 26 ml water in 150-ml beakers in Toshiba RC-4B automatic electric cookers for 20 min with excess (200 ml) water in the outer pan (Juliano *et al.*, 1965). The cooker was left undisturbed for 10 min after cooking, following which the cooked rice was drained and cooled in plastic bags. Measurements of hardness and stickiness of cooked rice were made with the Instron Model 1140 Food Tester according to Perez & Juliano (1979).

RESULTS AND DISCUSSION

Waxy rices from the Philippines, South Korea and Thailand

The analysis showed that preferred varieties have low gelatinisation temperature by alkali spreading or by final BEPT determination (Table 1). RD6 and Niaw San Pahtawng are preferred over RD4 in Thailand and Panpet 63 is inferior to Malagkit

TABLE 1

PROPERTIES OF RAW AND COOKED WAXY MILLED RICES FROM THREE COUNTRIES ARRANGED IN INCREASING GELATINISATION TEMPERATURE (BEPT)

Variety or line	*Alkali spreading value* (1·7% *KOH*)	(1·3% *KOH*)	*Final BEPT* (°C)	*Protein* (% *dry basis*)	*Neutral gel consistency* (*mm*)	*Amylograph visc.* (*BU*) *Peak*	*Consistency*	*Cooked rice* *Hardness* (*kg*)	*Stickiness* (*g/cm*) *Extruded*	*Not Extruded*
Philippines										
Malagkit Sungsong	6·4	6·0	62·5	7·4	76	440	65	3·5	432	492
IR29	7·0	4·2	63	8·8	52	560	100	5·6	264	—
UPL-Ri-1 (C229-1)	6·5	4·6	64	6·9	62	400	50	3·3	520	—
IR3464-75-1-1	7·0	5·8	62·5	6·4	60	680	65	4·2	428	450
IR4427-51-6-3	6·9	6·5	64	6·9	60	535	65	4·7	407	—
Panpet 63	2·3	2·0	77	7·3	35	870	80	5·9	—	210
C441-4	3·0	2·0	74	11·4	40	—	—	5·2	224	257
Thailand										
Niaw San Pahtawng	6·8	4·8	64	7·0	94	570[a]	120[a]	4·9	437	357
RD6	7·0	5·0	63·5	6·9	74	450[a]	90[a]	5·9	390	324
RD4	2·0	2·0	73·5	8·0	46	820[a]	80[a]	6·6	267	300
South Korea										
Tongil chal	7·0	5·8	62·5	10·0	70	505	65	4·9	—	330
Wx 219-3-5-3-1-2	4·2	3·0	70·5	11·6	66	755	90	6·8	287	260
Wx 219-3-5-5-2-3	4·0	3·0	70	11·2	65	750	75	6·3	272	240
Wx 202-13-9-3	3·2	2·2	73·5	9·4	66	795	95	5·2	224	257

[a] Data from Ngamchuen Kongseree, Rice Division, Dept. of Agriculture, Bangkok, Thailand.

Sungsong for making Filipino rice cakes (Antonio *et al.*, 1975) and puffed parboiled rice (Antonio & Juliano, 1974).

Neutral gel consistency showed good correlation with final BEPT among samples from Thailand and the Philippines but intermediate final BEPT samples from South Korea were similar in gel consistency to low and high final BEPT samples (Table 1). Amylograph peak viscosity and, to a lesser extent, consistency, tended to be higher for the intermediate and high final BEPT waxy rices. Similar results have been obtained for the alkali-viscogramme peak viscosity of waxy rice starches (Suzuki & Juliano, 1975).

The hardness and stickiness of cooked rice using the Instron food tester showed generally higher hardness readings and lower stickiness for the intermediate and high final BEPT samples than the low final BEPT samples (Table 1). However, some of the low final BEPT rices overlapped with the readings for the rices with higher final BEPT. For example, the sample of cooked IR29 rice, which has been reported to be inferior to Malagkit Sungsong in texture of cooked rice, was definitely harder and less sticky than cooked Malagkit Sungsong rice. IR29 had the hardest gel consistency among the low final BEPT Philippine waxy rices tested. These results on waxy rices confirmed our observations on non-waxy rices that among samples of similar amylose content, gel consistency and final BEPT are important eating quality indicators (Perez & Juliano, 1979).

A freshly harvested sample of a new Philippine variety, UPL-Ri-1, had a similar hardness score but was more sticky than Malagkit Sungsong. The IRRI line IR3464-75-1-1 had stickiness similar to that of Malagkit Sungsong but was slightly harder. Cooked rice of the IR4427-51-6-3 line had similar stickiness properties but was harder than cooked Malagkit Sungsong. These varieties are softer than the Thai waxy rice varieties, Niaw San Pahtawng and RD6.

Our previous studies have shown that waxy rices with high final BEPT have higher molecular weight amylopectins with higher gel viscosity (Perdon & Juliano, 1975). Such differences in molecular size presumably contribute also to differences in hardness as measured by resistance to extrusion of the cooked rice.

Variation in cooked rice hardness and stickiness was observed in samples of the same rice variety or line. A 1975 sample of UPL-Ri-1 with 7·8 % protein had cooked rice hardness of 5·0 kg and stickiness of 262 g/cm, which are at variance with the freshly harvested sample of the same variety (Table 1). Our previous results indicate that ageing has little effect on the texture of cooked waxy rice based on taste panel evaluation (Juliano *et al.*, 1969) and stickiness measurement with a beam balance technique (Villareal *et al.*, 1976). Two crops of four waxy rices showed more variation in the hardness than in the stickiness of cooked rice (Table 2). Many factors probably contribute to the variation in these properties of cooked rice among samples of the same rice selection or variety. For example, the higher protein cooked rice sample was not necessarily harder or less sticky than the lower protein sample of the same line.

TABLE 2
HARDNESS AND STICKINESS OF COOKED MILLED RICE OF TWO 1977 CROPS OF FOUR WAXY RICES MEASURED WITH AN INSTRON FOOD TESTER

Sample	*Protein (% dry basis)*		*Hardness (kg)*		*Stickiness (g/cm)*	
	Dry season	*Wet season*	*Dry season*	*Wet season*	*Dry season*	*Wet season*
IR3464-75-1-1	6·4	9·1	4·2	4·0	428	434
IR4427-51-6-3	7·8	8·3	4·7	3·8	407	447
IR4427-58-5-2	6·6	8·2	3·7	3·6	494	447
IR4445-63-1-2-2	7·7	8·6	4·9	3·9	407	407
Mean	7·1	8·6	4·4	3·8	434	434

Japanese rices

Japanese waxy rices of known cake quality (Palmiano & Juliano, 1972) presented an interesting contrast to the other waxy rices from the tropics and South Korea as they showed less differences in hardness and stickiness of cooked rice (Table 3). All the samples had low final BEPT and soft gel consistency. The samples of good cake-making quality had higher amylograph peak viscosity and final BEPT and softer consistency than those of poor cake-making quality. However, neutral gel consistency values were similar for all samples. Cooked rice from the good quality samples tended to have lower hardness values (softer) but had stickiness values similar to those of the poor quality samples of similar protein content such as Nakatamochi. The higher hardness and lower stickiness values for Norin No. 1 may be due in part to its higher protein content than the three other samples. Very low final BEPT such as the value of 58·5 °C for Norin No. 1 may also result in poorer quality cooked waxy rice at high BEPT. Perdon & Juliano (1975) showed a U-shaped relationship between final BEPT and intrinsic viscosity $[\eta]$ of amylopectin of waxy rices with minimum $[\eta]$ at about 68 °C.

TABLE 3
PHYSICO-CHEMICAL PROPERTIES OF RAW AND COOKED MILLED NIIGATA WAXY RICES DIFFERING IN CAKE QUALITY[a]

Property	*Koganemochi*[b]	*Nakatamochi*[b]	*Hatsunemochi*[c]	*Norin No. 1*[c]
Rice cake quality	Good	Poor	Good	Poor
Protein (% dry basis)	7·4	8·0	7·4	10·9
Amylograph visc. (BU)				
Peak	560	350	595	350
Consistency	35	80	45	65
Neutral gel consistency (mm)	86	90	81	86
Alkali spreading value (1·3% KOH)	2·2	4·7	2·8	4·3
Final BEPT (°C)	67·5	63	68·5	58·5
Cooked rice hardness (kg)	5·6	5·9	6·0	6·9
Cooked rice stickiness (g/cm)	326	347	370	267

[a] All data except neutral gel consistency and cooked rice properties from Palmiano & Juliano (1972).
[b] Obtained from and assessed for cake quality by Dr H. Kurasawa.
[c] Obtained from and assessed for cake quality by Dr S. Saito.

CONCLUSIONS

The hardness and stickiness of cooked rice were shown to be important eating quality indicators of waxy rices from South Korea, the Philippines and Thailand. Preferred varieties have soft, sticky, cooked rice and were verified to generally correspond to waxy rices with low final BEPT, soft neutral gel consistency, or both. Among four low final BEPT Japanese waxy rices, the preferred rices had relatively higher final BEPT (>65 °C).

ACKNOWLEDGEMENTS

We thank the Plant Breeding Department, IRRI, M. H. Heu, Seoul National University, Suweon, Korea, Ngamchuen Kongseree, Rice Division, Dept. of Agriculture, Bangkok, Thailand, A. M. del Mundo (Institute of Human Ecology) and P. S. Castillo, University of the Philippines at Los Baños, for the waxy rice samples.

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INDICATORS OF EATING QUALITY FOR NON-WAXY RICES†

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(Received: 26 June, 1978)

ABSTRACT

Although amylose content is the major determinant of the eating quality of milled rice, varieties of similar amylose content may differ in eating quality. Stickiness of cooked rice, as measured with an Instron food tester, gave a better correlation with amylose content than did hardness. Among rices of similar amylose content (high, intermediate or low) differences in hardness of cooked rice were shown to be generally related to differences in gel, and amylograph consistency, final gelatinisation temperature, or both. A higher rice concentration improved the differentiation among low-amylose samples for gel and amylograph consistency.

INTRODUCTION

Starch is the major constituent of milled rice and its characteristics differ widely among varieties, as reflected in such properties as the amylose: amylopectin ratio and final gelatinisation or birefringence end-point temperature (BEPT) (Juliano, 1972*a*, *b*). On the basis of amylose content, milled rice is classified as waxy (0–2% amylose, dry basis), low (10–20%), intermediate (20–25%) and high (>25%). Final BEPT is classified as low <70 °C; intermediate 70–74 °C; and high >74 °C (Juliano, 1972*b*). Our studies of rices that differ widely in amylose content indicate that amylose content has a major influence on the taste panel scores for cohesiveness, tenderness and gloss of boiled rice, regardless of the rice:water ratio used during cooking (Juliano *et al.*, 1965, 1972).

† Presented in part at the 61st Annual Meeting, American Association of Cereal Chemists Inc., New Orleans, La., USA, 5–8 October, 1976.

Fd. Chem. 0308-8146/79/0004-0185/$02·25

Varietal differences in eating quality have been reported for waxy rices and among non-waxy rices of similar amylose content (Juliano *et al.*, 1974). Among waxy and high amylose rices, gel consistency tests have been developed that correlate with texture and taste panel scores for cooked rice (Cagampang *et al.*, 1973; Juliano & Perdon, 1975; Perdon & Juliano, 1975). Recently, the texturometer (Kwon *et al.*, 1975; Endo *et al.*, 1976) and the Instron food tester instrument (Mossman & Fellers, 1976; Priestley, 1974; Lisch & Launay, 1975) were used to measure the texture of cooked rice. Because of the widespread preference in Southeast Asia for rice of intermediate amylose content (Juliano *et al.*, 1964; 1974), we studied the properties of representative intermediate amylose rices from Indonesia, Philippines and Thailand, rices that show extreme elongation on cooking (Juliano, 1972) and three japonica rices from Europe. Low-amylose rice samples from South Korea, Japan and Thailand were also studied. Our purpose was to establish indexes of eating quality among these non-waxy rices of similar amylose content. A similar study was made on waxy rices (Perez *et al.*, 1979).

MATERIALS AND METHODS

Materials

Rice samples were obtained from rice breeding programmes in each of the countries specified. The samples were received at IRRI either as milled or rough rice. Rough rice was dehulled with a Satake THU test husker and milled with a Satake TM-05 grain testing mill. Flours for amylograph, amylose and protein analysis were obtained with a cyclone sample mill with 40-mesh sieve. Flours for gel consistency were prepared in ten-grain lots for 40 sec in Wig-L-Bug amalgamators (Cagampang *et al.*, 1973). All samples were stored for at least six months after harvest before analysis to ensure adequate ageing (Villareal *et al.*, 1976).

Methods

Alkali spreading value was estimated according to the method of Little *et al.* (1958) on replicate six grains soaked in 10 ml 1·7% and 1·3 or 1·15% KOH for 23 h at 30 °C. Final gelatinisation or birefringence end-point temperature (BEPT) was measured photometrically on rice flour dispersed in water in a mortar and pestle and suspended in water in a Spectronic 20 colorimeter at 525 nm with heated test tube holder (Ignacio & Juliano, 1968). Amylose was measured colorimetrically at pH 4·5 and 608 nm using rice samples of known amylose content at pH 10 for the standard curve (Juliano, 1971). Protein was determined from micro-Kjeldahl N multiplied by the factor 5·95. Gel consistency was measured on duplicate 100, 110 or 120 mg flour samples in 2 ml 0·20 or 0·15N KOH as previously described (Cagampang *et al.*, 1973).

Amylograph pasting viscosity was determined using a Brabender

Viscoamylograph with 700 g.cm sensitivity cartridge and 40 g/360 ml water (10%), 44 g/356 ml water (11%), or 48 g flour/352 ml water (12%) (Juliano *et al.*, 1964). Amylograph consistency in Brabender units was calculated from the difference between viscosity when cooled to 50°C and final viscosity on cooking at 94°C.

Texturometer data on four Japanese rice samples were obtained at the National Food Research Institute (Japan) according to standard procedures (Endo *et al.*, 1976).

Cooked rice for measurement in an Instron Model 1140 food tester was prepared by cooking 20 g milled rice in a predetermined optimum amount of water (34, 38 and 42 ml for low-, intermediate- and high-amylose rices, respectively) in 150 ml beakers for 20 min in Toshiba RC4B automatic electric cookers with excess (200 ml) water in the outer pot. The cookers were left undisturbed for at least 10 min after cooking, following which the cooked rices were drained and cooled in plastic bags. Duplicate 17 g cooked rice samples were placed in the Ottawa Texture Measuring System cell, modified with four side liners to reduce the cell cross-section to 15% of the original and used with a 2·6 × 2·5 cm plunger. Each sample was pressed with 145 g of weight for 1 min before extrusion. Hardness was the maximum force (in kg) needed to extrude the rice through the cell's perforated base at a crosshead speed of 10 cm/min and the same chart speed. Hardness values were 15% of the values obtained with the standard cell. For the stickiness test, cooked rice (either extruded or whole) was pressed onto the platform with the OTMS plunger (6·9 × 6·9 cm) for 10 sec with a clearance of 0·4 mm, allowing the rice to squeeze out around the edges. Stickiness was expressed as g.cm, which is the product of the force in grammes required to lift the plunger and the distance in centimetres that the plunger traverses (Mossman & Fellers, 1976). This is measured directly by planimetry from the Instron chart paper. The chart speed was 100 cm/min and crosshead speed 5 cm/min.

RESULTS AND DISCUSSION

Stickiness and hardness of cooked rice and amylose content

Stickiness of cooked rice prepared with optimum cooking water was correlated negatively with amylose content using mainly IRRI samples (Fig. 1). Hardness of cooked rice showed a positive correlation but provided more spread of values at any amylose level than stickiness of cooked rice. The results suggest that varietal differences in the texture of cooked rices of similar amylose content are probably related to differences in hardness or tenderness of cooked rice since the range of stickiness values is narrow except among waxy rices. Stickiness and hardness of cooked rice are also significantly negatively correlated ($r = -0{\cdot}75^{**}$, $n = 26$). Previous taste-panel studies showed that scores for cohesiveness and tenderness of cooked rice are negatively correlated with amylose content of milled rice (Juliano *et al.*, 1965, 1972).

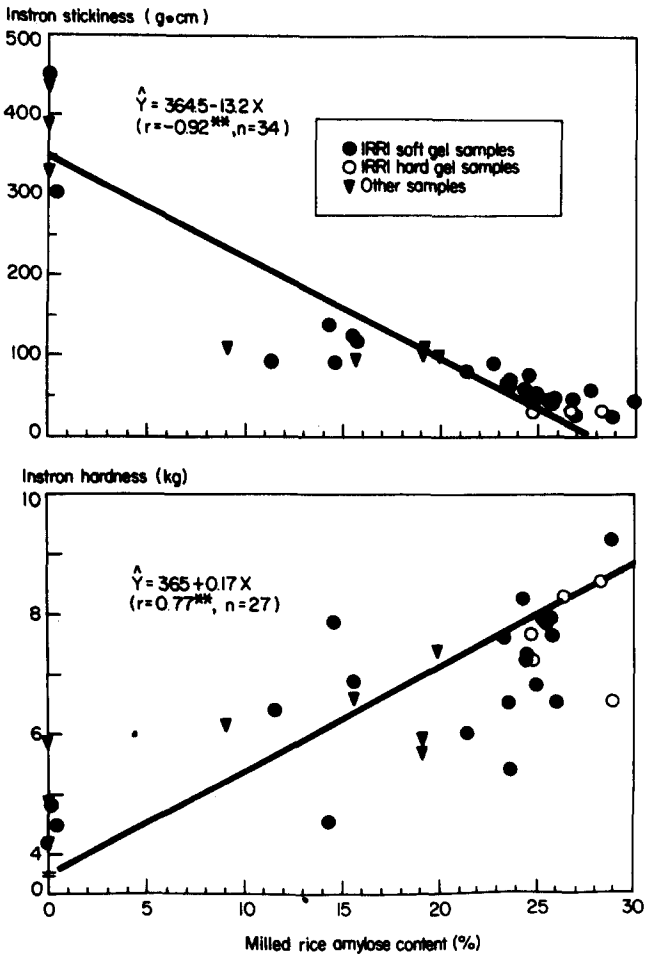

Fig. 1. Relationship between amylose content of milled rice and Instron stickiness and hardness of rice cooked with the optimum level of water (based on amylose content) and cooled.

High amylose rices

Among 20 high amylose rices in Fig. 1, of which 17 are IRRI rices, rices of the three gel consistency types overlapped in hardness and stickiness of cooked rice (Table 1). However, the soft gel consistency samples tended to have softer and more sticky cooked rices. These results are in agreement with previous observations that among high amylose rices, soft gel consistency is preferred over hard gel consistency (Juliano *et al.*, 1974). Soft gel consistency was associated mainly with intermediate final BEPT, indexed by alkali spreading value, and hard gel consistency was associated with low final BEPT. This association may be influenced in part by the

TABLE 1
HARDNESS AND STICKINESS OF THE COOKED RICE OF HIGH-AMYLOSE MILLED RICES DIFFERING IN GEL CONSISTENCY

Property	*Gel consistency type*		
	Hard (27–40 *mm*)	*Medium* (41–60 *mm*)	*Soft* (61–100 *mm*)
Number	8	6	6
Cooked rice hardness (kg)			
Range	7·2–9·2	6·8–7·9	6·5–7·6
Mean	8·2	7·5	6·9
Cooked rice stickiness (g.cm)			
Range	28–50	39–54	44–60
Mean	34	46	53
Alkali spreading value			
Range	5·0–7·0	3·5–7·0	3·0–5·0

fact that IRRI sources of dwarfing gene had high-amylose milled rices with low final BEPT and hard gel consistency (Suzuki & Juliano, 1975).

Intermediate-amylose rices

A study of optimum paste concentration for the gel consistency test for intermediate-amylose rices indicated that the standard concentration of 100 mg/2 ml for high amylose rices gave the best spread of values (Table 2). Higher viscosity, evidenced by harder gel consistency, was obtained at similar paste concentration in 0·15N KOH. Gel consistency in 0·15N KOAc (1 ml 0·3N KOH neutralised after starch dispersion with 1 ml 0·3N HOAc) was similar to that in 0·2N KOH. However, gels in 0·2N KOH were generally more stable to liquid phase separation than in 0·15M KOAc.

TABLE 2
PROPERTIES AND EFFECT OF RICE CONCENTRATION AND SOLVENT ON GEL CONSISTENCY VALUES (IN MILLIMETRES) OF THREE INTERMEDIATE-AMYLOSE MILLED RICES

Property	*C4–63G*	*IR2699–18–2*	*IR2071–636–5*
Protein (% dry basis)	7·7	7·8	10·5
Amylose (% dry basis)	23·0	24·5	23·5
Alkali spreading (1·7% KOH)	2·0	6·7	7·0
Final BEPT (°C)	75	67	67
Amylograph consistency (BU)	230	250	430
Gel consistency (mm)			
100 mg rice/2 ml 0·2N KOH	92	71	34
110 mg rice/2 ml 0·2N KOH	47	36	32
100 mg rice/2 ml 0·15N KOH	51	42	32
110 mg rice/2 ml 0·15N KOH	34	32	29
100 mg rice/2 ml 0·15N KOAc	94	76	32
Hardness of the cooked rice (kg)	5·9	7·3	6·5
Stickiness of the cooked rice (g.cm)	78	78	68

Amylograph consistency closely followed the gel consistency values for the three samples (Table 2). The C4-63G samples had the softest cooked rice but the IR2071-636-5 rice, with hardest gel consistency, gave only intermediate hardness value. Cooked IR2071-636-5 was also less sticky than the two other rices. A contributing factor may be its higher protein content.

A study of the physicochemical properties of five Indonesian rices showed that the poorest quality rice, Dewi Ratih, had high amylose content and the rest had intermediate amylose content (Table 3). The low rating for Pelita I-1 was not

TABLE 3
PHYSICOCHEMICAL DATA OF MILLED RICE OF FIVE INDONESIAN VARIETIES ARRANGED IN ORDER OF DECREASING EATING QUALITY, 1975–1976 CROP

Property	*Rojolele*[a]	*Seratus malam*	*Bengawan*	*Pelita I–1*	*Dewi Ratih*
Amylose (% dry basis)	23·8	24·0	24·4	23·4	28·3
Protein (% dry basis)	9·3	7·7	6·2	7·8	5·9
Alkali spreading value	5·9	6·0	6·0	3·6	5·9
Gel consistency (mm)	63	62	46	80	52
Amylograph consistency (BU)					
10% paste	280	260	270	270	465
11% paste	370	395	400	350	—
12% paste	410	405	530	385	—
Instron cooked rice					
Hardness (kg)	6·1	6·7	7·1	6·5	7·5
Stickiness (g.cm)	76	76	88	72	65

[a] Aromatic.

consistent with its soft gel consistency and the low amylograph consistency of its 12% paste. It was neither inferior to Seratus malam in hardness nor in stickiness of the cooked rice. The only difference noted was its lower alkali spreading value or higher final BEPT than the three other better quality rices. Among the three low final BEPT rices, Bengawan had higher amylograph consistency and harder gel consistency values than the others. It also had the hardest cooked rice. The premium variety, Rojolele, had the lowest hardness value of the three.

Final BEPT as an indicator of quality of intermediate amylose rices was also evident in intermediate amylose IRRI lines with BPI-121-407 or C4-63G as intermediate amylose parent. The two IR4570 and one IR7963 line derived from BPI-121-407 had similar properties to the parent—alkali spreading, gel consistency and hardness and stickiness of the cooked rice (Table 4). The two IR4215 and one IR9575 lines derived from C4-63G had similar properties to those of C4-63G. These two sets had similar gel consistency values and differed mainly in final BEPT (alkali test) and hardness of the cooked rice.

A survey of 16 intermediate amylose varieties indicated a range of hardness values of 5·2–8·2 kg and stickiness of 50–80 g.cm. Three intermediate amylose (23–25%) low

TABLE 4
COMPARISON OF PROPERTIES OF PROMISING IRRI LINES WITH C4-63G AND BPI-121-407 AS A PARENT

Variety or line	*Amylose content (% dry basis)*	*Protein content*	*Alkali spreading value (1·7% KOH)*	*Gel consistency (mm)*	*Cooked rice*	
					Hardness (kg)	*Stickiness (g.cm)*
IR4215-301-2[a]	24·7	7·3	2·5	100	6·4	122
IR4215-409-2[a]	22·7	7·5	2·5	80	5·5	93
IR4570-74-2[b]	24·1	7·7	7·0	94	7·6	75
IR4570-124-3[b]	24·4	8·4	7·0	80	7·2	89
IR7963-30-2[b]	25·1	9·4	6·9	73	7·9	90
IR9575[a]	21·4	9·7	2·5	82	6·0	84
BPI-121-407	24·6	10·1	6·9	96	7·6	117
C4-63G	23·0	7·7	2·0	92	5·9	78

[a] C4-63G is a parent.
[b] BPI-121-407 is a parent.

BEPT japonica varieties from Europe—Arlesienne, Ponta Rubra and Raffaello—had hardness values of 6·7–7·4 kg and stickiness of 52–66 g.cm, which were not different from those of indica varieties. Among four varieties that showed extreme elongation on cooking of pre-soaked grain (Juliano, 1972), Basmati 6129, Basmati 370, and Dum Siah had different values (hardness 7·0–8·0 kg; stickiness 50–57 g.cm) from those of D25-4 (hardness 5·2 kg; stickiness 82 g.cm).

Low-amylose rices

For low-amylose rices, the optimum gel concentration was not 100 mg but 110–120 mg/2 ml in two crops of South Korean rices (Table 5). Tongil is considered

TABLE 5
EFFECT OF RICE CONCENTRATION ON THE GEL AND AMYLOGRAPH CONSISTENCY VALUES OF LOW-AMYLOSE SOUTH KOREAN MILLED RICE AND INSTRON HARDNESS AND STICKINESS VALUES OF THEIR COOKED RICES

Property	1973 *crop*		1975 *crop*		
	Tongil	*Jinheung*	*Tongil*	*Jinheung*	*Yushin*
Amylose (% dry basis)	18·5	18·5	19·9	19·1	19·2
Protein (% dry basis)	8·4	6·5	8·1	8·1	9·1
Alkali spreading (1·7% KOH)	7·0	7·0	7·0	7·0	7·0
Alkali spreading (1·3% KOH)			3·5	6·0	3·8
Gel consistency (mm)					
100 mg/2 ml 0·2N KOH	100	100	86	89	100
110 mg/2 ml 0·2N KOH	91	100	66	82	84
120 mg/2 ml 0·2N KOH	77	86	54	76	66
Amylograph consistency (BU)					
10% paste	250	220	190	180	150
12% paste	—	—	425	290	285
Instron cooked rice					
Hardness (kg)	7·4	6·2	7·4	5·7	5·9
Stickiness (g.cm)	97	92	102	102	111

of poorer eating quality (more flaky) than Jinheung. Even amylograph consistency, which was similar with 10% pastes of the two rices, showed differences with 12% pastes in agreement with gel consistency data. This was understandable because the amylograph method with 10% paste was developed principally for corn and wheat starches, which have a high amylose content.

Hardness of the cooked rice closely followed the gel consistency at 120 mg/2 ml and amylograph consistency in both sets of samples (Table 5). Stickiness of the cooked rice again was not different for Tongil and Jinheung in both sets—in contrast to hardness values. Final BEPT, as indexed by alkali spreading in 1·3% KOH, was higher in Tongil than in Jinheung but was probably not related to hardness difference since Yushin had a similar alkali spreading value to Tongil but similar hardness of the cooked rice to Jinheung. Both Tongil and Yushin are indica × japonica crosses and an alkali test in 1·15% KOH can distinguish between japonica and indica × japonica hybrids (Maniñgat & Juliano, 1978).

Four Japanese rices with known texturometer readings of the cooked rice were assessed for Instron hardness and stickiness of the cooked rice. Ranking of hardness values for the four varieties was the same by both methods (Table 6). However, ranking of stickiness with the Instron method did not tally with cohesiveness, adhesiveness, or adhesive power with the texturometer. However, Kinpa had the least adhesiveness, cohesiveness and adhesive power values with the texturometer and the lowest stickiness value by Instron tester. Further comparison of the two methods is required in order to confirm these results.

Comparison of Instron values with grain properties showed that the stickiness values followed closely the amylose content (Table 6) with the highest amylose

TABLE 6
PHYSICOCHEMICAL PROPERTIES OF FOUR MILLED RICES FROM FUKUI, JAPAN, 1976 CROP[a]

Property	*Kinpa*	*Honenwase*	*Koshihikari*	*Koshinishiki*
Amylose (% dry basis)	19·1	16·7	17·3	16·2
Protein (% dry basis)	6·1	7·5	5·7	6·8
Alkali spreading value (1·15% KOH)	6·0	5·0	4·9	5·0
Gel consistency (mm)	55	68	66	66
Amylograph consistency (BU)[b]	185	185	65	85
Cooked rice Instron				
Hardness (kg)	6·1	6·8	5·9	6·0
Stickiness (g.cm)	72	89	81	94
Cooked rice Texturometer				
Hardness	2·03	2·11	1·97	1·98
Cohesiveness	0·36	0·38	0·36	0·36
Adhesiveness	0·03	0·05	0·09	0·04
Adhesive power	0·47	0·79	0·95	0·68
Hardness/adhesiveness ratio	68	42	20	50

[a] Rice samples ex. Fukui Agr. Expt. Station and Texturometer data from Dr S. Chikubu and Mr I. Endo, Nat. Food Res. Inst., Tokyo, Japan.
[b] 12% paste.

sample, Kinpa, being the least sticky. Gel and amylograph consistency was not simply related to hardness. Kinpa should have had the hardest cooked rice based on gel and amylograph consistency but its hardness rating was not the highest. Honenwase gave the hardest cooked rice, which was consistent with its high amylograph consistency but not with its soft gel consistency. Kinpa had a higher final BEPT, based on alkali test, than the three other samples. Among the three samples with similar final BEPT and gel consistency, the two softest samples had the lowest amylograph consistency.

A survey of 18 low-amylose varieties gave a range of cooked rice hardness of 4·5–9·9 kg and stickiness of 72–112 g.cm. The hardness values showed more overlap with values for intermediate-amylose rice than stickiness values which were relatively higher than for intermediate amylose rices. Waxy rices also overlapped with low-amylose rice more in hardness (3·3–6·8 kg) than in stickiness (224–520 g.cm) of the cooked rice (Perez *et al.*, 1979). Indica and japonica low-amylose rices also overlapped in hardness and stickiness of cooked rice and their values were not simply related to final BEPT differences.

CONCLUSIONS

Although both stickiness and hardness of cooked rice measured by the Instron food tester correlated with amylose content, a wider range of hardness was observed at any amylose level. Among high-amylose IRRI rices, those with a soft gel consistency tended to give a softer and more sticky cooked rice. Varietal differences among rices with low (9–20 %) or intermediate (20–25 %) amylose content were associated with differences in hardness of the cooked rice rather than with stickiness differences. Among intermediate amylose rices, soft cooked rices were associated with samples with soft gel consistency and low amylograph consistency (12 % paste) or with differences in final BEPT. Among low-amylose rices, hardness of the cooked rice was related mainly to differences in gel and amylograph consistency, modified by using 20 % higher rice levels than the standard procedures. Thus, among non-waxy rices of similar amylose content, gel and amylography consistency or gelatinisation temperature were shown to be important eating quality indicators associated with the hardness of cooked rice. These properties were also shown to be indicators of the eating quality of waxy rice (Perez *et al.*, 1979).

ACKNOWLEDGEMENTS

Rice samples were provided by H. M. Beachell, Co-operative Central Research Institute for Agriculture—IRRI Programme, Bogor, Indonesia; P. S. Castillo, University of the Philippines at Los Baños; Plant Breeding Department and

International Rice Testing Programme, IRRI; B. R. Jackson, Rockefeller Foundation, Bangkok, Thailand; Chin Thiam Hon, Agricultural Research Centre, Kuching, Sarawak, East Malaysia; M. Shafi Chaudry, Rice Research Station, Rasht, Iran; The Botanist, Rice Research Institute, Kala Sha Kaku, Pakistan; B. D. Webb, Agricultural Research Service, USDA, Beaumont, Texas, USA; H. L. Carnahan, California Co-operative Rice Research Foundation, Inc., Biggs, Calif., USA; J. N. Rutgers, Dept. of Agronomy and Range Sciences, Univ. Calif., Davis, Calif., USA; M. H. Heu, Seoul National University, Suweon, Korea; S. Chikubu, National Food Research Institute, Tokyo, Japan; P. Feillet, Laboratoire de Technologie des Blés Durs et du Riz, INRA Montpellier, France; G. Baldi, Centro di Ricerche sul Riso, Mortara, Italy; M. Rosero, Centro Internacional de Agricultura Tropical, Cali, Colombia; Instituto dos Cereais, Lisbon, Portugal; Myo Thant, Agricultural Research Institute, Gyogon, Insein, Burma.

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SOME FACTORS AFFECTING THE RATE OF PROTEASE CATALYSED REACTIONS

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(Received: 4 July, 1978)

ABSTRACT

The rates of hydrolysis of casein catalysed by bacterial proteases were determined at different pH values and temperatures. The bacterial proteases Maxatase and Alcalase were active in the pH range 6·7–9·0 with a maximum rate at pH 6·7 when the temperature was 37°C. However, even at pH 9·0, activity is still quite satisfactory. The reaction rates of the two enzymes at 45°C were higher than those at 37°C when the pH value was 8·2. The rate of the Maxatase catalysed reaction was more than that of Alcalase at 45°C. The activation energies were calculated and, as expected, it was found that the higher the enzyme reaction rates, the lower were the activation energies.

INTRODUCTION

The hydrogen ion concentration (pH value) and the temperature are important factors which affect rates of enzyme catalysed reactions (Dixon & Webb, 1964; Fairley & Kilgour, 1966) and Hagihara (1960) has grouped proteases into three categories: (a) acid proteases, active about pH 3, (b) neutral proteases, active about pH 6–7 and (c) alkaline proteases, active about pH 8–10. Therefore both the pH and the temperature ranges over which proteases are active are very important factors in determining the type of protease which will be most suitable for particular applications. For example, protease used in detergents (SDIA, 1971; Kame *et al.*, 1973) should be active at alkaline pH values and high temperatures. There are commercially available proteases that are recommended for use in detergents.

In this paper we are interested in evaluating two commercially available proteolytic enzymes to be used in the detergent industry. The effects of pH value and

Fd. Chem. 0308-8146/79/0004-0197/$02·25

temperature on the reaction rates of the bacterial proteases Alcalase and Maxatase were investigated. The rates of casein proteolysis were measured using an oscillator densimeter technique which was found to be easy and accurate.

MATERIALS AND METHODS

Source of materials

Alcalase and Maxatase were obtained as thermostable bacterial proteolytic enzyme preparations produced as granulates from a special strain of *Bacillus* for use in detergents at high temperatures and alkaline pH values. These enzyme preparations were kindly provided by Novo Industry A/S, Bagsvaed, Denmark and Gist-Brocades N.V. Industry, Delft, The Netherlands respectively.

Methods

The measurement of the reaction rate was based on the change of density of the substrate–enzyme mixture which was measured by a digital densimeter DMA 02C manufactured by A. Paar Graz, Austria (El-Saied, 1980; Kratky *et al.*, 1973). The principle of the technique is that the hydrolysis of casein solution by protease consumes water which causes a reduction in the volume of the casein–protease mixture. Thus the density increases according to the reaction:

$$\text{Casein} + \text{water} \xrightarrow{\text{protease}} \text{amino acids} + \text{peptides}$$

The casein used was dissolved in distilled water of known pH value to a concentration of 5% and pre-incubated at the desired temperature. The enzyme preparation was dissolved in distilled water of known pH value by using a magnetic stirrer for 30 min and also pre-incubated at the desired temperature. Distilled water was adjusted to the desired pH value with NaOH (0·1N). The dried oscillator was filled from a mixture of 5 ml casein solution and 1 ml protease solution (0·8–2 mg/ml). The first density measurement was carried out after 8 min—the time required to achieve thermal equilibrium. The increase in density was measured periodically at intervals of 0·5 min for 30 min. The density (d) was calculated according to the equation: $d = At^2 + B$ where t was the period in seconds of the oscillator which was filled with the sample and A and B were constants determined by two calibration measurements using air and distilled water (Timmermans, 1950). The dependence of density on the time of enzymic reaction was plotted by means of a computer (Hewlett-Packard Model 9810A) equipped with a plotter for different concentrations of protease (Fig. 1). Enzyme reaction rates ($\Delta d/\Delta t$) using different protease concentrations were calculated for each variable studied.

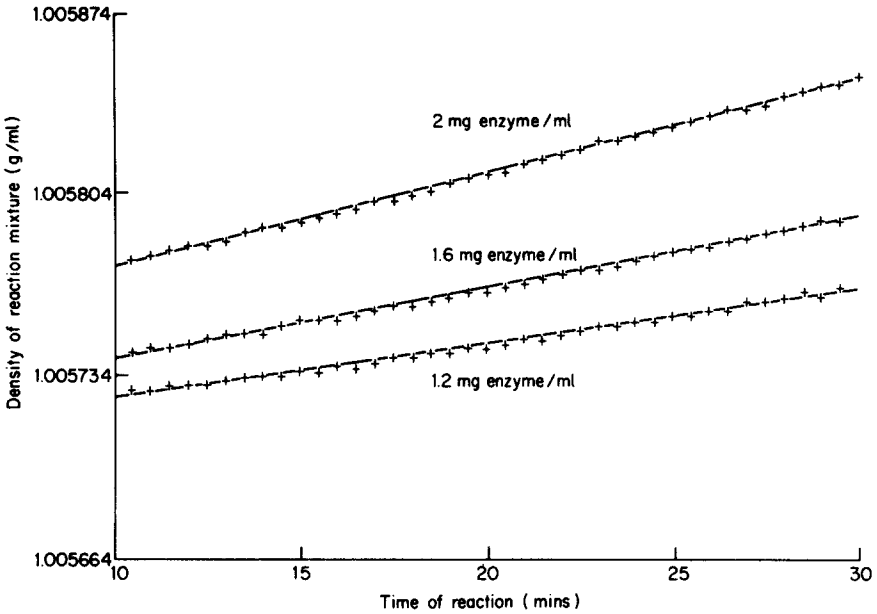

Fig. 1. Effect of enzyme concentration on density of reaction mixture using Maxatase at pH 9·0 and 37°C.

The activation energy of the enzymes was calculated using the Arrhenius equation:

$$\ln \frac{k_1}{k_2} = \frac{-\Delta E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where k_1 and k_2 are the rates of enzyme reactions at temperatures T_1 (37°C) and T_2 (45°C), ΔE is the activation energy and R is the gas constant.

RESULTS AND DISCUSSION

Effect of pH

The enzyme reaction rates of the bacterial proteases Alcalase and Maxatase were carried out at pH values of 6·7 and 9·0. The results at 37°C are shown in Tables 1 and 2.

It is clear from these results that bacterial protease was more active at pH 6·7. These results are in agreement with those of Fukumoto *et al.* (1957), who found that the optimum pH value for protease produced from *Bacillus subtilis* was 6·0–8·0, and with those of O'Brien & Campbell (1957), who reported that the optimum pH value for protease produced from *B. stearothermophilus* was 6·9–7·2.

TABLE 1

EFFECT OF pH VALUE UPON THE RATE OF HYDROLYSIS OF CASEIN CATALYSED BY THE BACTERIAL PROTEASE ALCALASE

(Δd = density difference between consecutive points in Fig. 1)
(Δt = time as abscissa corresponding to Δd in Fig. 1)

pH 6·7		*pH* 9·0	
Amount of enzyme (mg/ml)	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$	*Amount of enzyme (mg/ml)*	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$
1·2	3·26	1·2	2·08
2·0	5·08	1·6	2·84
		2·0	3·70

TABLE 2

EFFECT OF pH VALUE UPON THE RATE OF HYDROLYSIS OF CASEIN CATALYSED BY THE BACTERIAL PROTEASE MAXATASE

pH 6·7		*pH* 9·0	
Amount of enzyme (mg/ml)	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$	*Amount of enzyme (mg/ml)*	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$
0·8	2·28	1·2	2·16
1·2	3·22	1·6	2·91
		2·0	3·83

TABLE 3

EFFECT OF TEMPERATURE ON THE RATE OF BACTERIAL PROTEASE ALCALASE CATALYSED REACTION AT pH 8·2

37 °C[a]		45 °C	
Amount of enzyme (mg/ml)	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$	*Amount of enzyme (mg/ml)*	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$
0·8	1·84	0·8	3·20
1·6	3·48		
2·0	4·43	2·0	7·30

[a] Data taken from El-Saied (1980).

TABLE 4

EFFECT OF TEMPERATURE ON THE RATE OF BACTERIAL PROTEASE MAXATASE CATALYSED REACTION AT pH 8·2

37 °C[a]		45 °C	
Amount of enzyme (mg/ml)	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$	*Amount of enzyme (mg/ml)*	$\frac{\Delta d}{\Delta t}\left(\frac{\mu g}{ml\ min}\right)$
0·8	1·96	0·8	3·77
1·6	3·76	1·6	6·54
2·0	4·57		

[a] Data taken from El-Saied (1980).

Effect of temperature

The reaction rates at 37 °C and 45 °C were determined at pH 8·2 for Maxatase and Alcalase. The results are presented in Tables 3 and 4.

From these results, the rates of reaction of the two enzymes at 45 °C were higher than that at 37 °C. However, the rate of the Maxatase catalysed reaction at 45 °C was higher than that of Alcalase at the same temperature.

The activation energies of the bacterial proteases Alcalase and Maxatase are shown in Table 5.

TABLE 5
ACTIVATION ENERGIES OF ALCALASE AND MAXATASE

Enzyme	*Activation energy (kcal/mole)*
Alcalase	13·10
Maxatase	14·80

These results indicate that the activation energies of the commercial enzymes Alcalase and Maxatase were found to be in good agreement with that for pure proteolytic enzymes reported in the literature (Bender *et al.*, 1964) (12·0 $\pm$ 1·0 kcal/mole).

From this study we can conclude that the optimum pH values of commercial proteolytic enzymes used in the industry are in agreement with those of pure bacterial enzymes (Fukumoto *et al.*, 1957; O'Brien & Campbell, 1957). The results obtained using the digital oscillator technique were reproducible and reliable and in good agreement with the results reported in the literature (Bender *et al.*, 1964).

CONCLUSIONS

This study has shown clearly that the commercial bacterial proteases Alcalase and Maxatase were quite stable in the pH range 6·7–9·0 with a maximum pH value of 6·7 when the temperature was 37 °C. Moreover, the activity of both enzymes at 45 °C was higher than that at 37 °C when the pH value was 8·2. However, at 45 °C the activity of Maxatase was higher than that of Alcalase—a fact which may be due to its structure. Industrial applications for proteolytic enzymes in which the hydrolysis of protein is carried out at moderate to high temperatures over a broad pH range are the production of washing compounds, gelatine, leather, meat, fish and silk.

ACKNOWLEDGEMENTS

The author expresses his thanks to the International Seminar for Research and Education in Physical Chemistry and Chemical Engineering (which is an international organisation financed by the Deutsche Akademische Austauschdienst)

and to Professor Dr Marcil Loncin and Dr Ing. Horst Weisser (Institut für Lebensmittelverfahrenstechnik der Universität Karlsruhe) for their deep interest in this work.

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EDIBLE MUSHROOMS AS PRODUCERS OF AMYLASES

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(Received: 4 July, 1978)

ABSTRACT

Five varieties of mushroom—Absidia blakesleeana, Agaricus bisporus, Lentinus edodes, Peziza auburouniv *and* Polyporus sulphureus—*were qualitatively examined for their ability to hydrolyse starch. Quantitative estimation was made to compare the amylolytic activity of the five types of mushroom propagated on a synthetic medium with that of* Aspergillus niger *NRRL* 337.

Lentinus edodes *proved to be the most promising strain for amylase production. The amylolytic activity of* Lentinus edodes *reached its maximum* (621 *U/ml*) *after seven days of incubation, the dry mycelium yield being* 7·5 *g/l with a protein content of* 34%.

Lentinus edodes *was then propagated on various extracts of food wastes—malt sprouts, navel orange peel, potato peel, rice bran, corn steeping water and rice steeping water. Malt sprouts extract proved to be a promising medium for* Lentinus edodes *propagation as well as for amylase production.*

INTRODUCTION

Amylases are hydrolytic enzymes involved in the degradation of starch and starch-like polysaccharides. Underkofler (1954), Rose (1961) and Reed (1966) reported that great numbers of microorganisms were able to produce amylases—for example, *Aspergillus* spp., *Rhizopus* spp., *Penicillium* spp. and *Bacillus* spp. Commercial production of microbial amylase is possible by several organisms such as the moulds *Aspergillus niger*, *A. oryzae* and *A. flavus* and the bacteria *Bacillus subtilis*, *B. stearothermophilus* and *B. coagulans*. For maximum yield of microbial enzymes, a potent mould strain must be selected and the optimum cultural conditions for

Fd. Chem. 0308-8146/79/0004-0203/$02·25

production of the enzyme by the selected strain must be developed (Underkofler, 1954; Rose, 1961 and Miligi *et al.*, 1976). Microorganisms for enzyme production can be grown on cheap carbon sources, including discarded food materials which are not in forms suitable for human consumption. Most fungi are capable of utilising the complex mixture of organic compounds which occurs in most wastes. They also have the advantage that they can be harvested by simple filtration methods which are not applicable to bacteria or yeast (Worgan, 1976).

No previous studies of amylase production with edible mushrooms have been carried out. Accordingly, the present work aimed to assess the amylolytic activity and mycelium yield during mushroom propagation on food waste extracts.

MATERIALS AND METHODS

Organisms

Five cultures of edible mushroom—*Absidia blakesleeana* NRRL 1304, *Agaricus bisporus* IARP_5, *Lentinus edodes* IFRI 2571 c, *Peziza auburoniv* IFRI No. 154 and *Polyporus sulphureus* IFRI 964—were used in the present work with *Aspergillus niger* NRRL 337.

Waste materials

Malt sprouts, wheat bran, potato peel, navel orange peel, rice germ, rice steeping water and corn steeping water were used.

Synthetic medium

The medium recommended by Mahmoud *et al.* (1973) for fungal amylase production was prepared. Portions of the medium (100 ml) were transferred to Erlenmeyer flasks (*ca.* 500 ml), plugged and sterilised at 121 °C for 20 min.

Preparation of inoculum

Potato dextrose agar slants were inoculated with the active culture of the fungi, then incubated at 30 °C for 72 h. From each slant one loopful was transferred by streaking to every petri dish containing potato dextrose agar and incubated for 72 h at 30 °C after which the spores were transferred to sterile water to prepare a spore suspension. The number of spores in the suspension ($2{\cdot}6 \times 10^8$/ml) was determined by the indirect dilution technique for cell count (De Moss & Bard, 1957).

Plant wastes extraction

Suitable amounts (100 g) of the raw material under test were boiled with distilled water (500 ml) for one hour, filtered through cheesecloth and diluted to one litre. Portions of the extract (100 ml) were transferred to Erlenmeyer flasks (*ca.* 500 ml) and sterilised at 121 °C for 20 min.

Mycelium yield

The fungal growth was removed by filtration through cheesecloth, washed twice with 100-150 ml distilled water and dried on a filter paper at 60 °C for 12 h. The dry weight of mycelium was obtained.

Qualitative test

The ability of mushroom cultures to hydrolyse starch was examined by a preliminary test on starch agar according to the method described by Peltier & Beckord (1945).

Quantitative determination

The amylolytic activity of the culture filtrate was determined colorimetrically according to the method described by Pmston (1964).

Ash and total nitrogen

The methods of the Association of Official Analytical Chemists (1970) were followed.

RESULTS AND DISCUSSION

Selection of the most promising mushrooms

The qualitative tests indicated that the five varieties of mushroom had the ability to hydrolyse starch. Accordingly, their amylolytic activity was quantitatively determined after being cultured on the synthetic medium. The amylolytic activity of the mushrooms under test was compared with that of *Aspergillus niger* NRRL 337 (a strain recommended by NRRL for amylase production).

The results, illustrated in Fig. 1, indicate that the five tested mushrooms differed in potency of amylolytic activity. The highest activity was attained in the case of *Lentinus edodes* after seven days of incubation while the least activity was detected in the culture filtrate of *Polyporus sulphureus* after eight days of incubation. The amylolytic activity demonstrated in the culture filtrate of *Aspergillus niger* after six days of incubation was generally higher than that obtained in all the tested mushrooms, being about twofold that detected in *L. edodes* upon cultivation for the same period. The results also revealed that the amylolytic activity demonstrated in the culture filtrate of *L. edodes* after seven days of incubation was more than twofold that detected on the fifth, seventh and eighth days of incubation in *Polyporus sulphureus*, *Absidia blakesleeana* and *Peziza auburouniv*, respectively.

As *Lentinus edodes* and *Agaricus bisporus* proved to be promising for amylase production, experiments were carried out to assess the pH change, the amylolytic activity, the mycelium yield and the protein content during their propagation on a synthetic medium.

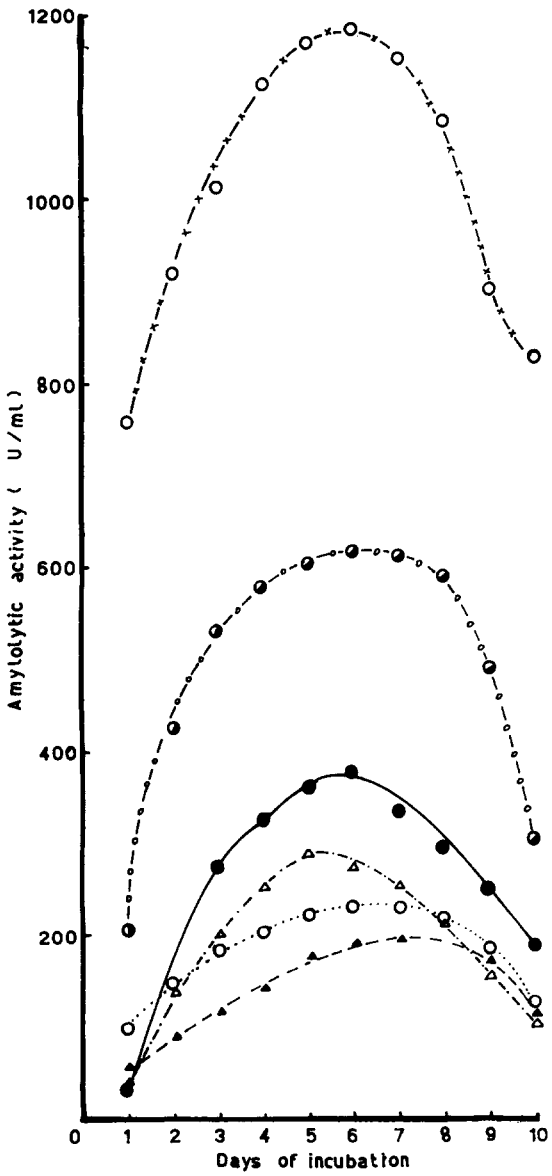

Fig. 1. Amylolytic activity of the culture filtrate of five mushrooms propagated on a synthetic medium compared with that of *Aspergillus niger* (NRRL 337). (○-×-×-○ *Aspergillus niger*; ◐-○-○-◑ *Lentinus edodes*; ●—●—● *Agaricus bisporus*; △-·-·-△ *Polyporus sulphureus*; ○···○ *Absidia blakesleeana*; ▲--▲ *Peziza auburouriv.*

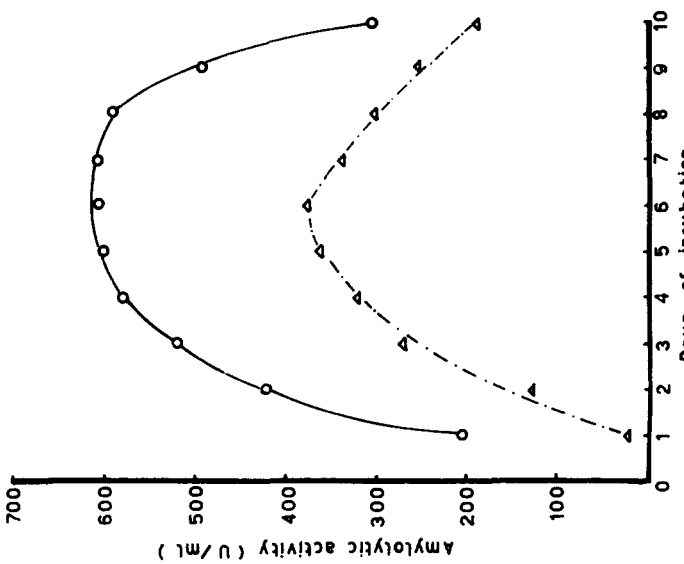

Fig. 3. Amylolytic activity of the culture filtrate of *Agaricus bisporus* (△-·-·-△) and *Lentinus edodes* (○——○) propagated on a synthetic medium.

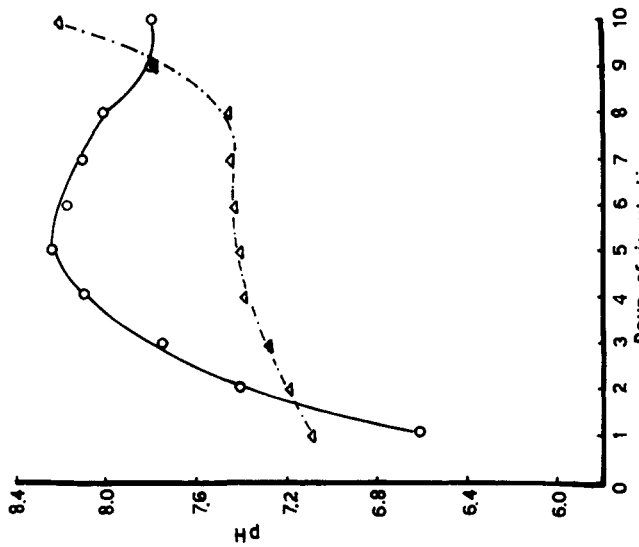

Fig. 2. pH changes during propagation of *Agaricus bisporus* (△-·-·-△) and *Lentinus edodes* (○——○) on a synthetic medium.

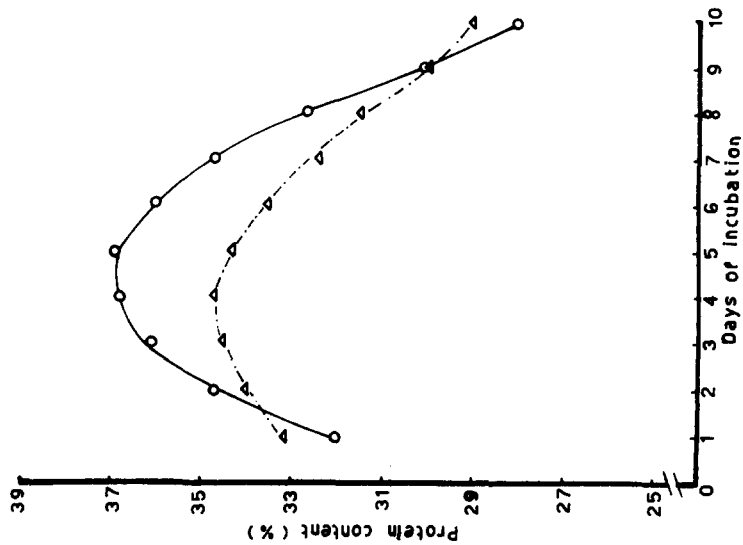

Fig. 5. Protein content of *Agaricus bisporus* (△-·-·-△) and *Lentinus edodes* (○——○) mycelium propagated on a synthetic medium.

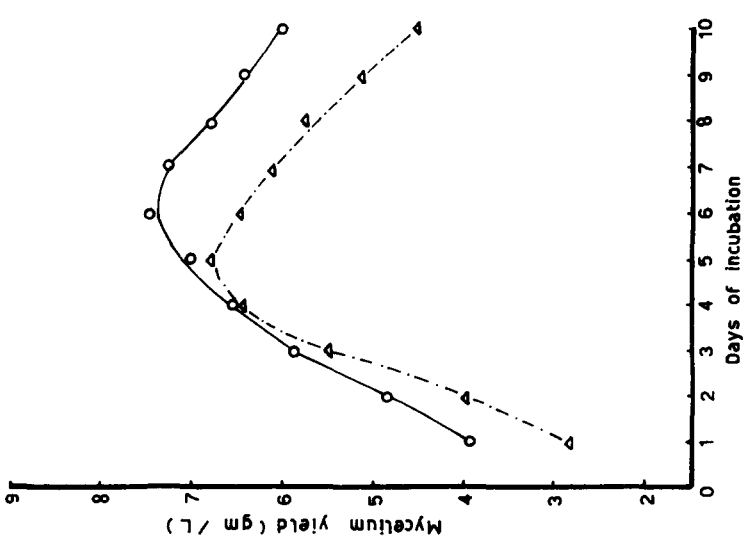

Fig. 4. Mycelium yield of *Agaricus bisporus* (△-·-·-△) and *Lentinus edodes* (○——○) propagated on a synthetic medium.

The results, illustrated in Fig. 2, indicate that a gradual but slight increase was detected in the pH of the culture filtrate of *Lentinus edodes* up to the fifth day of incubation, after which a very slight decrease was reported, while in the case of *Agaricus bisporus* a slight increase in the pH was reported during the incubation period.

The amylolytic activity of the culture filtrate increased during *Agaricus bisporus* and *Lentinus edodes* propagation, reaching its maximum on the fourth to seventh days. Upon incubation up to ten days, a gradual decrease in the activity was noted, as shown in Fig. 3. The increase in the amylolytic activity achieved in the culture filtrate was associated with that of the mycelium yield, as illustrated in Fig. 4. A slight decrease in the mycelium yield was reported after the fifth and sixth days of incubation in the case of *Agaricus bisporus* and *Lentinus edodes*, respectively.

The mycelium protein content in the case of *Agaricus bisporus* and *Lentinus edodes* ranged between 28·0 and 34·6 % and 29·0 and 36·7 %, respectively during the growth period of ten days. The maximum protein content was attained on the fourth day of incubation in the case of *Agaricus bisporus* and on the fifth day in the case of *Lentinus edodes*, as can be seen from Fig. 5. Generally the protein content of *Lentinus edodes* slightly exceeded that of *Agaricus bisporus*.

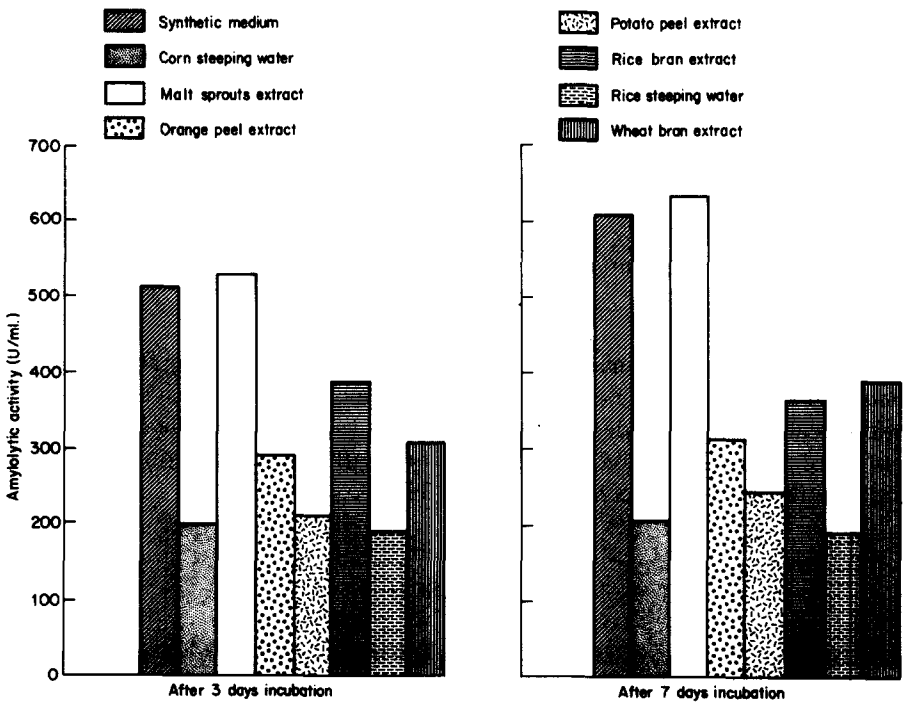

Fig. 6. Amylolitic activity of *Lentinus edodes* propagated on different media.

The amylolytic activity detected in the culture filtrate of *Lentinus edodes* was about twofold that of *Agaricus bisporus*. The mycelium yield and the protein content of *Lentinus edodes* exceeded that of *Agaricus bisporus*.

Propagation on waste extracts

Lentinus edodes was chosen for further study. The results, illustrated in Fig. 6, reveal that the amylolytic activity detected in the culture filtrate differed greatly according to the type of medium upon which *Lentinus edodes* was propagated. Propagation of *Lentinus edodes* on malt sprouts extract showed the highest amylolytic activity of all the media used, either after three or seven days of incubation (the period during which the mycelium growth and amylolytic activity reached the maximum levels as previously shown in Fig. 3). A slight increase in the amylolytic activity occurred when the incubation period increased to seven instead of three days, upon propagation on the media shown in Table 1. The results of the

TABLE 1

AMYLOLYTIC ACTIVITY, MYCELIUM YIELD AND PROTEIN CONTENT OF *Lentinus edodes* PROPAGATED ON DIFFERENT MEDIA

Type of media	*After three days of incubation*			*After seven days of incubation*		
	Amylolytic activity (*U/ml*)	*Mycelium yield* (*g/l*)	*Protein content* (%)	*Amylolytic activity* (*U/ml*)	*Mycelium yield* (*g/l*)	*Protein content* (%)
Synthetic medium	521·3	5·89	34·9	619·0	7·00	32·3
Corn steeping water	198·6	Weak growth	—	207·3	2·54	31·06
Malt sprouts extract	530·5	9·61	34·50	628·7	6·50	32·60
Orange peel extract	282·4	3·40	33·55	315·0	3·60	33·19
Potato peel extract	212·0	2·90	30·05	248·0	2·80	30·55
Rice bran extract	385·0	4·40	34·72	418·0	5·00	33·12
Rice steeping water	183·1	6·20	32·23	191·6	7·60	32·33
Wheat bran extract	301·6	3·98	31·90	495·9	3·17	30·89

present study reveal that malt sprouts extract medium is promising for growing *L. edodes* for amylase production after three days of incubation. The highest mycelium yield was also attained under the same conditions as shown in Table 1. The protein content of the mycelium did not seem to vary greatly upon cultivation on different media as it was always between 30% and 34·9%.

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EFFECT OF A PROTEOLYTIC ENZYME PRODUCED BY *CLOSTRIDIUM PERFRINGENS* UPON PORCINE MUSCLE

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(Received: 5 July, 1978)

ABSTRACT

Purified proteolytic enzyme preparations isolated from actively growing cultures of Clostridium perfringens *(ATCC* 13124—*Type A) were added to aseptic porcine muscle and incubated at* 37 °*C. Samples were removed after one, two and four days incubation. The sarcoplasmic and myofibrillar proteins were extracted and subjected to both conventional and SDS-polyacrylamide disc gel electrophoresis to assess the changes due to the enzyme treatment. The enzyme preparation caused little change in the sarcoplasmic protein fraction but caused some proteolysis in the myofibrillar extract. Comparison of the protein profile from muscle incubated with the purified enzyme preparation and that incubated with the actively growing organisms* per se *showed that the organisms caused more degradation, suggesting that they produced additional enzymes causing proteolysis of porcine muscle.*

INTRODUCTION

Clostridium perfringens is known to produce several toxins (enzymes) which affect living tissue. Oakley *et al.* (1946) determined that the ability to soften muscle was one attribute of *C. perfringens* filtrates which led to the identification of a collagenase produced by that organism. Recent research by Kameyama & Akama (1971) indicated that the collagenase acts primarily upon the connective tissue supporting the muscle fibres and secondarily upon the muscle fibre itself. Other enzymes produced by *C. perfringens* which may affect the muscle structure are a lecithinase, which acts upon the sarcoplasmic reticulum (Strunk *et al.*, 1967), a proteinase (Oakley *et al.*, 1948; Bidwell, 1950) and a hyaluronidase, which causes depolymerisation of the ground substance (Ispolatovskaya, 1971).

In this study, an enzyme preparation from culture filtrates of *C. perfringens* was

Fd. Chem. 0308-8146/79/0004-0213/$02·25

used to determine enzymatic action upon individual muscle proteins. The effects of the enzyme preparation were observed after incubation with aseptic muscle samples for periods up to four days. The sarcoplasmic and myofibrillar proteins were then isolated from the incubated muscle and changes in the isolated protein fractions were followed by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Culturing of organisms and measurements of enzyme activity

Clostridium perfringens (ATCC 13124—Type A) was cultured in Difco thioglycolate broth and transferred to either a peptone medium supplemented with Difco Bacto Beef (Murata *et al.*, 1956) or a synthetic amino acid mixture for enzyme culturing as described by Hapchuk & Pearson (1978). The transfers were grown under anaerobic conditions at 37 °C and enzyme activity was followed using a modification of the azocoll method of Kameyama & Akama (1970) as outlined by Hapchuk & Pearson (1978).

Purification of the enzyme

The steps followed in purification and concentration of the enzyme fraction are outlined in detail by Hapchuk & Pearson (1978). The final preparation, utilised in the current investigation, had a specific activity of 79 azocoll units per milligramme protein following 159-fold purification and 12% recovery (Hapchuk & Pearson, 1978). The active fractions were combined, lyophilised and held at −20 °C until used for studying their effects upon breakdown of the muscle proteins.

Muscle degradation

Aseptic samples of pig muscle were obtained as described by Hasegawa *et al.* (1970). Ground aseptic muscle was inoculated by 0·77 units of enzyme per gramme of muscle dissolved in 0·02M Tris-HCl-5mM $CaCl_2$, pH 7·5. Similar samples were inoculated with the same amount of 0·02M Tris-HCl-5mM $CaCl_2$, pH 7·5, to give a final concentration of 0·2mM Ca^{++} in the muscle. The inoculated samples and uninoculated controls were incubated at 37 °C for one, two or four days.

Extraction of muscle proteins

At the end of each incubation period the sarcoplasmic and myofibrillar proteins were extracted using the method of Helander (1957) with modifications as described previously by Hapchuk *et al.* (1976).

Electrophoretic techniques

Analytical disc-gel electrophoresis of the sarcoplasmic extract was performed in an alkaline system using the technique described by Gabriel (1971). SDS-gel

electrophoresis of both the sarcoplasmic and myofibrillar extracts was accomplished according to the method of Weber & Osborn (1969). The molecular weights of the band components in SDS gels were determined by comparison with a curve constructed using the following protein standards: myoglobin (17,800 daltons), chymotrypsinogen (25,700 daltons), pepsin (35,000 daltons), ovalbumin (43,000 daltons), bovine serum albumin (68,000 daltons) and phosphorylase *a* (94,000 daltons). Disc-gel electrophoresis of the myofibrillar proteins in urea was performed using a modification of the method of Rampton (1969). From three to seven replicates of each sample were prepared using both SDS- and disc-gel electrophoresis.

The position and intensity of the protein bands in all gels were monitored using a Kontes Chromaflex K-495000 densitometer with a modified sample plate. The relative mobility (R_M) was defined as the distance of protein migration divided by the distance of bromophenol blue dye migration. The areas under the peaks were determined by triangulation and were expressed as a percentage of total protein area.

Total protein

Total protein concentration of the myofibrillar and sarcoplasmic extracts was measured by the method of Lowry *et al.* (1951).

Statistical analysis

Analysis of variance for unequal subclass numbers using the procedure described by Steel & Torrie (1960) was utilised to determine statistical significance. Duncan's multiple range test was applied to the data to test for differences between treatment means at the 5% level of significance (Steel & Torrie, 1960). Thus, significance herein will refer only to $P = <0{\cdot}05$.

RESULTS AND DISCUSSION

Changes in extractable sarcoplasmic and myofibrillar proteins

Changes in the quantity of the sarcoplasmic and myofibrillar proteins during incubation are shown in Fig. 1. The amount of extractable protein declined in all samples during incubation, decreasing most in the myofibrillar fraction but also showing a marked decline in the sarcoplasmic extract. The amount of extractable sarcoplasmic protein declined from 92 to 55 mg/g for the control to 39 mg/g for the Ca^{++}-containing sample and to 34 mg/g for the enzyme treated sample during four days incubation. The myofibrillar proteins declined from an initial value of 82 mg/g for the control to 20 mg/g after four days incubation compared with corresponding values of 19 and 29 mg/g for the Ca^{++}-containing control and the enzyme-treated samples held at 37 °C for the same period of time.

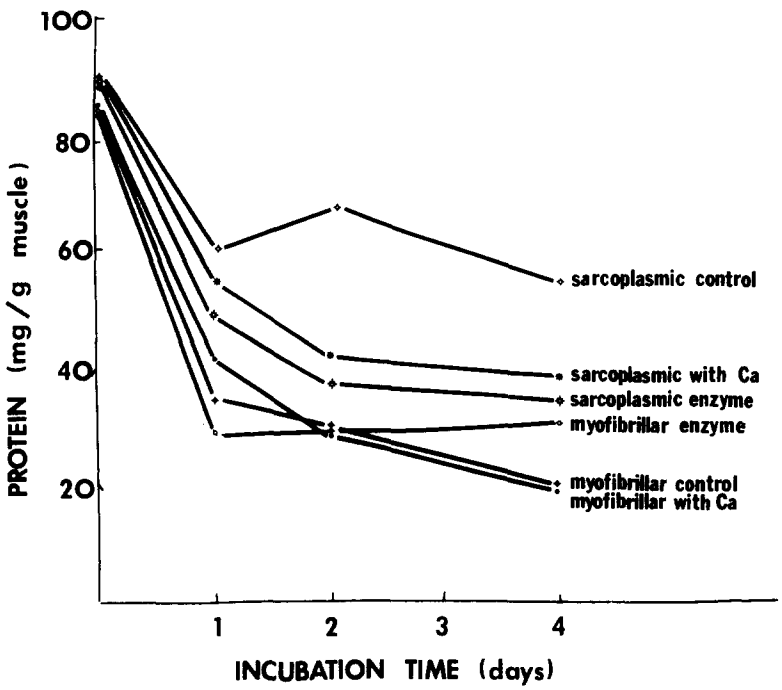

Fig. 1. Changes in the amount of extractable sarcoplasmic and myofibrillar protein as influenced by treatment during incubation at 37°C.

The decreased content of extractable sarcoplasmic and myofibrillar protein may be due, in part, to the increase in NPN which was reported to occur during incubation of muscle in an earlier communication from our laboratory (Hapchuk *et al.*, 1976). However, the increase in NPN was insufficient to account for all of the decrease in the protein fractions, increasing from 6·6 to 8·0 mg N/g muscle during incubation of the control and from 6·2 to 8·8 mg N/g muscle in a sample incubated with *C. perfringens* organisms (Hapchuk *et al.*, 1976). Thus, it appears likely that incubation at 37°C may also cause some denaturation of the proteins, which could also contribute to the decreased content of sarcoplasmic and myofibrillar proteins. This is not surprising in view of the relatively high temperature of incubation.

Changes in components of the sarcoplasmic protein fraction

Disc-gel electrophoresis in 8M *urea*—The position and relative intensity of the protein bands from the sarcoplasmic extract after conventional disc-gel electrophoresis in the presence of 8M urea are shown in Table 1. Incubation resulted in significant changes in the proteins with R_M values of 0·10, 0·25, 0·30, 0·48 and 0·60 in both the enzyme-treated and Ca^{++}-containing samples. The differences between the enzyme-treated and the Ca^{++}-containing samples were not significant at any

TABLE 1

RELATIVE MOBILITIES AND PEAK AREAS IN PERCENTAGE OF TOTAL AREA FOR THE SARCOPLASMIC PROTEINS AFTER DISC-GEL ELECTROPHORESIS IN 8M UREA

Treatment	*Incubation in days*	*Peak areas (percent of total protein area)*[a,b]					
R_M values		0·60	0·48	0·38	0·30	0·25	0·10
Control	0	7^{a}	4^{a}	—[a]	—[a]	42^{a}	48^{a}
	1	7^{a}	3^{a}	—[a]	—[a]	38^{b}	$51^{a,b}$
	2	$10^{a,b}$	$2^{b,c}$	—[a]	—[a]	29^{c}	59^{d}
	4	$12^{b,c}$	$2^{b,c}$	2^{a}	—[a]	25^{c}	60^{d}
Ca^{++}-containing	1	7^{a}	3^{a}	—[a]	9^{b}	26^{c}	$54^{b,c}$
control	2	7^{a}	2^{b}	2^{a}	—[a]	$22^{c,d}$	66^{e}
	4	$13^{b,c}$	—[d]	6^{b}	—[a]	$16^{d,e}$	65^{e}
Enzyme	1	7^{a}	4^{a}	—[a]	8^{b}	26^{c}	$56^{c,d}$
	2	10^{b}	$1^{c,d}$	3^{a}	—[a]	28^{c}	$58^{c,d}$
	4	14^{c}	—[d]	3^{a}	—[a]	11^{e}	72^{f}

[a] Any two means in the same column followed by the same superscript are not significantly different as determined using Duncan's multiple range test at the 5% level.
[b] Mean values of — indicate the absence, or negligible amounts, of the component.

time, which indicates that any differences between the action of enzyme and the Ca^{++} treatment upon the sarcoplasmic proteins are not detectable using disc-gel electrophoresis.

A few differences were noted between the control and the enzyme- and Ca^{++}-treated samples, notably in the low mobility proteins with R_M 0·25 and 0·10. After four days' of incubation, the control contained a larger percentage of the R_M 0·25 component and a lower percentage of the R_M 0·10 fraction than the enzyme- and Ca^{++}-treated samples.

SDS disc-gel electrophoresis—Table 2 shows the changes in the sarcoplasmic proteins during incubation as measured by SDS-gel electrophoresis. Significant changes were noted during incubation in all the protein/subunit bands measured, although differences between the Ca^{++}-containing control and the enzyme-treated muscle were not evident in all cases. The percentage of polypeptides with a molecular weight of 30,000 daltons declined significantly after four days' incubation with added Ca^{++} being lower than the enzyme-treated sample incubated for the same length of time. A decline was also observed in the enzyme-treated muscle by the first day of incubation, but was not observed until the second day of incubation in the Ca^{++}-containing control.

After one day of incubation, the enzyme-treated samples exhibited greater increases than the controls in the protein/subunit bands with molecular weights of 52,000 and 66,000 daltons, but further incubation resulted in comparable percentages for both the enzyme-treated and the Ca^{++}-containing samples. In all other instances, the changes noted were observed in both the enzyme-treated samples and the Ca^{++}-containing control, which suggests that most of the changes occurring during incubation were a result of added Ca^{++} and the incubation time

TABLE 2
MOLECULAR WEIGHTS AND PEAK AREAS IN PERCENTAGE OF TOTAL AREA FOR THE SARCOPLASMIC PROTEINS AFTER SDS-GEL ELECTROPHORESIS

Treatment	*Incubation in days*	*Peak areas (percent of total protein area)*[a,b]						
Molecular weight $\times 10^{-3}$ (daltons)		17	30	38	45	52	66	90
Control	0	1^{a}	16^{a}	$18^{a,b}$	$27^{a,b}$	$14^{a,b}$	14^{a}	9^{a}
	1	$4^{a,b}$	15^{a}	$19^{b,c,d}$	$25^{a,b}$	$13^{b,c}$	$20^{b,c}$	4^{b}
	2	$5^{b,c}$	$13^{a,b}$	$19^{b,c}$	23^{a}	15^{a}	$23^{c,d}$	1^{c}
	4	7^{c}	14^{a}	$21^{c,d}$	$25^{a,b}$	12^{c}	$22^{b,c}$	—[c]
Ca^{++}-containing control	1	8^{c}	17^{a}	$20^{b,c}$	29^{b}	$12^{b,c}$	15^{a}	2^{c}
	2	8^{c}	9^{c}	$21^{c,d}$	28^{b}	$13^{b,c}$	$20^{b,c}$	—[c]
	4	11^{d}	1^{d}	27^{e}	36^{c}	—[c]	24^{d}	—[c]
Enzyme	1	$4^{a,b}$	8^{c}	15^{a}	$26^{a,b}$	25^{f}	$21^{b,c}$	1^{c}
	2	$6^{b,c}$	$10^{b,c}$	22^{d}	28^{b}	13^{b}	20^{b}	1^{c}
	4	$5^{b,c}$	7^{c}	27^{e}	33^{c}	4^{d}	24^{d}	—[c]

[a] Any two means in the same column followed by the same superscript are not significantly different as determined using Duncan's new multiple range test at the 5% level.
[b] Mean values of — indicate the absence, or negligible amounts, of the components.

and temperature. This is in agreement with the work of Bullen & Chushner (1962) who found that muscle injected with $CaCl_2$ exhibited considerable breakdown. Work by Henderson *et al.* (1970) also indicated that the presence of Ca^{++} ions initiates muscle destruction.

Overall results upon incubation of muscle with the enzyme preparation isolated from *C. perfringens* indicate that only minor changes occurred in the protein/subunits of the sarcoplasmic proteins. However, addition of Ca^{++} ions caused considerable degradation. Some of the damage observed may be associated with activation of the Ca^{++}-activated protease in muscle described by Busch *et al.* (1972) and more recently characterised by Dayton *et al.* (1976*a*, *b*).

Changes in components of the myofibrillar protein fraction

Disc-gel electrophoresis in 8M *urea*—The relative mobilities and the percentage of total myofibrillar protein in each peak as separated by polyacrylamide gel electrophoresis in the presence of 8M urea are shown in Table 3. Some components of the peaks have been tentatively identified by comparison with the relative mobilities reported by Rampton (1969). These are troponin at R_M 0·84, reduced tropomyosin at R_M 0·58, actin at R_M 0·44, oxidised tropomyosin at R_M 0·30 and myosin at R_M 0·10. Although the proteins migrated as single bands, some of them were probably contaminated with other proteins as it is difficult to get good separations, particularly with the slowly migrating proteins.

Incubation caused a significant increase in the percentage of the R_M 0·10 (myosin) band both for the control and the Ca^{++}-treated samples, but the enzyme-treated sample was significantly lower than the other two treatments after four days'

TABLE 3

RELATIVE MOBILITIES AND PEAK AREAS AS PERCENTAGE OF TOTAL AREA FOR MYOFIBRILLAR PROTEINS AFTER DISC-GEL ELECTROPHORESIS IN 8M UREA

Treatment	*Incubation in days*	*Peak areas (percent of total protein area)*[a]				
R_M values		0·84	0·58	0·44	0·30	0·10
Identification[b]		Troponin	Reduced tropomyosin	Actin	Oxidised tropomyosin	Myosin
Control	0	14^{a}	$10^{a,b}$	34^{a}	$20^{a,b,c,d}$	22^{a}
	1	7^{c}	7^{b}	$25^{b,c}$	$15^{c,d}$	$46^{d,e}$
	2	$11^{a,b}$	13^{a}	18^{d}	24^{a}	$34^{a,b,c,d}$
	4	$6^{c,d}$	$10^{a,b}$	$21^{c,d}$	25^{a}	$39^{b,c,d,e}$
Ca^{++}-containing	1	$9^{b,c}$	12^{a}	$30^{a,b}$	14^{d}	$36^{b,c,d,e}$
control	2	$7^{b,c}$	6^{b}	$28^{a,b}$	$16^{b,c,d}$	$41^{c,d,e}$
	4	3^{d}	$10^{a,b}$	$25^{b,c}$	14^{d}	49^{e}
Enzyme	1	$8^{b,c}$	$9^{a,b}$	$31^{a,b}$	$23^{a,b}$	$35^{a,b,c,d}$
	2	$9^{b,c}$	$10^{a,b}$	$30^{a,b}$	$21^{a,b,c}$	$31^{a,b,c}$
	4	$6^{c,d}$	$9^{a,b}$	32^{a}	$16^{b,c,d}$	$28^{a,b}$

[a] Any two means in the same column followed by the same superscript are not significantly different as determined using Duncan's multiple range test at the 5% level.
[b] Identifications were made according to Rampton (1969).

incubation. This suggests that the enzyme caused some breakdown during incubation, although there was little change in comparison with the 0 day control. The protein at R_M 0·30 (oxidised tropomyosin) showed little change during incubation except that this fraction declined in both the Ca^{++}-treated and the enzyme-treated samples. The percentage of the band at R_M 0·44 (actin) declined during incubation for four days in the control and Ca^{++}-treated samples but remained the same in the enzyme-treated sample. This suggests that the enzyme in some way enhanced the extraction of the protein. Neither the band at R_M 0·58 (reduced tropomyosin) nor that at 0·84 (troponin) behaved significantly differently during incubation, with the former showing little change and the latter decreasing to about the same extent in all samples.

Disc electrophoresis in 8M urea revealed that the quantitative changes in the myofibrillar proteins as a result of incubation of muscle with the enzyme preparation were relatively minor, except for some apparent decrease in myosin. In general, autolysis *per se* (control samples) and the addition of Ca^{++} ions caused as much change in the different proteins as the enzyme treatment followed by incubation.

SDS-disc gel electrophoresis—Identification of some of the proteins was accomplished by using their molecular weights as reported by other workers (Table 4). Although some of the peaks presumably contained more than one protein, others appeared to be relatively pure and their molecular weights give a fairly positive identification.

Table 5 shows the percentages of the different molecular weight proteins and polypeptide subunits in the myofibrillar extract as determined by SDS-disc gel electrophoresis. The 15,000 dalton component was not present in the control,

TABLE 4
MOLECULAR WEIGHTS OF SOME MYOFIBRILLAR PROTEINS AS DETERMINED WITH SDS-GEL ELECTROPHORESIS SHOWING COMPARISONS WITH LITERATURE VALUES

Calculated molecular weights of unknown peaks	*Tentative identification*	*Reported molecular weights of known proteins*	*Citation*
19000	Troponin C	18000	Wilkinson *et al.* (1972)
	Myosin light chain	16000; 14000	Starr & Offer (1971)
		18000	Sender (1971)
		18000; 16000	Offer *et al.* (1973)
		16000; 17500; 22000	Scopes & Penny (1971)
		18500–19500	Patterson & Strohman (1970)
24500	Troponin I	23000	Wilkinson *et al.* (1972)
		24000	Offer *et al.* (1973)
31000	Unidentified	30000	Hay *et al.* (1973)
			Offer *et al.* (1973)
35000	Tropomyosin	34000–35000	Scopes & Penny (1971)
		36000	Hay *et al.* (1973)
		32000	Offer *et al.* (1973)
	Troponin T	35000	Offer *et al.* (1973)
		38000	Sender (1971)
		37000	Wilkinson *et al.* (1972)
44000	Actin	41500	Scopes & Penny (1971)
		49000	Hay *et al.* (1973)
		44000	Sender (1971)
		41700	Offer *et al.* (1973)
70000	Unidentified	55000; 75000	Scopes & Penny (1971)
95000	α-actinin	90000	Scopes & Penny (1971); Offer *et al.* (1973)
		115000	Hay *et al.* (1973)
		102000	Sender (1971)
120000	Unidentified	105000	Scopes & Penny (1971)
		110000	Starr & Offer (1971)
145000	C-protein	140000	Offer *et al.* (1973)
200000	Myosin heavy chain	200000	Offer *et al.* (1973)
		207000	Sender (1971)
		210000	Hay *et al.* (1973)
		195–200000	Patterson & Strohman (1970)

although it was present in the Ca^{++}-treated sample after one day's incubation, but then disappeared. This component, for which no identification was assigned, was present in the enzyme-treated sample from one to four days' incubation. The absence of this protein/polypeptide in the control and the large amount in the enzyme-treated sample indicates that incubation with the enzyme accelerated its formation. The band with a molecular weight of 31,000 also increased significantly in the enzyme-treated sample after incubation for four days.

There were no significant changes in the percentage of the component with a molecular weight of 19,000, which would be either myosin light chains or troponin C or a combination of these proteins. The peaks at molecular weights of 44,000 daltons (actin) and 145,000 (probably C-protein) both decreased significantly during

TABLE 5

MOLECULAR WEIGHTS AND PEAK AREAS AS PERCENTAGE OF TOTAL AREA FOR MYOFIBRILLAR PROTEINS SEPARATED BY SDS-GEL ELECTROPHORESIS

Treatment	*Incubation in days*	*Peak areas (percent of total protein area)*[a,b]										
10^{-3} × molecular weight (daltons)		15	19	24·5	31	35	44	70	95	120	145	200
Control	0	—[a]	9^{a}	$6^{a,b}$	6^{a}	17^{a}	$14^{a,c}$	$5^{a,b}$	5^{a}	5^{a}	6^{a}	27^{a}
	1	—[a]	$7^{b,c}$	13^{e}	$8^{a,b}$	33^{c}	$15^{c,d}$	2^{a}	—[c]	—[b]	9^{d}	12^{b}
	2	—[a]	8^{b}	$11^{d,e}$	6^{a}	39^{c}	$17^{c,d}$	3^{a}	—[c]	—[b]	9^{d}	7^{c}
	4	—[a]	$7^{b,c,d}$	9^{c}	$9^{a,b,c}$	$37^{d,e}$	$21^{e,f}$	3^{a}	3^{a}	—[b]	8^{c}	$5^{c,d}$
CA^{++}-containing control	1	4^{b}	$7^{b,c,d}$	$11^{d,e}$	16^{d}	26^{b}	$18^{d,e}$	$8^{b,c}$	4^{a}	—[b]	7^{a}	$1^{e,f}$
	2	—[a]	$7^{c,d}$	$11^{d,e}$	$11^{b,c}$	$34^{c,d}$	$18^{d,e}$	8^{c}	—[c]	—[b]	$7^{a,c}$	$4^{c,d,e}$
	4	—[a]	6^{d}	4^{a}	$9^{a,b,c}$	$36^{c,d,e}$	23^{f}	12^{d}	—[c]	—[b]	3^{d}	$1^{e,f}$
Enzyme	1	7^{c}	10^{a}	13^{e}	18^{d}	26^{b}	10^{b}	4^{a}	8^{b}	—[b]	4^{b}	$1^{e,f}$
	2	6^{c}	$8^{b,c}$	$11^{c,d}$	13^{c}	27^{b}	17^{d}	9^{c}	—[c]	—[b]	7^{a}	$3^{d,e,f}$
	4	6^{c}	$7^{b,c}$	7^{b}	25^{e}	28^{b}	$12^{a,b}$	12^{d}	—[c]	—[b]	4^{b}	—[f]

[a] Any two means in the same column followed by the same superscript are not significantly different as determined using Duncan's multiple-range test at the 5% level.
[b] Mean values of — indicate the absence or negligible amounts, of the components.

incubation with the enzyme for four days at 37 °C compared with the control and the control with added Ca^{++} ions. This indicates that the enzyme was causing some breakdown of these two proteins.

The protein/subunits with molecular weights of 95,000 (α-actinin) and 120,000 (unidentified) decreased significantly during incubation for all treatments. Thus, the changes appeared to be due to autolysis rather than the action of the added enzyme. The peaks at 24,500 (troponin I) and 70,000 daltons (unidentified) showed essentially the same pattern regardless of treatment. The protein/polypeptide with a molecular weight of 35,000 daltons (presumably either tropomyosin or troponin T or a combination of both) increased during incubation for one day in all samples, but the enzyme treatment lowered the level of this component after incubation for either two or four days.

Although the 200,000 dalton component (myosin) decreased in all samples during incubation at 37 °C, the decrease was significantly greater in those samples to which the enzyme and Ca^{++} were added. Since the values for the enzyme- and Ca^{++}-treated samples were not significantly different, results suggest that the added Ca^{++} ions were responsible for the decline in this component. It is suggested that the added Ca^{++} ions may activate the sarcoplasmic muscle protease described by Busch *et al.* (1972) and cause proteolysis of this protein band during subsequent incubation with both the Ca^{++}-treated and the enzyme-treated samples.

Results indicated that the enzyme treatment caused significant changes in the 15,000 (unidentified), 31,000 (unidentified), 35,000 (tropomyosin and/or troponin T), 44,000 (actin) and 145,000 (C-protein) dalton components. The added enzyme had no measurable effect upon the other protein bands, although addition of Ca^{++} ions accelerated proteolysis of the 200,000 dalton component.

Action of isolated enzyme and Clostridium perfringens *organisms*

Although the low molecular weight component of 15,000 daltons was observed with both the enzyme-treated and the *C. perfringens*-treated samples (Hapchuk *et al.*, 1976), the intact organisms caused more extensive proteolysis. Earlier work in this laboratory showed that *C. perfringens* organisms preferentially degraded troponin, causing extensive proteolysis (Hapchuk *et al.*, 1976), whereas the 35,000 dalton peak corresponding to troponin T and/or tropomyosin increased as a result of the enzyme treatment in this investigation. SDS-gel electrophoresis demonstrated that the enzyme preparation also degraded actin (44,000) which was shown to occur on incubation with the viable organisms using disc-gel electrophoresis in urea but not by SDS electrophoresis in earlier work from this laboratory (Hapchuk *et al.*, 1976).

Overall results indicate that viable *C. perfringens* organisms produce several enzymes, some of which were lost during the purification of the preparation used in this study. Thus, the enzyme preparation used in this study did not cause as much proteolysis on incubation with muscle as *C. perfringens* organisms *per se*.

ACKNOWLEDGEMENTS

The research reported herein is a portion of a Ph.D. thesis of the senior author submitted to Michigan State University in partial fulfilment of the requirements for the degree. This investigation was supported in part by Public Health Service Research Grant No. FD-00097 from the Food and Drug Administration.

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PHYSICO-CHEMICAL CHANGES IN PHILIPPINE *CARABAO* MANGOES DURING RIPENING

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(Received: 15 July, 1978)

ABSTRACT

Physico-chemical changes in Philippine Carabao *mangoes were measured through a seven-day ripening period at ambient conditions* (28–32 °*C and* 64–67 % *RH*). *Among these were changes in colour, texture, moisture, pectin, starch, total soluble solids, total carotenoids, ascorbic acid, titrable acidity and pH. The ripening changes observed are discussed and compared with known information on similar changes in other mango varieties.*

INTRODUCTION

The mango is among the choicest of fruits in the Philippines. Total production increased from 152,000 tons in 1970 to 331,000 tons in 1976 (Bureau of Agricultural Economics, 1970–1976). Although mangoes are available all year round in various parts of the country, their peak season starts in late February and lasts until the start of the rainy season in June.

Increasing agricultural production of the fruit requires parallel development in methods of post-harvest handling and processing. At the present time, local mango processing technology is still the subject of much laboratory research and the control of post-harvest ripening and deterioration limits optimum distribution of the fruit in the fresh form.

The objective of this work is to provide basic information on the physico-chemical changes in *Carabao* mangoes during ripening. Among these are the changes in colour, texture, moisture, pectin, starch, total soluble solids, total carotenoids, ascorbic acid, titrable acidity and pH. Such post-harvest chemical changes have been reported by de Leon & de Lima (1967) for the local *Pico* variety. Information of

Fd. Chem. 0308-8146/79/0004-0225/$02·25

this nature is expected to help in the development of processing and post-harvest handling methods for the optimum utilisation of this important crop.

EXPERIMENTAL PROCEDURES

Procurement of samples

The study was conducted in May and June of 1974 and 1975. In 1974, 140–150 kg of fresh mature fruits were purchased from a local market. The fruits were reportedly newly harvested from the province of Zambales (approximately 200 km from Manila). In 1975, 270 kg of fruit were picked randomly by laboratory personnel from ten trees in a mango orchard in Nueva Ecija (approximately 100 km from Manila). The fruits were transported to the laboratory on the same day that they were purchased and/or harvested.

The above two methods of sampling would be typical of the methods that would be open to potential fruit processors in securing the fruit.

Method of ripening and sampling

Samples were laid out on a laboratory table and allowed to ripen at existing ambient conditions (28–32 °C and 64–67 % relative humidity).

On a daily basis, ten fruits were selected randomly from the batch for physical and chemical analysis. The colour and texture were observed and measured on the unpeeled fruits. They were then peeled and the pulp sliced thinly. The peel and the seed were weighed and discarded.

Approximately 250 g of the sliced fruit were dried for starch analysis. Drying was done in a forced draft oven at 70 °C until a moisture content of about 10 % was reached. The rest of the slices were homogenised in a Waring blender from which samples for total soluble solids determination and other chemical analyses were taken.

Physico-chemical analysis

The method described by Lustre *et al.* (1976) was used for the preparation of sample and analysis of starch.

Total carotenoids were extracted by blending the fruit pulp with methanol and petroleum ether (1:1:2) in a Waring blender, washing the ethereal layer with water after separation by centrifugation and reading the optical density at 440 nm in a Beckman Spectronic 20 (Dharkar *et al.*, 1966).

The Bolin and Book modification (Bolin & Book, 1947) of the Roe and Osterling colorimetric method was used for the estimation of total ascorbic acid.

Titrable acidity (expressed as per cent anhydrous citric acid) was determined by titration with 0·1N sodium hydroxide using phenolphthalein as indicator (AOAC, 1970).

Pectin was determined from 20 g of the mango purée following the procedure described by Lustre *et al.* (1976) for *Saba* bananas.

The soluble solids of the purée, expressed in degree Brix, was determined with the use of the Zeias Opton hand sugar refractometer.

A Fisher pH meter (Accumet 220) was used to measure the pH of the purée.

The texture of the whole fruit was measured with the aid of a precision penetrometer (Precision Scientific Co., Chicago, Illinois, USA). The penetrometer measures the depth to which a standard cone penetrates a given material under prescribed conditions of weight, time and temperature. In the mangoes, the depth of penetration was determined at room temperature by releasing the cone assembly and allowing it to drop for 15 sec at the tip, centre and stem end of the top and bottom surfaces of the fruit. The whole fruit with the peel was used in the measuring procedure. The total weight of the cone was 102·5–0·05 g and the total weight of its movable attachments, 47·5 $\pm$ 0·05 g. Its hardened steel tip, which penetrates the fruit, had an outside diameter of 0·329 in at its base and 0·016 in at its tip.

The moisture content was determined by drying 5 g of the blended pulp in a vacuum oven at 70 °C and a vacuum of not more than 30 in Hg for 16 h (Lustre *et al.*, 1976).

RESULTS AND DISCUSSION

Carabao mangoes studied in this work took seven days to ripen, from the green mature to the yellow-ripe stage. The sixth day was judged to be representative of the table-ripe stage. During this time, the peel had turned from green to golden-yellow and on the sixth day all fruits had turned yellow. This non-uniform change in fruit colour is typical of mangoes and other tropical fruits.

The analysis reported here, although subject to this limitation, still serves as the most practical guide to a fruit processor or trader as the fruits sampled for analysis were typical of the normal batch of fruits that would be available to him in the market.

Physico-chemical changes during the ripening of mangoes at ambient conditions are shown in Table 1. The results, presented for each year, are the average of duplicate analyses. The data for each year represent two methods of sample procurement from two different sources and are both presented to give an indication of the range of values manifested by two different batches of fruit from two sources.

PHYSICO-CHEMICAL CHANGES

(*a*) *Starch*

The starch content and its degradation during ripening are shown in Table 1 and Fig. 1.

TABLE 1

PHYSICO-CHEMICAL CHANGES IN *Carabao* MANGOES DURING RIPENING

Physico-chemical properties	*Year*	*Days after harvest*						
		1	2	3	4	5	6	7
Starch (%)	1974	11·41	10·43	8·94	6·24	2·32	0·71	0·34
	1975	11·38	9·08	3·09	0·90	0·54	0·0	0·0
Total soluble solids	1974	7	9	10	12	17	18	18
(%)	1975	10	11	15	18	19	19	19
Total carotenoids	1974	0·62	0·29	0·48	0·69	1·20	1·88	0·91
(mg %)	1975	0·82	0·87	1·07	1·46	1·62	2·29	2·75
Ascorbic acid	1974	78·12	65·62	68·75	59·38	50·00	28·44	23·75
(mg %)	1975	61·56	59·50	55·00	48·25	41·18	41·88	37·81
Titrable acidity	1974	1·9	2·3	1·9	1·5	0·7	0·4	0·2
(%)	1975	1·9	1·8	1·3	0·9	0·6	0·5	0·4
pH	1974	3·4	3·3	3·4	3·5	4·0	4·3	4·4
	1975	3·9	3·8	4·0	4·2	4·6	4·9	5·0
Pectin (%)	1974	0·37	0·44	0·46	0·51	0·75	0·76	0·75
	1975	0·53	0·61	0·66	0·75	0·78	0·76	0·90
Moisture (%)	1974	79·81	79·70	79·47	79·68	80·34	80·54	80·10
	1975	79·16	78·88	78·69	78·43	81·83	79·27	78·92
Texturemeter reading	1974	13·4	15·7	20·1	21·6	23·2	35·7	47·2
(mm)	1975	—	11·0	11·6	16·0	25·0	30·8	36·5
Colour of fruit	1974	Green	Green	Green	Yellowish-green	Greenish-yellow		Dark yellow
	1975	Green	Green	Green	Yellowish-green	Greenish-yellow		Dark yellow

(%) = g/100 g fresh banana.
(mg %) = mg/100 g fresh banana.

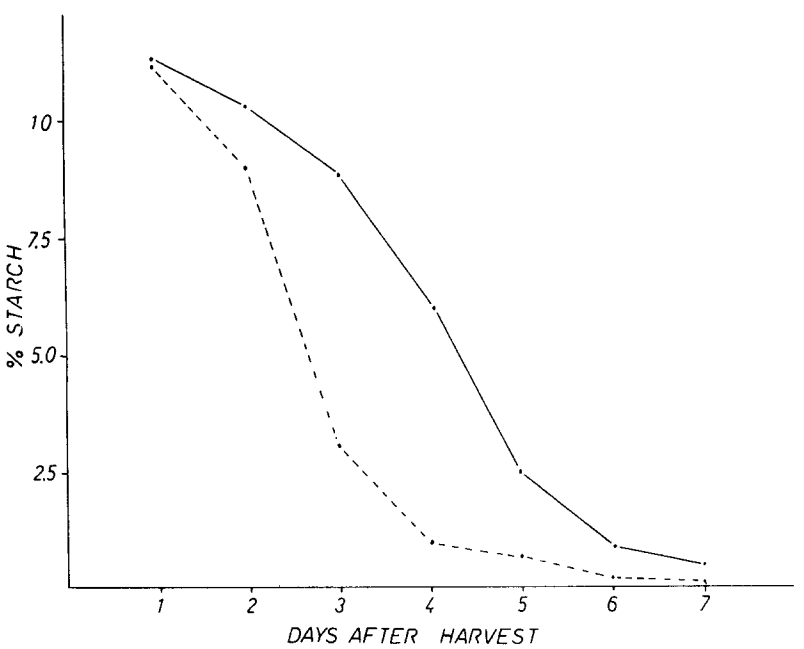

Fig. 1. Changes in starch of *Carabao* mangoes during ripening. ——, 1974; – – –, 1975.

As shown in Table 1, the *Carabao* mango had approximately 11% starch at harvest. This was degraded rapidly during the seven-day ripening period due to the hydrolysis of starch to sugars which accompanies fruit ripening. At the table-ripe stage, i.e. six days after harvest, the *Carabao* mangoes contained negligible amounts of starch.

As seen from Fig. 1, the maximum rate of starch hydrolysis occurred between the third and fifth day of ripening for the 1974 crop and between the second and fourth day of ripening for the 1975 crop. The difference in time at which the onset of rapid starch hydrolysis was initiated was probably due to differences in maturity and/or in the number of immature samples between the two batches of fruit. In spite of this difference, however, the maximum rate of starch degradation was similar—at approximately 3 to 4% per day—for both batches of fruit.

(*b*) *Total soluble solids*

The total soluble solids was 7% and 10% at harvest for the 1974 and 1975 crops, respectively, as shown in Table 1. This increased during ripening to 18 and 19% on the seventh day after harvest.

As shown in Fig. 2, the maximum rate of soluble solids formation occurred between the third and fifth days for the 1974 crop and between the second and fourth days for the 1975 crop. These same periods covered the time at which the maximum rate of starch degradation occurred in the ripening fruit. This is not unexpected as sugars are known to be produced in fruits during ripening, from the degradation of starches.

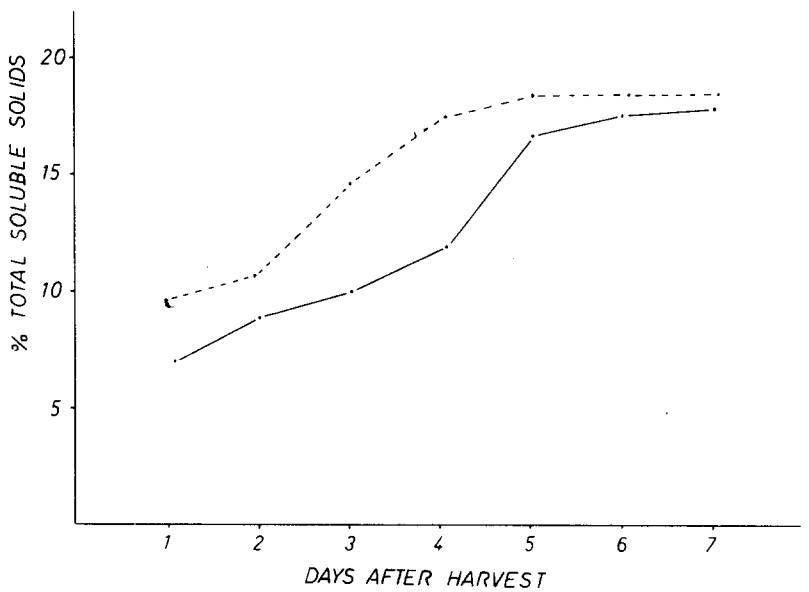

Fig. 2. Changes in total soluble solids of *Carabao* mangoes during ripening. ——, 1974; – – –, 1975.

The maximum rate of formation of total soluble solids, as seen in Fig. 2, is about the same at 3·5% per day for both batches of fruit.

Figure 2 also shows that, after the fifth day of ripening, soluble solids increased only slightly and started to level off. Prolonging the ripening period beyond this time will therefore not result in significant further increases in percent total soluble solids in the fruit.

(*c*) *Total carotenoids*

Mango pulp turned from whitish-yellow to yellow, on ripening. This change in colour is due to the increase in carotenoids, the major colour pigment in ripe mango pulp (Hulme, 1971).

As shown in Table 1, total carotenoids at harvest were 0·62 and 0·82% for the 1974 and 1975 crops, respectively. This increased rapidly after the second day of harvest, reaching a level almost threefold higher than the original, at the table-ripe stage, six days after harvest. As shown in Fig. 3, carotenoid concentration in the pulp of the 1975 crop showed an increasing trend even at the ripe stage. An unexpected drop occurred for the 1974 crop on the seventh day of ripening and this was probably due to the variability in maturity at this stage of ripeness, within fruits sampled for analysis.

The increase in total carotenoids was found to correlate well with the known

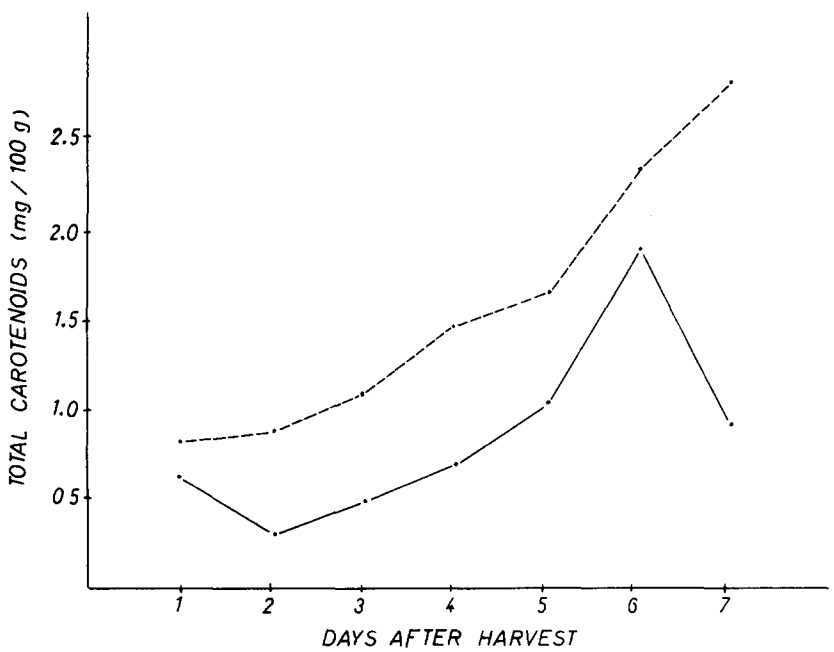

Fig. 3. Changes in total carotenoids of *Carabao* mangoes during ripening. ——, 1974; – – – –, 1975.

softening of the fruit during ripening. Calculation of the correlation coefficient between total carotenoid content and texture, as measured with a penetrometer, at the various stages of ripeness gave a value of 0·95 at the 1% level of significance. Softening of the unpeeled fruit gives an indication of the extent of yellowing of the pulp.

A high correlation coefficient was also found between total carotenoids and total soluble solids. A value of 0·79, significant at the 5% level, was obtained. Thus the extent of yellowing of the pulp was also a good measure of the extent of formation of total soluble solids in the fruit.

(*d*) *Ascorbic acid*

Table 1 shows that *Carabao* mango, in the green stage, is a good source of ascorbic acid. The fruit contained 78·12 and 61·56 mg per cent ascorbic acid for the 1974 and 1975 crops, respectively, on the first day of ripening. The acid was degraded during ripening and only 28·44 and 41·89 mg per cent ascorbic acid remained at the table-ripe stage (fifth day of ripening).

The concentration of ascorbic acid in the fruit at each ripening stage varied greatly between the 1974 and 1975 crops, indicating inherent variability between fruits coming from two sources. However, the extent at which the acid was degraded during ripening was very similar for both crops, up to the fifth day after harvest. At this ripening stage, when the fruit was near the table-ripe stage, an average of 50% of the acid had been degraded.

Ascorbic acid is important in citrus fruits like the mango, both for its nutritive properties and for the role it is suspected to play in fruit juice discoloration and browning.

(*e*) *Pectins*

In some mango products such as jams and fruit juices, the amount of pectins in the fruit could be important. As seen in Table 1, the fruit contained 0·37 and 0·53 mg per cent pectins at harvest (1974 and 1975 crops, respectively). This increased progressively during ripening, such that on the seventh day of ripening the original pectin content of the green fruit had almost doubled.

(*f*) *Moisture*

Water is a major constituent of mango pulp. As shown in Table 1, freshly bought and harvested fruit contained 79·16 and 79·81% moisture for the 1974 and 1975 crops, respectively. This changed little throughout the ripening period.

(*g*) *Titrable acidity and pH*

The mango is a sour fruit when unripe. As such it has been used as a pickled appetiser and as an acid condiment in home-cooked meals. As the fruit ripens, the acid flavour decreases and is tempered by the gradual sweetening of the pulp. The primary acid in mangoes is reported to be citric acid (Hulme, 1971).

Table 1 shows that the fruit had a titrable acidity of 1·9 % and pHs of 3·4 and 3·9 at harvest. As the fruit ripened, per cent titrable acidity decreased rapidly to an average of 0·3 while the pH rose to 4·4 and 5·0 for the 1974 and 1975 crops, respectively. There was an 84 % decrease in acidity and a 1 unit increase in pH during seven days of ripening. The figure also shows that changes in fruit acidity occurred at their maximum rate, after the second day of ripening, which was about the same ripening stage during which marked changes in other chemical constituents of the fruit were found to occur.

COMPARISON WITH OTHER MANGO VARIETIES

Carabao mangoes are well liked not only for their golden-yellow colour but for their sweet, juicy and uniquely pleasant flavour. The fruits examined in this work had an average weight of 220·5 g, of which 13 % constituted the peel and 13·4 % the seed.

Hulme (1971), in a tabulated summary of the chemical composition of six varieties of Florida mangoes and three varieties of Indian mangoes, reported a minimum starch content in the green fruit of 4·2 % and a maximum of 10·6. Elahi & Khan (1973) reported a starch content of 15 % at harvest for Pakistani mangoes. As shown in Table 1, the *Carabao* mango has a starch content of about 11 % at harvest which is comparable to the starch levels reported for the different varieties of the fruit.

For per cent total soluble solids, Hulme (1971) reported a range of 11 to 16·2 %. The *Carabao* mango, as shown in Table 1, contained 17 and 19 % total soluble solids at a similar stage of ripening.

The pH of mangoes during ripening was reported (Hulme, 1971) to rise from 3 to 5·2 and total acidity to vary from 0·13 to 0·71 %. In Pakistani mangoes (Elahi & Khan, 1973), acidity varied from 0·003 to as high as 2·96 %. The values found in *Carabao* mangoes ranged from an average of 0·3 to 1·9 %.

Another mango variety produced on a commercial scale in the Philippines and used widely in processing is the *Pico* variety. A comparison of the chemical composition of these two varieties at the green and table-ripe stages is shown in Table 2.

Table 2 shows that after one day of ripening, the *Carabao* mango variety had a higher starch content than the *Pico* variety, indicating a greater ability to accumulate starch during growth. It had an average starch content of 11·38 % compared with 5·2 for the *Pico* variety.

In both the green and ripe fruits, the *Carabao* variety had a higher content of total carotenoids but less ascorbic acid. It was also slightly less acidic.

Total soluble solids was comparable for both varieties when the fruit was unripe but was slightly higher in the *Carabao* variety when the fruit was ripe.

The above differences, although not primarily responsible for the marked

TABLE 2
COMPARISON OF THE CHEMICAL COMPOSITION OF *Carabao* AND *Pico* MANGOES

Chemical composition (fresh basis)	*Carabao variety*[a]	*Pico variety*[b]
Starch (%)	11·39—green fruit	5·2 —green fruit
	0·35—ripe fruit	0 —ripe fruit
Total soluble solids (%)	8·5 —green fruit	8·6 —green fruit
	18·5 —ripe fruit	16·9 —ripe fruit
Total carotenoids (mg %)	0·72—green fruit	0·05—green fruit
	2·08—ripe fruit	0·99—ripe fruit
Ascorbic acid (mg %)	69·84—green fruit	98·0 —green fruit
	35·11—ripe fruit	52·0 —ripe fruit
Total titrable acidity (%)	1·9 —green fruit	2·58—green fruit
	0·4 —ripe fruit	0·28—ripe fruit
pH	3·6 —green fruit	2·8 —green fruit
	4·7 —ripe fruit	4·0 —ripe fruit

Green fruit—One day after harvest.
Ripe fruit—The average of the values six days after harvest for the *Carabao* variety (table-ripe stage).
—The average of the values seven days after harvest for the *Pico* variety (reportedly[b] the stage at which organoleptic scores were highest).
[a] Indicated values are the average for the 1974 and 1975 crops taken from Table 1.
[b] De Leon & De Lima (1967).

differences in flavour between the two types of local mango, provide background information on the variety that would be better suited for specific needs.

SUMMARY

The *Carabao* mango, one of the choicest of Philippine fruits, goes from the green-mature to the yellow-ripe stage in seven days at ambient conditions (28–32 °C and 64–67 %RH). In the green-mature stage, it was found to contain approximately 11 % starch and 8 % total soluble solids. The other major constituent was water, which was present to the extent of 79 to almost 80 %. On ripening, there was little change in moisture content. The conversion of starch to sugar left the fruit with an almost negligible starch content and 18 to 19 % total soluble solids, on the seventh day of ripening.

The most significant chemical change accompanying ripening was the degradation of starch and the formation of soluble solids. Starch decreased at the maximum rate of 3 to 4 % per day and soluble solids increased at the maximum rate of 3·5 % per day on the second to third day of ripening.

The ripe fruit was also found to be a good source of carotenoids, averaging 2·08 mg per cent at the sixth day of ripening. Ascorbic acid was present in significant amounts in the green fruit but was degraded by almost 50 % of its original concentration during ripening. From an average of 69·84 mg per cent ascorbic acid at harvest, only 35·11 mg per cent remained at the table-ripe stage.

Total acidity decreased from an average of 1·9 to 0·3 % and pH increased from 3·7 to 4·7, from the green to the table-ripe stage of ripening.

Physico-chemical changes in *Carabao* mangoes during ripening, found in this work, were similar to those of other mango varieties that have been reported in the literature. Total soluble solids content of 17 to 19 % is, however, higher than the range of 11 to 16·2 % that has so far been reported for the total soluble solids in other varieties of this fruit. In addition, compared with the local *Pico* variety, at comparable stages of ripeness, *Carabao* mangoes (at the green stage) have a higher starch content but less ascorbic acid and are slightly less acidic.

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Technical Note

The Structural Basis for Bitterness in the *Isodon* Diterpenes

Chemical examinations of the plants of the genus *Isodon* (Labiatae) have produced a number of biologically active diterpenoids (Fujita *et al.*, 1976*a*; Kubo *et al.*, 1977*c*). Antitumour (Fujita *et al.*, 1976*b*), bacterial (Kubo *et al.*, 1974*c*) and insect growth inhibitory (Taniguchi *et al.*, 1979) activities were found to come from the α-methylene moiety of these compounds.

These *Isodon* bitter principles also showed antifeedant activity against the larvae of African army worms *Spodoptera exempta* and *S. littoralis* (Taniguchi *et al.*, 1979). Interestingly, the army worm antifeedants taste 'bitter' to humans whilst non-active compounds do not.

Recently we proposed a qualitative theory to relate the bitter taste of these diterpenoid compounds to their chemical structure. For a compound in this series to taste bitter, it must contain at least one 'bitter unit', where the 'bitter unit' consists of hard acid (AH) and hard base (B) moieties positioned so that they can form an intramolecular hydrogen bond (Kubota & Kubo, 1969). For example, isodonal (1) (Kubo *et al.*, 1974*a*; Kubota & Kubo, 1967), possessing an α-hydroxyl group at C-11 (R-configuration) tastes quite bitter, whereas trichodonin (2) (Kubo *et al.*, 1974*a*; Kubota & Kubo, 1968), having a β-hydroxyl group at C-11, is tasteless. The closest orbital distance between the 11-hydroxyl proton (AH) and the 6-aldehydic oxygen (B) is about 1 Å for the bitter isodonal and about 3 Å for the tasteless trichodonin (2). This suggests that only isodonal has the correct spatial geometry to complex with a similar hard acid/hard base unit that must exist in the taste bud receptor site. While this theory cannot be extended to all bitter-tasting compounds, it does effectively predict bitter taste in many terpenoid compounds.

Our continuing investigation of this series has led to the isolation of five new bitter compounds (3) to (7), all of which have closely related chemical structures including a full *ent*-kaurene skeleton. Recently similar compounds have been isolated as bitter principles from *Englerastrum scandens* (Nomoto *et al.*, 1976). These compounds are

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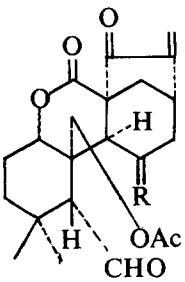
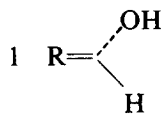
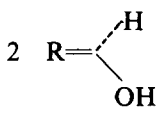
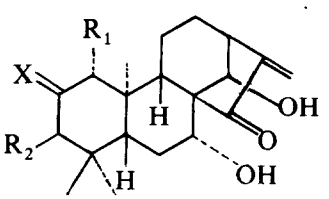


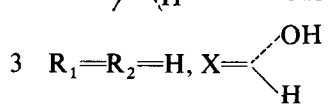


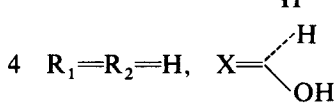


ideal for chemical structure/bitter taste studies since the rigid structure of the *ent*-kaurene system permits the placement and spacial arrangement of chemical groups on this skeleton to be accurately determined.

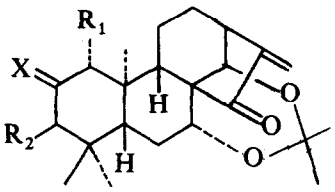
X-ray analysis of mebadonin (3) (Hirotsu *et al.*, 1973) shows that the 7α- and the 14β-hydroxyl form an intramolecular hydrogen-bond which is probably the 'bitter unit' of this compound. In order to test whether this moiety is responsible for the bitterness, umbrosin A (4), umbrosin B (5) (Kubo *et al.*, 1974*b*), kamebanin (6) (Kubo *et al.*, 1977*a*) and isodomedin (7) (Kubo *et al.*, 1977*b*), i.e. compounds containing the







5 R_1=R_2=H, X=O
6 R_1=OH, R_2=H, X=H_2
7 R_1=OH, R_2=OAc, X=H_2

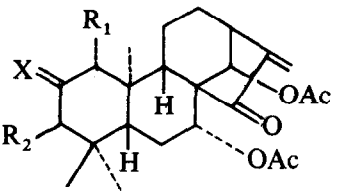


8 R_1=R_2=H, X= 

9 R_1=R_2=H, X=O

10 R_1=OH, R_2=H, X=H_2

11 R_1=OH, R_2=OAc, X=H_2



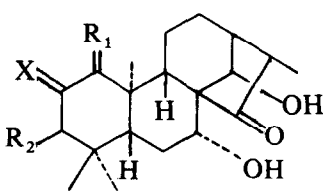
12 R_1=R_2=H, X= 

13 R_1=OAc, R_2=H, X=H_2

14 R_1=R_2=OAc, X=H_2

same 7α- and 14β-hydroxyl groups as mebadonin (3), were converted to acetonides (8) to (11) and acetates (12) to (14). These derivatives were tasteless. In contrast, it was found that other derivatives of (4) to (7) where the 7α- and the 14β-hydroxyl groups remain unchanged (15) to (20) were still bitter. Furthermore, other *ent*-kaurenoids (21) to (23) that have similar chemical structures but lack this 'bitter unit' are tasteless.

From the information so far obtained it appears that the presence of functional groups removed from the 'bitter unit', such as hydroxyl, acetoxyl, carbonyl and double bonds have little direct relation with the bitter taste of these compounds. Compound (24), which only has the 7α- and the 14β-hydroxyl groups ('bitter unit') on its *ent*-kaurene skeleton, is not as bitter as the more highly substituted parent compound (6). Removal of functional groups will change the physical properties of



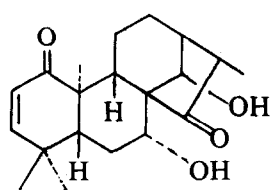
15 R_1=H_2, R_2=H, X= 

16 R_1=  , R_2=H, X=H_2

17 R_1=  , R_2=OAc, X=H_2

18 R_1=O, R_2=H, X=H_2

19 R_1=H_2, R_2=H, X=O



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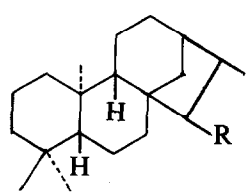
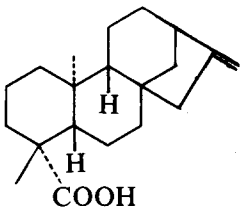


21 R=OH

22 R=H



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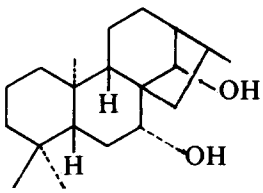

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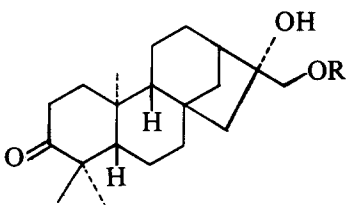
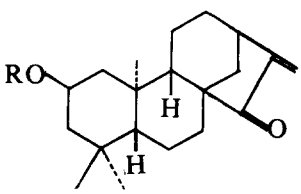
these compounds and affect their transport to the receptor site rather than their binding to the receptor site.

It is interesting to note that some *ent*-kaurene glucosides, specifically sugereoside (25) (Ichikawa *et al.*, 1973) and creticoside (26) (Chen & Murakami, 1971) are bitter, but their aglycones—(27) and (28) respectively—are tasteless. In this case the bitter



25 R=gluc
27 R=H



26 R=gluc
28 R=H

unit is likely to involve the sugar moiety. The detailed study will be reported elsewhere.

ACKNOWLEDGEMENT

The authors are grateful to Professor Koji Nakanishi for valuable discussion.

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BOOK REVIEWS

Structure–Activity Relationships in Human Chemoreception. By M. G. J. Beets. Applied Science Publishers Ltd, London. 1978. xii × 408 pp. Price: £25·00.

This excellent text gives us an up-to-date and truly chemical discourse on structure/activity relationships including a complete understanding of the stepwise chemical advances of the last decade. The author was the second president of the European Chemoreception Research Organisation and no one could be better qualified to set the entire subject into perspective. Dr Beets divides his book into two main sections, the first dealing with the fundamental concepts of chemoreception from an absolutely theoretical point of view and the second with the major findings in the areas of taste and olfaction. Throughout the book there is a constant theme of logical conceptual development and it is this underlying philosophy which is a genuinely original contribution to current thinking in this fascinating subject. Dr Beets describes what he considers to be the two main alternative views of the receptor—a general concept and a special concept. The former seems to be preferred by the author, despite the precise chiral specifications required for taste according to, say, the Shallenberger sweetness hypothesis. Dr Beets develops the concept of spatial overlap of enantiomers as a means of explaining their sensory differences. Whilst perfectly logical in itself, this idea could lead to slight misleading of the reader. On p. 65, for example, there is the sentence, 'strictly speaking, the mirror image relationship between enantiomeric molecules exists only in a single set of mutual orientations'. Actually enantiomorphism is a manifestation of intrinsic molecular asymmetry and therefore persists in all orientations. Nevertheless, the author is correct in emphasising orientation as perhaps the most important, yet often disregarded, factor in chemoreception.

Sadly, one omission in the discussion of olfaction and taste is the problem of temporal effects. This subject is now becoming of great practical and theoretical

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importance and the reviewer would have been anxious to hear Dr Beets' views on this subject in relation to structure and alignment of molecules.

Dr Beets points out that much of the research effort on olfaction and taste up to now has depended too much on which stimuli were 'on the shelf'. He earns all the more credit for extracting all the relevant information from the literature and fashioning it so well into this potent and lively book. It is packed with valuable information, possesses a full and entirely up-to-date bibliography and is thoroughly recommendable to all food scientists, psychologists, physiologists, chemists, biochemists and pharmacologists interested in the chemoreception of taste and olfaction.

G. G. BIRCH

Xylitol. Ed. by J. N. Counsell. Int. Symposium organised by Roche Products Ltd. May, 1977. Applied Science Publishers Ltd, London. xiv + 191. Price: £12·00.

This useful little book summarises the properties of xylitol, a naturally occurring sugar alcohol of potential technological value. The book contains chapters on the occurrence, properties and value of xylitol in confectionery and food technology, the biochemistry of xylitol and its tolerance by the human organism, exocrine secretions after administration of xylitol and three entire chapters devoted to different aspects of xylitol and dental caries.

Xylitol is more widespread in nature than is generally known. Many species of fruit and vegetable contain more xylitol than vitamin C, for example, and a rich natural source is yellow plum (935 mg/100 g). The substance is made commercially by hydrogenating xylose obtained from Birchwood chips and the pure crystalline product contains no detectable amount of xylose.

Xylitol is already used in chewing gum, toothpaste and other products but its legal status as a food ingredient in the UK is uncertain as it is neither a carbohydrate sweetener nor an artificial sweetener. Neither is it a humectant or other type of food additive in the strict sense of the term. Therefore, as the Editor himself so succinctly puts it (p. 28), 'What is it?'.

Undoubtedly the most interesting property of xylitol for the confectionery manufacturer is its lack of cariogenicity and this, together with its good sweetness and cooling effect in the mouth, render it very attractive for food purposes. This book is well produced, the discussion after each chapter is lively and informative (particularly the section on sweetness addiction) and the book is highly recommendable to all interested in carbohydrates and sweeteners as well as being of general value to food scientists, technologists, nutritionists and dental scientists.

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G. G. BIRCH

OLIGOSACCHARIDE FORMATION DURING HYDROLYSIS OF LACTOSE WITH *SACCHAROMYCES LACTIS* LACTASE (MAXILACT^{R}): PART 1—QUANTITATIVE ASPECTS

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(Received: 15 July, 1978)

ABSTRACT

The formation of oligosaccharides during hydrolysis of lactose with Saccharomyces lactis *lactase (Maxilact^{R}) was followed at different lactose concentrations. The amount of oligosaccharides formed increased with the lactose concentration and had its maximum value (5–13 % by weight of total sugar) when about 65–75 % of the sugar was hydrolysed to monosaccharides. The oligosaccharides were hydrolysed on prolonged incubation with active enzyme.*

INTRODUCTION

The hydrolysis of lactose in milk and other dairy products is likely to become an important process in the future. One reason for this is the well-documented intolerance for lactose, due to low intestinal lactase activity in older children and adults, common in most populations.

Milk is a very good source of essential nutrients and could, with this process, be consumed in larger quantities by people with low lactase activity. Lactose hydrolysis can also be advantageous in many industrial processes (Tumerman *et al.*, 1954; Sampey & Neubeck, 1955; Holsinger & Guy, 1974; Thompson & Brower, 1974; Thompson & Gyuricsek, 1974; Bouvy, 1975).

For some time there has been available on the market a β-galactosidase isolated from the yeast *Saccharomyces lactis* in the form of an odourless and tasteless, free-flowing powder. The enzyme is sold under the name Maxilact^{R} by Gist-Brocades,

Fd. Chem. 0308-8146/79/0004-0243/$02·25

Delft, The Netherlands. In comparison with other commercial β-galactosidases, it has a relatively low price. Its optimum pH is around 6·5–7·0, the normal pH of milk. The mechanism of the enzymic hydrolysis of lactose with lactase is generally of a transgalactosidic nature (Wallenfels & Malhotra, 1961). This means that the enzyme transfers the galactose moiety of a β-galactoside to an acceptor containing a hydroxyl group. This acceptor can be water, and in that case the hydrolysis of one molecule of lactose yields one molecule of free glucose and one of free galactose. The acceptor can, however, also be another lactose molecule and in that case a trisaccharide containing two galactose molecules is formed. If such trisaccharides in turn act as acceptors, tetrasaccharides are formed. When the glucose and galactose concentrations rise later on during the hydrolysis, the probability that these monosaccharides become acceptors increases. Other disaccharides (allolactose, galactobiose) are then formed.

In the present study the formation of oligosaccharides (trisaccharides and higher saccharides) at various lactose concentrations was studied. The formation of disaccharides other than lactose could not be detected with the separation technique used.

MATERIALS AND METHODS

Maxilact 40000^R was obtained from Gist-Brocades NV, Delft, The Netherlands.

All chemicals used had analytical grade purity and the buffer used for the incubations was 0·05M potassium phosphate, pH 6·8.

UHTST-sterilised milk (trade name: H-lättmjölk, fat content 0·5%) was purchased from Mjölkcentralen, Enköping, Sweden and had a lactose concentration of 5%.

Incubations

The enzyme concentration in all incubations was 40 mg Maxilact^R per litre.

The hydrolysis of lactose in buffer was studied at three different concentrations of lactose: 5, 10 and 20% (w/v). To permit long incubation times a bacteriostatic agent, methyl-*p*-oxybenzoic acid, was added to a concentration of 0·1% (w/v). This compound did not influence the Maxilact^R activity.

The incubation in UHTST-treated milk was carried out according to an aseptic method described by Dahlqvist *et al.* (1976).

Samples were collected at various times and the enzyme was inactivated by immersing the test tube in boiling water for 5 min before sugar analysis.

Quantitative paper chromatography of sugars

The paper chromatography was performed by the descending technique on Whatman No. 1 paper which had previously been cut into nine separate strips, held

together by intact paper. The strips were 1·5 cm wide and 1 cm apart. The lower end of the paper was serrated to allow the solvent to drip off. Two duplicates of the samples to be analysed were applied on four of the strips. Two strips were used as blanks, i.e. no sugar was applied.

On the remaining three strips a marker was applied which, after the run, was developed for 10 min at 110 °C with aniline phthalate reagent (1.66 g phthalic acid, 0·91 ml aniline, 5 ml aq. dest. and 95 ml acetone). One of the samples was used as a marker.

On each strip sugar solution was applied in portions of 5 μlitres until the total sugar amount was about 1 mg. After drying the chromatogram was equilibrated and run for 18 h with ethyl acetate: acetic acid: water (9:2:2 v/v). Three to five centimetre long pieces of the paper containing the sugars to be determined, and corresponding blanks from the strip with no sample applied, were carefully eluted for 20 min at room temperature in test tubes with 5 ml 0·05M potassium phosphate buffer, pH 6·8.

The total amount of sugar in each spot and in the corresponding blank was determined by the anthrone method, performed as described by Scott & Melvin (1953) but with heating for 7·5 min at 100 °C instead of 16 min at 90 °C. Lactose was used as a standard. Through careful handling of the paper strips during elution the blanks were low. With the anthrone method used galactose gives lower readings than the same amount of glucose. It must therefore be observed that since the oligosaccharides probably contain more galactose than glucose, due to the reaction mechanism, the percentage of disaccharides—and of oligosaccharides in particular—is somewhat underestimated, in spite of the use of lactose as a standard (*maximum* 17% underestimation). The monosaccharide spots, on the other hand, containing more glucose, are overestimated (*maximum* 13% overestimation).

The monosaccharide spots were also analysed specifically. Glucose was determined with a commercial glucose oxidase reagent, GLOX novum^{R}, purchased from AB Kabi, Stockholm, Sweden. The reagent was dissolved in 0·5M Tris buffer, pH 7·0, instead of water in order to inhibit a trace of lactase present. To one millilitre of a suitable dilution of the eluate two millilitres of GLOX novum reagent were added and kept at 37 °C for 60 min. The absorbance at 450 nm was determined in a spectrophotometer. Galactose was determined by a sensitive fluorimetric method as follows: one millilitre of eluant diluted to contain about 5 μg galactose was transferred to a fluorimeter cuvette. Two millilitres of 1M Tris buffer, pH 8·6, and 50 μlitres NAD solution (10 mg/ml) were added. The fluorescence was measured in a filter fluorimeter (primary filter with maximum transparency at a wavelength of 325 nm, and secondary filter transparent for wavelengths >405 nm). When a stable reading was obtained, 10 μlitre galactose dehydrogenase (5 mg/ml, obtained from Boehringer Mannheim, catalogue No. 15095) was added and the fluorescence was measured intermittently until a plateau was reached (~60 min). For standardisation a 5 μlitre galactose solution (0·696 mg/ml) was then added and the fluorescence was followed until a second plateau was reached.

RESULTS

Incubation with lactose in buffer

(1) *Lactose concentration* 5% (*w/v*): The hydrolysis was followed for 24 h. As seen in Fig. 1, the oligosaccharide concentration reached its highest value after 4 h while the concentration of lactose was still relatively high. The galactose concentration was lower than the glucose concentration. The difference was greater than expected from the amount of oligosaccharides, indicating that disaccharides with two galactose molecules (galactobiose) were probably also formed.

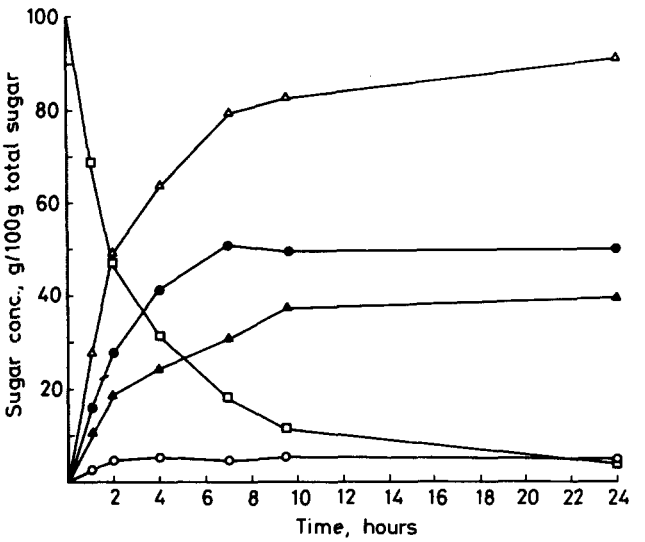

Fig. 1. Incubation with 5% lactose in 0·05M potassium phosphate buffer, pH 6·8. Forty milligrammes Maxilact® per litre. The incubation temperature was 24°C. (△——△: total monosaccharides, ●——●: glucose, ▲——▲: galactose, □——□; disaccharides, ○——○: oligosaccharides).

(2) *Lactose concentration* 10% (*w/v*): The hydrolysis was here followed for 14 days to see whether the oligosaccharides could be hydrolysed on prolonged incubation. On the fourteenth day the enzyme concentration was raised from 40 mg/litre to 400 mg/litre by the addition of fresh enzyme.

As compared with the hydrolysis at 5% lactose concentration the oligosaccharide formation was now larger. This is to be expected since the higher lactose concentration increases the probability of lactose becoming an acceptor of the galactose transferred by the enzyme. As shown in Fig. 2, the oligosaccharides were not hydrolysed by the enzyme on prolonged incubation. The disaccharide fraction also remained stable at about 10%. A large portion of fresh enzyme added on the fourteenth day, however, was able to hydrolyse the sugars completely during 24 h,

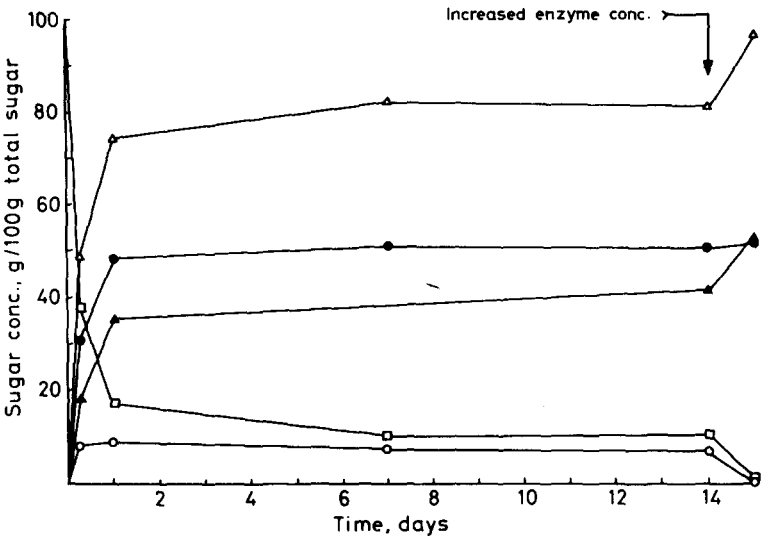

Fig. 2. Incubation with 10% lactose in 0·05M potassium phosphate buffer, pH 6·8. Forty milligrammes Maxilact[R] per litre. The incubation temperature was 24°C. At the fourteenth day the enzyme concentration was increased to 400 mg/litre. (△——△: total monosaccharides, ●——●:glucose, ▲——▲: galactose, □——□: disaccharides, ○——○: oligosaccharides).

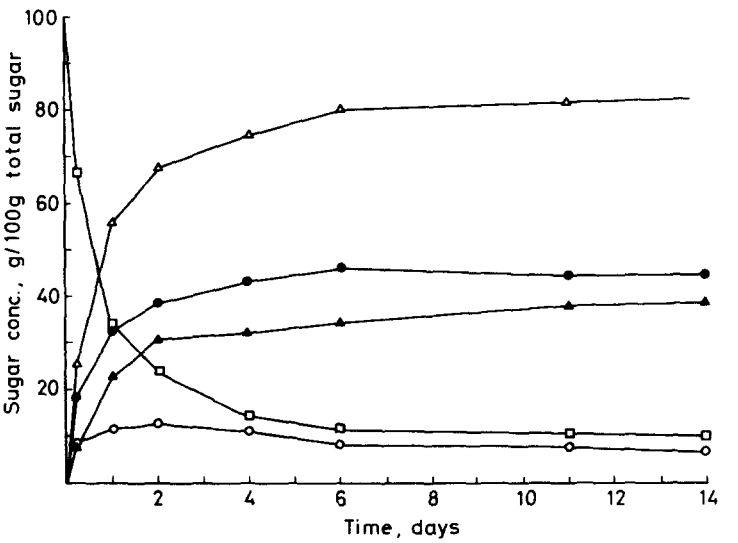

Fig. 3. Incubation with 20% lactose in 0·05M potassium phosphate buffer, pH 6·8. Forty milligrammes Maxilact[R] per litre. The incubation temperature was 24°C. (△——△: total monosaccharides, ●——●: glucose, ▲——▲: galactose, □——□: disaccharides, ○——○: oligosaccharides).

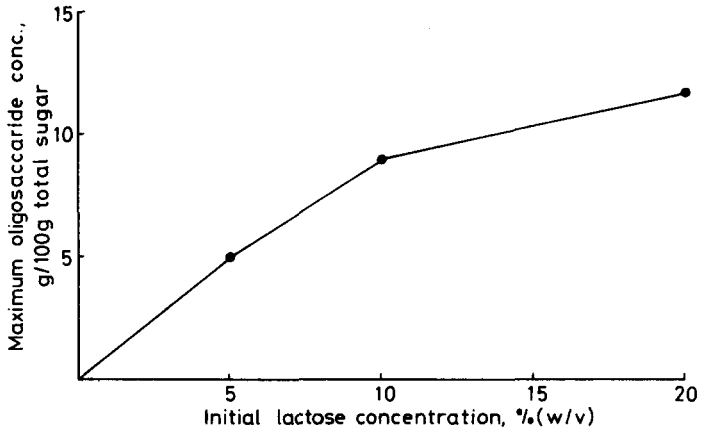

Fig. 4. Maximum oligosaccharide formation in relation to lactose concentration. The values are obtained from the maximum values of oligosaccharides in Figs. 1, 2 and 3. (△——△: total monosaccharides, ●——●: glucose, ▲——▲: galactose, □——□: disaccharides, ○——○: oligosaccharides).

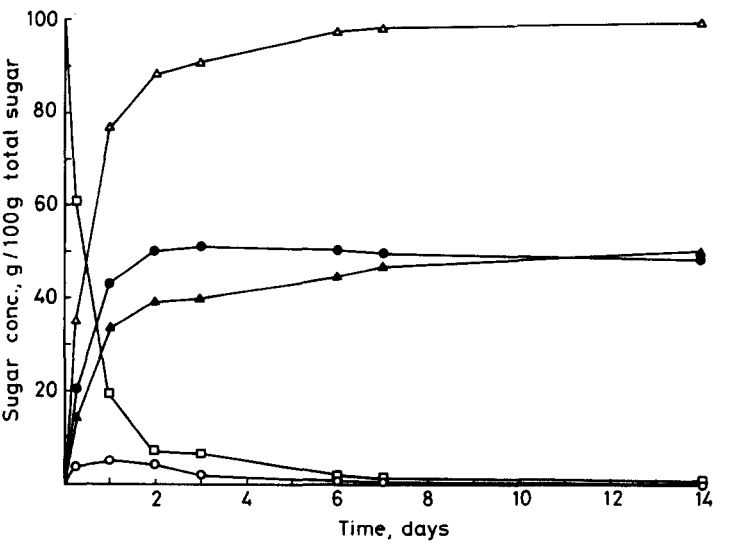

Fig. 5. Incubation in sterilised milk containing 5% lactose. Five millilitres of water containing 40 mg Maxilact[R] were sterilfiltered into one litre of milk. The incubation temperature was 24 °C. (△——△: total monosaccharides, ●——●: glucose, ▲——▲: galactose, □——□: disaccharides, ○——○: oligosaccharides).

indicating that the original amount of enzyme had been inactivated during the long incubation time. This experiment also showed that the oligosaccharides originally formed could be hydrolysed by the enzyme.

(3) *Lactose concentration* 20% (*w/v*): The oligosaccharide formation was even larger than in the 10% incubations. The oligosaccharide percentage reached its highest value after about two days when about 75% of the lactose had been hydrolysed (Fig. 3). After that there was a slight decrease in both oligosaccharides and disaccharides. Complete hydrolysis was not obtained, however, due to loss of enzyme activity.

(4) *Relationship between oligosaccharide formation and lactose concentration:* The oligosaccharide formation during hydrolysis did not increase linearly with the lactose concentration. As seen in Fig. 4, the maximum oligosaccharide formation was about 5% of the total sugars in the incubation with 5% lactose in the buffer, about 8·5% in a 10% lactose buffer and about 12·5% in a 20% lactose buffer.

The maximum amount of oligosaccharides formed was, in all cases, reached when about 65–76% of the sugar was present as monosaccharides.

Incubation with UHTST-sterilised milk

The hydrolysis of lactose in milk initially showed the same pattern as in buffer (Fig. 5). After 6–7 days, however, the oligosaccharides were almost completely hydrolysed and 98% of the sugar content occurred as free monosaccharides. Thus, an almost complete hydrolysis of both lactose and transglycosidation products was obtained.

DISCUSSION

The formation of oligosaccharides as by-products during the enzymic hydrolysis of lactose was more pronounced at high lactose concentrations, as would be expected. When 20% lactose solutions were incubated, up to 13% of the total sugars could be recovered as oligosaccharides. Apparently the loss of the lactase activity on prolonged incubation was the reason why the oligosaccharide content remained stable. After the addition of fresh enzyme the oligosaccharides were hydrolysed again.

Roberts & Pettinati (1957, 1958) determined the amounts of oligosaccharides formed during enzymic lactose hydrolysis as the difference between the total sugars and the sum of monosaccharides and lactose. At 5% lactose they obtained a maximum of 22% oligosaccharides. At 10% lactose they obtained 23% oligosaccharides and at 20% lactose 30% oligosaccharides. A maximum oligosaccharide production of 44·6% of the total sugars was achieved at a concentration of 35% lactose. Thus these authors reported considerably more oligosaccharide formation than found in our study. This could be due to the fact that

another enzyme, *Saccharomyces fragilis* lactase, was used. A considerable formation of disaccharides other than lactose, which were not analysed in our study, could also be of importance in explaining the higher figures obtained by the above-mentioned authors.

Wierzbicki & Kosikowski (1973*b*) obtained a low oligosaccharide formation (1 to 2 %) when using β-galactosidase from *Aspergillus niger* on acid whey containing 4 % lactose and with a pH of 4·5. These values are very low, in particular if compared with those of Roberts & Pettinati (1957, 1958). It appears that the choice of enzyme and medium, buffer, milk or whey, and perhaps the pH, are all important factors determining to what degree oligosaccharide formation will occur.

The hydrolysis of lactose in milk produced smaller amounts of oligosaccharides and these were virtually completely hydrolysed during prolonged incubation without any new enzyme additions. Apparently the lactase retained its activity for a much longer time in milk than in a lactose–buffer solution. It should be noted, however, that if the degree of hydrolysis in milk is brought only to 65–75 % (sum of free monosaccharides) at least 5 % of the total sugars will occur as oligosaccharides (2·5 g/litre milk).

Lactose hydrolysis in concentrated milk and whey would be advantageous from an industrial point of view, due to the smaller tanks required if the batch process is used. More oligosaccharide formation is, however, to be expected at higher concentrations, unless the incubation is carried out for a very long time. The oligosaccharides can cause intestinal discomfort mainly in the form of flatulence, as demonstrated elsewhere (Asp *et al.*, unpublished data).

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VIRUCIDAL ACTIVITY OF ORGANIC ACIDS

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(Received: 31 July, 1978)

ABSTRACT

The virucidal activity of many organic acids against the type species of some viral groups (Herpesvirus, Orthomyxovirus, *and* Rhabdovirus) *has been evaluated.*

The results thus obtained suggest that the activity of the different chemical compounds essentially depends on the functional groups present in the molecule.

Some hypotheses on the way in which organic acids can inhibit cellular infection caused by enveloped viruses are also suggested.

INTRODUCTION

We have, for a long time, carried out experiments in order to evaluate the virucidal activity of chemical compounds with known bacteriostatic and bactericidal activity. Our attention was mainly focused on those compounds which, because of their non-toxic activity at the current range of concentrations, are mainly used in the foodstuff industry for the cleaning and disinfection of premises, equipment, containers and tools. We have thus obtained indications about the activity of quaternary ammonium compounds, non-ionic, anionic and amphoteric surface active substances against type species of the viral groups most frequently involved in human and animal pathology (Poli *et al.* 1978*a*; Poli *et al.* 1978*b*).

Recently, we thought it interesting to study the virucidal activity of the organic acids which are ever more frequently used as additives for the preservation of foodstuffs, above all as antimicrobials, fungistatics, acidulants and antioxidants. The organic acids most currently used to this effect in food technology are benzoic

Fd. Chem. 0308-8146/79/0004-0251/$02·25

acid (Chichester & Tanner, 1975; Tressler & Joslyn, 1961; Vaughn *et al.*, 1969), sorbic acid (Chichester & Tanner, 1975; Gooding *et al.*, 1955; Grubb, 1957; Melnick *et al.*, 1956; Von Schelhorn, 1953), propionic acid (Chichester & Tanner, 1975; Molin, 1964), acetic acid (Chichester & Tanner, 1975; Desrosier, 1959; Garibaldi, 1968; Levin & Fellers, 1940), succinic acid (Bennet & Ewart, 1962; Feuge & Ward, 1960), fumaric acid (Feuge & Ward, 1958; Gardner, 1975; Meusel & Brunn, 1968), lactic acid (Gardner, 1975; Iveson *et al.*, 1954; Kuhrt & Swicklik, 1962), malic acid (Gardner, 1966; Gardner, 1975) and citric acid (Gardner, 1975; Gooding *et al.*, 1949; Hall, 1961; Miles Chemical Co.).

Moreover, as we had an ample choice of organic acids with different structural features (mono- and poly-carboxylic aliphatic or aromatic acids, with or without other polar functional groups), we could obtain from their different virucidal effects, empirical information on the relationships between chemical structure and activity.

MATERIALS AND METHODS

Organic acids

The chemical compounds tested, with their structures and acidic strength values (pK_a), are shown in Table 1. After a preliminary trial to detect the activity range of the various organic acids under test, the final experiment was carried out by mixing equal amounts of the viral suspensions and the acids in 0·1M aqueous solution, if soluble, or in saturated solution, if slightly soluble.

Viruses and cellular lines

After some preliminary trials carried out on five viral groups (*Herpesvirus*, *Orthomyxovirus*, *Enterovirus*, *Adenovirus* and *Rhabdovirus*) already used in previous experiments (Poli *et al.* 1978*a*; Poli *et al.*, 1978*b*), the final test was performed only on the type species of the viral groups susceptible to the organic acids (i.e. enveloped viruses)—*Herpesvirus*, *Orthomyxovirus* and *Rhabdovirus*.

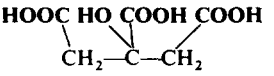
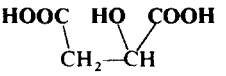
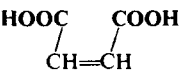
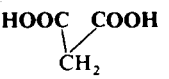
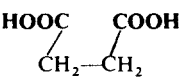
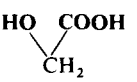
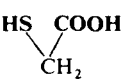
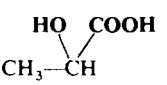
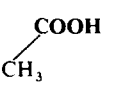
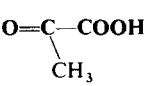
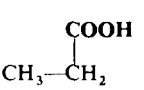
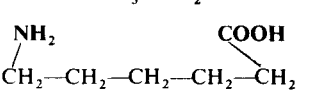
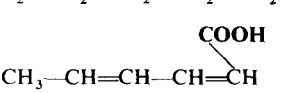
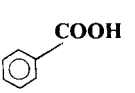
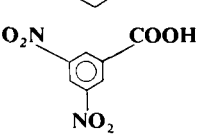
Herpesvirus: The HF-WR 260 ATCC strain of *Herpes simplex* virus type 1 was cultivated on monolayers of rabbit kidney cells (RK 13 cell line), grown in MEM (Minimum Essential Medium—EAGLE with EARLE salts) containing 10% of foetal bovine serum.

The procedure to prepare viral stocks is similar for all viruses: infected monolayers exhibiting over 75% cytopathic effect were quickly frozen and thawed three times to disrupt infected cells; the cell debris were removed by centrifugation at 3·300 *g* for 15 min and the supernatant, which represents the viral stock used in the definitive test, was assayed for virus infectivity, distributed in small aliquots and stored at −80°C until use.

Orthomyxovirus: The AWSN strain of influenza virus was cultivated in the

TABLE 1

CHEMICAL STRUCTURE AND VIRUCIDAL ACTIVITY OF ORGANIC ACIDS

Name	*Structure*[a]	pK_a[b]	*Virucidal activity*		
			Herpesvirus	Orthomyxovirus	Rhabdovirus
Citric		3·08	+ +	+ +	+ +
Malic		3·40	+ +	+ +	+ +
Fumaric[c]		3·02	+ +	+ +	+ +
Malonic		2·80	+ +	+ +	+ +
Succinic		4·17	+ +	+ +	+ +
Glycolic		3·83	+ +	+ +	+ +
Thioglycolic		3·55	+	+	+ +
Lactic		3·87	+	+ +	+ +
Acetic		4·76	+	+ +	−
Pyruvic		2·48	+ +	+ +	+ +
Propionic		4·48	+	+	−
ε-Amino caproic		4·43	−	−	−
Sorbic[c]		4·85	−	−	−
Benzoic[c]		4·19	±	±	−
3,5-Dinitro benzoic		2·82	−	−	−

[a] The functional groups are shown in bold type.

[b] For the polycarboxylic acids only pK_{a1} is reported.

[c] Used in saturated solutions.

allantoic cavity of SPF chick embryos of 10 days; eggs were incubated at 36 °C for 40 h after infection and all viable eggs were chilled in the refrigerator (4 °C) overnight before collection of allantoic fluid. After evaluation of viral titre by the haemoagglutination test (320 HAU/0·2 ml) the pooled allantoic fluid was distributed in small aliquots and stored at −80 °C until use. The final test was conducted by inoculating the virus on monolayers of baby hamster kidney cells (BHK 21 cell line), grown in MEM containing 10% of inactivated foetal bovine serum. The presence of the virus was detected three days after the infection by the haemadsorption reaction.

Rhabdovirus: The '1145/67' strain of vesicular stomatitis virus (VSV) was cultivated on monolayers of rabbit cornea cells (SIRC cell line), grown in ELH (Earle's Lactoalbumin Hydrolysate) containing 10% of foetal bovine serum.

Evaluation of virucidal activity

Experiments were carried out at room temperature; 1 ml of each organic acid was mixed from time to time with 1 ml of the virus stock suspension to be tested. After 10 min the mixture was rapidly diluted to stop the inherent toxicity of the organic acid to the cells (dilution to 10^{-8} in MEM containing 5% of inactivated foetal bovine serum). For control, 1 ml of every virus stock suspension was mixed with 1 ml of MEM or ELH with 5% of inactivated foetal bovine serum and dilutions were made after 10 min. Five wells of the microtitre plate, containing a confluent monolayer, were simultaneously inoculated, with a special multichannel pipette, with 50 μl of every dilution prepared.

After virus adsorption time (60 min at 37 °C) the inoculum was withdrawn from each well and 0·1 ml maintenance medium was added (MEM or ELH with 5% of inactivated foetal bovine serum). Thereafter the microtitre plates were placed at 37 °C in a CO_2 incubator. The cultures were observed daily through the ninth day for the appearance of CPE.

Instead, for evaluating the Influenza virus titre, the haemadsorption reaction was carried out on the cell monolayers three days after infection.

The virucidal activity of the organic acids is evaluated on the basis of titre reduction of the virus/disinfectant mixture (residual virus) compared with the control virus suspension. More specifically, a compound is considered (a) very active (+) when it inactivates more than 99·9% of the virus (i.e. a virus reduction of $\geq 10^3$ $TCID_{50}$); (b) poorly active (±) if the virus inactivation ranges from 99% to 99·9% (i.e. a virus reduction between two and three logarithms) and (c) inactive (−) if inactivation is less than 99% (i.e. a virus reduction of less than two logarithms). Finally (++) indicates the complete inactivation of the virus, i.e. a viral titre reduction up to $10^{0·5}$ $TCID_{50}$. (Because of the inherent toxicity of the organic acids tested it was impossible to detect the presence or absence of virus in the undiluted virus/disinfectant mixture; for this reason the highest titre fall is expressed as $\leq 10^{0·5}$ $TCID_{50}$.

RESULTS

The evaluation of the virucidal activity of the assayed organic acids against the type species of the three groups under test (*Herpesvirus*, *Orthomyxovirus*, *Rhabdovirus*) gave the results shown in Table 1. In particular it is possible to point out that:

(1) All the polycarboxylic acids (citric, malic, fumaric, malonic and succinic acids) exhibit a high virucidal activity (+ +) against the three viral groups tested.

(2) The monocarboxylic acids show a different virucidal activity depending on:
(a) the length of the aliphatic chain (a longer chain results in a reduced activity which becomes nil with the sorbic acid while slightly decreasing from glycolic to lactic acid, only in the case of *Herpesviruses* and from acetic to propionic acid only in the case of *Orthomyxoviruses*;
(b) the other functional groups in the molecule: the hydroxylic group (OH), as well as the carbonylic group (CO), but to a lesser extent the thiol group (SH), are responsible for the marked activity of the chemical compounds. The benzene ring also has a reducing effect on the activity of the acids but to a lesser extent than a long chain; on the other hand, the activity is inhibited in the presence of nitro groups (NO_2) in the benzene ring.

DISCUSSION AND CONCLUSIONS

The preliminary tests on the type species of the five viral groups have revealed that, in the concentrations used in the conclusive experiment, organic acids cannot inhibit the so-called 'naked' viruses because of the absence of the envelope (*Entervirus*, *Adenovirus*) whereas they are very active on the viruses provided with the envelope (*Herpesvirus*, *Orthomyxovirus*, *Rhabdovirus*).

For this reason the research was limited to the last viral groups only, but also extended to those organic acids which, although not used in food technology, are carriers of different functional groups so that they could allow a better interpretation of the possible structure/activity relationship.

When analysing the results reported in Table 1, derived from the final experiment (i.e. against the enveloped viruses only) a conclusion can be drawn that the virucidal activity is proportional to the molecular polarity. In fact, the acids containing only one COOH group are less active than those containing an additional COOH group and/or OH groups (hydrogen bond donors); the SH group, which is not a hydrogen bond donor, does not show a decisive influence on the virucidal activity (thioglycolic acid is much more active than acetic acid in the case of the *Rhabdovirus*

while the latter is slightly more active in the case of *Orthomyxovirus*). On the other hand, the nitro groups (NO_2) on the benzene ring of 3,5-dinitrobenzoic acid, when compared with benzoic acid, account for the slightly lower virucidal activity, probably because its water solubility is further reduced.

The preliminary test results and the above observations lead to some hypotheses about the mechanisms by which organic acids inhibit cellular infections caused by the viruses tested. First of all the envelope, whose integrity is indispensable to accomplishing the first two phases of the cellular infection, is likely to be the first viral structure attacked by the organic acids. (Although investigations have been carried out mainly on *Orthomyxovirus* and *Togavirus* envelopes, it seems certain that most of the information obtained generally applies to all other viral envelopes (Bächi *et al.*, 1969; Blough & Tiffany, 1975; Compans & Dimmock, 1969; Klenk, 1974; Klenk *et al.*, 1972; Landsberger *et al.*, 1973; Landsberger *et al.*, 1971; Rifkin *et al.*, 1972; Sefton & Burge, 1973; Stanley & Haslam, 1971; Untermann & Simon, 1974): the envelopes are morphologically represented by the typical structure of the cellular membrane and chemically composed by proteins, lipids and glycoproteins (Blough & Tiffany, 1975; Klenk, 1974); the latter are the constituents of the typical surface projections (spikes) which expose a hydrophilic end, carbohydrate-rich, and are anchored by a hydrophobic segment in the lipid layer (Rifkin *et al.*, 1972; Sefton & Burge, 1973; Stanley & Haslam, 1971; Untermann & Simon, 1974).)

Furthermore, from the structure/activity relationship observed it is clear that the interaction of the organic acids is related to the formation of new ion–dipole, dipole–dipole and hydrogen bonds which replace the original bonds. That is why we assume that the glycoprotein structures of the envelope are the ones attacked by organic acids: the steric disposition of the spikes is altered by the breakage of the weak bonds. It follows that any slight variation in the steric disposition of the glycoprotein chains makes the specific adsorption of the viral particle to the cellular membrane ineffective.

Examining the differences in the behaviour of some of the organic acids towards the three viruses tested, it is possible to infer, based on the above-mentioned hypothesis, that the glycidic portion of the spikes is the most involved; in fact the *Herpesvirus* and the *Rhabdovirus*, respectively the poorer (1·5%) and the richer (13%) in this component (Fenner *et al.*, 1974; Knudson, 1973; Roizman & Spear, 1971) are not very susceptible to the more polar acids (lactic and thioglycolic acid) and to the less polar acids (acetic, propionic and benzoic acids) respectively.

The results of this experiment confirm that the infectivity of the viruses provided with an envelope (*Herpesvirus*, *Orthomyxovirus* and *Rhabdovirus*), which have already proved to be more sensitive to chemical agents than the so-called 'naked' viruses, can also be easily inhibited by the interaction of some chemical compounds, such as organic acids, which probably do not disrupt the whole membrane but only alter the steric disposition of the specific viral receptors.

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USE OF PEROXIDE VALUE AND CARBONYL VALUE TO DETERMINE THE ONSET OF RANCIDITY IN MAYONNAISE

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(Received: 1 August, 1978)

ABSTRACT

Mayonnaise stored at 20 °C showed a maximum peroxide value well before the onset of rancidity. It is suggested that changes in peroxide value could be used to determine the potential shelf life of mayonnaise. Carbonyl value could not be used as a predictive tool as it did not show any marked change until rancidity was present.

INTRODUCTION

Mayonnaise is essentially an oil/water emulsion prepared from vegetable oils, vinegar and egg yolk, with other minor ingredients added for flavouring. The oils used in mayonnaise normally contain a high proportion of unsaturated fatty acids which are highly susceptible to autoxidation. As a consequence, the development of oxidative rancidity is the most important cause of spoilage of mayonnaise during marketing (Weiss, 1970). Autoxidation is due to the addition of atmospheric oxygen to the unsaturated fatty acids and results in the formation of hydroperoxides which themselves possess little off-flavour. However, they are readily degraded to low molecular weight carbonyl compounds which are responsible for the production of off-flavours in fatty foods (Lea, 1962).

The levels of peroxides and volatile carbonyl compounds in fats and oils have been determined by numerous workers. They have found that peroxide value increases as oxidation commences but eventually the rate of hydroperoxide decomposition exceeds the rate of hydroperoxide formation so that the peroxide value attains a maximum and then declines. The carbonyl value begins to rise at a later stage but continues to rise during the onset of rancidity (Hoffman, 1962). While there is considerable data on these changes during the oxidation of fats and oils, very little

Fd. Chem. 0308-8146/79/0004-0259/$02·25

information is available on mayonnaise, particularly on the relation of these changes to organoleptic acceptance. Since mayonnaise is a formulated emulsion it could be expected to behave differently from simple fats and oils and hence in this paper we have determined the peroxide and carbonyl values during storage and have related these changes to the onset of rancidity.

MATERIALS AND METHODS

Mayonnaise freshly prepared for the retail market was obtained in sealed 500 g jars from a commercial manufacturer in Sydney, Australia. The oil used in the formulation was refined sunflower oil. The samples were stored at 20 °C and at various intervals two jars were opened and each was analysed for peroxide value and carbonyl value and assessed for organoleptic acceptance. Peroxide value was determined by iodometric addition and titration against sodium thiosulphate as described by Pearson (1970). The sample for the estimation of carbonyl value was obtained by a steam distillation of mayonnaise for 2 h and extraction of the aqueous extract with pentane. The carbonyl value was determined by a colorimetric assay of the 2,4-dinitrophenylhydrazones as described by Henick *et al.* (1954). The values were quantified by comparison with a standard curve prepared from hexanol. The onset of rancidity was determined by an experienced industrial panel of mayonnaise tasters who were presented on each occasion with a sample of fresh mayonnaise and the experimental sample and asked to comment on the presence of rancidity.

RESULTS AND DISCUSSION

The data in Fig. 1 show that the peroxide value attained a maximum after about 15 days and then declined to a relatively low value. The level of volatile carbonyls increased as the mayonnaise aged but remained low until about 30 days when the carbonyl value markedly increased. No taste panel member detected any rancidity until 25 days but all tasters detected rancidity at 30 days. Since the panel was very experienced with mayonnaise tasting, they would have detected the presence of rancid flavours before most consumers. It would therefore seem that a maximum storage life of 30 days at 20 °C could be ascribed to the mayonnaise used in this study.

Since the maximum peroxide value occurred before the onset of rancidity, measurement of peroxide value could possibly be used as an index of the potential storage life in non-rancid mayonnaise samples. A rising peroxide value would indicate that the mayonnaise still had a considerable potential shelf life while a decreasing peroxide value would indicate that rancidity would occur within a short period. Carbonyl value would appear to be less useful in predicting the onset of

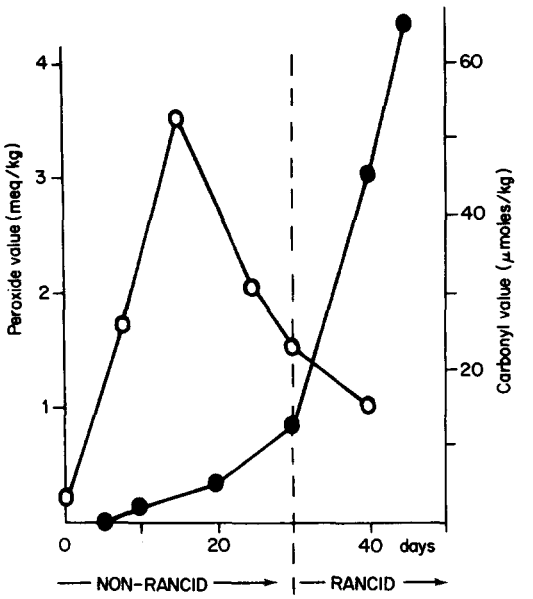

Fig. 1. Changes in peroxide value (○) and carbonyl value (●) in mayonnaise during storage at 20 °C in relation to the development of rancidity.

rancidity as readings remained relatively low up to the actual onset. The carbonyl value could only be used to determine the degree of severity of rancidity present.

ACKNOWLEDGEMENT

We wish to thank Master Foods of Australia Pty Ltd for assistance and co-operation throughout the project.

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THE NUTRITIVE VALUE OF SOME NIGERIAN LEAFY GREEN VEGETABLES—PART 1: VITAMIN AND MINERAL CONTENTS

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(Received: 10 August, 1978)

ABSTRACT

Ten leafy green vegetables commonly eaten by peasant Nigerians have been analysed for their vitamin and mineral contents. The range of thiamine, riboflavin, pyridoxine and ascorbic acid contents was as follows (in mg per 100 g DM): 0·06–0·27, 0·46–2·07, 1·08–1·95 *and* 20·6–160, *respectively. The range of values of mineral elements assayed were sodium:* 0·034%–0·28%, *potassium:* 1·43%–6·10% *calcium:* 1·08%–3·62%, *magnesium:* 0·45%–2·22%, *manganese:* 0·015%–0·115%, *iron:* 0·03%–0·059%, *copper:* 0·001%–0·0025%, *zinc:* 0·0006%–0·0135%, *sulphur:* 0·23%–0·59%, *phosphorus:* 0·21%–0·71% *and chlorine:* 0·14%–0·67% *on a dry matter basis. The implication of these results for the rural population is discussed.*

INTRODUCTION

Leafy green vegetables form a substantial proportion of the diets of most peasant Nigerians. Fafunso & Bassir (1975) reported that an average Nigerian consumes about 65 g of fresh vegetables daily.

Previous studies on some edible Nigerian leafy green vegetables have shown that most contain appreciable amounts of certain minerals and vitamins (Oke, 1964, 1966; Oyenuga, 1968; Munro & Bassir, 1969; Oyejola & Bassir, 1975). However, these studies were incomplete as they supplied information on a limited number of nutrients in a comparatively few varieties of edible leafy green vegetable.

For the studies reported here, we selected some of those varieties of leafy green vegetable that are commonly eaten by the peasant population of the Cross River State of Nigeria.

Fd. Chem. 0308-8146/79/0004-0263/$02·25

EXPERIMENTAL

The leafy green vegetables

Ten varieties of edible leafy green vegetable were selected and grown on the Departmental Experimental Farm without fertiliser treatments. The edible portions harvested (usually in the morning) from four-week old plants were quickly transported in plastic bags to the laboratory for processing.

Processing of the edible leaves

The edible portions of the leaves were carefully washed with tap water and allowed to drain dry at room temperature. Care was taken to avoid physical damage to the leaves and prevent loss of nutrients by leaching. The clean leaves were then dried in an oven pre-set at 60 °C (Oyenuga, 1968) for 12 h. The dried vegetables were pulverised in a steel-bladed small sample laboratory grinding mill. The powder was packed in air-tight polythene bags and stored in deep freeze.

Vitamin assay

The vitamins assayed included thiamine, riboflavin, pyridoxine and ascorbic acid. Thiamin and riboflavin were extracted from the vegetable powders and assayed by the methods of the Association of Vitamin Chemists (1966). The fluorescence of thiochrome when excited at 365 nm and measured at 435 nm (Joslyn, 1970) formed the basis of thiamine estimation. The characteristic fluorescence of riboflavin at 563 nm when excited at 475 nm (Michelson & Yamamoto, 1958) was used in its assay. In both cases a Perkin-Elmer fluorescence spectrophotometer (model 204) was used. Pyridoxine was estimated colorimetrically by the method of Bina *et al.* (1943) on an SP 600 spectrophotometer. Ascorbic acid was extracted by the method of Freebarn (1959) and estimated volumetrically using the 2,6-dichlorophenol indophenol dye method of the Association of Vitamin Chemists (1966).

Mineral analysis

All the mineral elements (except phosphorus, sulphur and chlorine) were analysed by means of an atomic absorption spectrophotometer (Perkin-Elmer model 403). The method of Walsh (1971) was used in all cases. Phosphorus was estimated in the wet digests of the vegetable powders as the phosphomolybdate complex on the SP 600 spectrophotometer by the method of Yuen & Pollard (1955). Sulphur was estimated gravimetrically by the dry ashing–barium sulphate method of Chapman & Pratt (1961). Chlorine was estimated volumetrically by the method of Humphries (1956).

RESULTS AND DISCUSSION

The results of the vitamin assay (Table 1) show that the ascorbic acid contents of the vegetables varied widely between 20·6 and 160·2 mg/100 g DM. Most of these

TABLE 1
VITAMIN CONTENTS OF THE VEGETABLES

Local name	*Botanical name*	*mg per 100 g dry matter*			
		Thiamin	*Riboflavin*	*Pyridoxine*	*Ascorbic acid*
Ikong etighi	*Abelmosobus esculentus*	0·060 ± 0·002	1·04 ± 0·06	1·89 ± 0·08	46·95 ± 1·66
Etinyong	*Corchorus olotorius*	0·11	0·81 ± 0·02	1·48 ± 0·07	62·10 ± 4·78
Mmongmmogi-kong	*Talimun triangulare*	0·10 ± 0·01	0·93 ± 0·01	1·93 ± 0·09	29·37 ± 2·04
Inyang afia	*Amaranthus hybridus*	0·130 ± 0·002	1·05 ± 0·05	1·08 ± 0·09	52·46 ± 1·19
Etinkene	*Piper guineense*	0·110 ± 0·003	0·46 ± 0·03	1·95 ± 0·05	27·01 ± 1·76
Ntong	*Ocimum basillicum*	0·160 ± 0·002	0·62 ± 0·01	1·86 ± 0·08	20·58 ± 2·12
Ikong nnangi	*Cucurbita pepo*	0·150 ± 0·001	1·40 ± 0·07	1·57 ± 0·07	78·98 ± 1·77
Etidot	*Vernonia amygdalina*	0·170 ± 0·007	0·94 ± 0·03	1·96 ± 0·03	20·72 ± 0·92
Ikong ubong	*Telfairia occidentalis*	0·080 ± 0·002	2·07 ± 0·12	1·55 ± 0·08	160·17 ± 6·42
Utasi	*Marsdenia latifolia*	0·270 ± 0·008	1·56 ± 0·04	1·45 ± 0·05	99·10 ± 3·49

Mean of three determinations ± standard deviation.

vegetables are important sources of dietary ascorbic acid. The nutritional importance of ascorbic acid has assumed greater dimensions since the recognition of its role in the therapy of atherosclerosis (Ginter *et al.*, 1969; Milenkov & Mitkov, 1969), respiratory diseases (Williams & Deason, 1967; Preshaw, 1972 and Cheraskin *et al.*, 1973) and prickly heat (Hindson, 1970). The thiamine contents of the vegetables were generally low, varying between 0·06 and 0·27 mg (per 100 g DM). The riboflavin and pyridoxine contents are comparatively higher in most of the vegetables, varying between 0·46 and 2·07 mg per 100 g DM. These findings are important when considered in the light of a recent survey (Ekpo, 1970) which confirmed the prevalence of symptoms of vitamin B deficiency in parts of the Cross River State of Nigeria. Although the studies of Umoh & Bassir (1977) have related the low consumption of these vitamins to losses encountered during traditional cooking methods, greater consumption of those varieties of the vegetables shown to be rich in these vitamins could nevertheless alleviate the vitamin deficiency symptoms in the affected areas.

However, it might be necessary to augment dietary supplies of these vitamins, especially thiamin, by food fortification. Our studies have shown that most of the vegetables are rich in calcium and iron (Table 2) with values ranging between 1·08 and 3·62 % and 0·03 % and 0·059 % (on a dry matter basis), respectively. The value of these vegetables as dietary sources of these elements could be adversely affected by high contents of phytates, oxalates and cyanogenic glycosides reported in some Nigerian vegetables (Oke, 1966 and Munro & Bassir, 1969). However, it has been observed by Oke (1966) and Bassir (1969) that traditional preparatory methods rid the vegetables of most of the oxalate and cyanogenic glycosides. It is important to note that the value of the vegetables as a source of dietary iron is subject to the bio-availability of their iron contents. Bio-availability studies on the iron in these vegetables have been carried out (Ifon, 1977) and will form the subject of a subsequent paper.

TABLE 2
MINERAL CONTENTS OF THE VEGETABLES

Vegetables	% *Dry weight*										
	Na	*K*	*Ca*	*Mg*	*Mn*	*Fe*	*Cu*	*Zn*	*S*	*P*	*Cl*
Abelmoschus esculentus	0·092 ± 0·002	1·86 ± 0·02	3·62 ± 0·13	1·14 ± 0·01	0·025 ± 0·005	0·047 ± 0·002	0·0013	0·0115 ± 0·002	0·59 ± 0·14	0·37 ± 0·03	0·440 ± 0·006
Corchorus olitorius	0·034 ± 0·002	3·83 ± 0·08	1·26 ± 0·01	0·59 ± 0·10	0·016 ± 0·001	0·042 ± 0·001	0·0014 ± 0·0001	0·0078 ± 0·0002	0·30 ± 0·04	0·71 ± 0·03	0·24 ± 0·01
Talinum triangulare	0·28 ± 0·08	6·10 ± 0·20	2·40 ± 0·01	2·22 ± 0·02	0·035 ± 0·005	0·041	0·0010 ± 0·0001	0·0093 ± 0·0008	0·23 ± 0·02	0·34	0·510 ± 0·003
Amaranthus hybridus	0·068 ± 0·003	4·29 ± 0·09	2·78 ± 0·03	1·45 ± 0·01	0·029 ± 0·001	0·050 ± 0·002	0·0011	0·0135 ± 0·0020	0·27 ± 0·01	0·67 ± 0·01	0·170 ± 0·003
Piper guineense	0·074 ± 0·002	3·90 ± 0·13	2·08 ± 0·02	1·01 ± 0·01	0·025	0·045 ± 0·001	0·0019 ± 0·0005	0·0094 ± 0·0001	0·30 ± 0·03	0·21 ± 0·01	0·670 ± 0·001
Ocimum basillicum	0·045 ± 0·001	3·90 ± 0·13	2·48 ± 0·03	0·53 ± 0·03	0·015 ± 0·001	0·054 ± 0·001	0·0025 ± 0·0010	0·0060 ± 0·0001	0·23 ± 0·02	0·44 ± 0·02	0·350 ± 0·005
Cucurbita pepo	0·057 ± 0·002	3·33 ± 0·08	2·82 ± 0·07	0·86 ± 0·02	0·036 ± 0·001	0·059 ± 0·001	0·0012 ± 0·0001	0·0115 ± 0·0020	0·49 ± 0·01	0·71 ± 0·01	0·340 ± 0·016
Vernonia amygdalina	0·034 ± 0·002	3·75	1·08 ± 0·02	0·45 ± 0·01	0·016 ± 0·001	0·030 ± 0·002	0·0023 ± 0·0003	0·0079 ± 0·0001	0·31 ± 0·03	0·41 ± 0·02	0·560 ± 0·016
Telfairia occidentalis	0·048 ± 0·003	2·45 ± 0·05	1·71 ± 0·05	0·65 ± 0·02	0·025 ± 0·001	0·034 ± 0·001	0·0014 ± 0·0001	0·0065 ± 0·0005	0·40 ± 0·01	0·61 ± 0·01	0·180 ± 0·005
Marsdenia latifolia	0·039 ± 0·001	1·43 ± 0·08	2·29 ± 0·09	0·98 ± 0·03	0·115 ± 0·005	0·03	0·0015	0·009 ± 0·001	0·36 ± 0·06	0·24 ± 0·01	0·140 ± 0·007

Mean of two determinations ± standard error.

The low sodium contents in the vegetables, ranging between 0·034 and 0·28 % (on a dry matter basis) suggest the possibility of incorporating these vegetables into the diets of obese patients. The comparatively high content of sulphur in some of the leafy vegetables is important as sulphur has recently been implicated in the detoxication of cyanide in local foodstuffs (Oke, 1973).

The results of these studies have justified the wide consumption of these leafy green vegetables. Greater cultivation and consumption of these varieties of leafy green vegetable could be recommended as a measure for combating specific nutrient deficiencies among the peasant population in Nigeria.

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BROAD BEAN LIPOXYGENASE—PART 1: PARTIAL PURIFICATION AND CHARACTERISATION

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(Received: 15 August, 1978)

ABSTRACT

From locally grown large broad beans, active lipoxygenase preparations A, B and C were obtained by ammonium sulphate fractionation, Sephadex G-150 chromatography and Sephadex G-50 and Sephadex G-150 chromatography, respectively. All preparations were almost completely inhibited by 2×10^{-5}M *diethyldithiocarbamate. The inhibitors EDTA, 8-hydroxyquinoline 2,2-dipyridyl and 1:10 phenanthroline were found to inhibit preparations of different purities at various millimolar concentrations. Crude extracts and preparation C were found to be activated by calcium ions while preparation B was not. Further characterisation of preparation C showed that its optimum pH was about 6 and that it oxidised linoleic acid, methyllinoleate and trilinolein. The ester and triglyceride activity of the preparation could also be stimulated by calcium ions, such activations being greatest at pH 7. Amongst the soybean lipoxygenase isoenzymes so well characterised in the literature, isoenzyme 2 is activated by calcium ions, has an optimum pH near 6·5 and oxidises both linoleic acid and methyllinoleate. The preliminary results obtained, therefore, suggest that preparation C has isoenzyme 2 as an essential component.*

INTRODUCTION

Great emphasis has been placed in recent years on upgrading the use of legumes either alone or mixed with cereals to meet the growing shortage of good quality proteins, especially in the under-developed countries (Siegel & Fawcett, 1976). Broad bean, *Vicia faba* L. var. minor (peterm) Beck, is widely cultivated in Europe and Asia and has a great potential consumer appeal in the Middle East. It contains about 23% protein (FAO, 1970) which is of good nutritional quality. Its greater use

Fd. Chem. 0308-8146/79/0004-0269/$02·25

in the diet could be promoted by developing inexpensive, acceptable, nutritious and easily prepared food products containing it.

Lipoxygenase (linoleate: O_2 oxidoreductase, EC 1.13.1.13) catalyses the hydroperoxidation of poly-unsaturated fatty acids and esters containing a *cis*, *cis*-1,4-pentadiene system. Its presence may affect the storage and processing of food since the hydroperoxidation and/or subsequent decomposition of food products can affect taste, odour and colour (Smith & Circle, 1972; Grosch, 1972).

Broad beans are a good source of lipoxygenase (Beaux & Drapron, 1974; Al-Obaidy, 1975). It has been found that processed broad beans develop rancidity very soon after grinding (Eskin & Henderson, 1974).

While a lot of work has been done on the lipoxygenases from a variety of plant materials, especially soybeans, the enzyme from broad beans has received little attention. Beaux & Drapron (1974) isolated the enzyme from *Vicia faba* L. and studied some of its physico-chemical properties. Eskin & Henderson (1974) partially purified the enzyme from buffered crude extracts of acetone powder by pH 5·0 treatment, heat treatment and ammonium sulphate treatment. They provided evidence for the existence of two types of lipoxygenases in fava beans, neither of which was inhibited by cyanide. However, their two preparations showed markedly differing responses to calcium ions. Despite a long-held belief to the contrary, soybean lipoxygenase is claimed to contain a prosthetic group (Pistorius & Axelrod, 1974). The present investigation was undertaken in order to learn more about the nature of lipoxygenase enzymes in broad beans with a view to understanding their mechanism of action and role in the development of rancidity.

MATERIALS AND METHODS

All chemical determinations reported in this study were made at least in duplicate. All reagents employed were chemically pure. All solutions were made in deionised water.

Raw materials

Dried broad beans (*Vicia faba* L. var. Major) were purchased from the local market and stored in a cold room at 10 °C in cellophane bags until used. All experiments were carried out on the same batch of beans.

Preparation of crude water extracts

Ten grammes of dried broad bean powder (20 mesh) were extracted with 10 ml of deionised water for 20 min with continuous stirring by a magnetic stirrer at a moderate speed. The mixture was strained through cheese-cloth and centrifuged for 30 min at 5000 rpm. The supernatant was divided into small vials and kept frozen at −20 °C until used.

Sephadex gel chromatography

Sephadex G-50 and G-150 were employed in this study. The column dimensions used in all chromatographic separations were 2·5 × 4·0 cm. The procedure employed for the preparation of the gel and the packing of the column was exactly according to that given by Pharmacia Fine Chemicals Co. Further information regarding the separation techniques is included in the accompanying graphs.

Ion exchange chromatography

Active enzyme preparations from the gel filtration eluates were concentrated by ultrafiltration (50 ml cells, X M50 membrane and 4–5 kg/cm^2 pressure) and applied on ion exchangers.

The columns of the ion exchangers, DEAE-Cellulose DE-23, DEAE-Sephadex A-50 and CM-Sephadex C-50, were prepared according to the method described by Pharmacia Fine Chemicals Co. In the case of DEAE-Cellulose, the method proposed by Whitaker (1972) was also tried.

For elution from the ion exchange columns, a NaCl gradient was used (Arens *et al.*, 1973). With CM-Sephadex, the elution buffer was acetate, pH 4·8 (0·05M) while with DEAE-Sephadex and DEAE-Cellulose, phosphate buffer pH 7·0 (0·01M) was employed.

Protein determination

Protein in samples was determined by measuring the absorbances at 280 nm and 260 nm and applying the following formula (Whitaker, 1972):

$$\text{Protein concentration (mg/ml)} = 1{\cdot}55\ A_{280} - 0{\cdot}76\ A_{260}$$

Measurement of enzyme activity

Two methods were employed for the determination of enzymic activity:

(a) A modified form of the Surrey (1964) method. The modification involved the initiation of reaction in a spectrophotometric cell containing Tween-20 solubilised substrate and noting the increase in absorbance at 234 nm with time. Reaction rates were calculated from the slope of the straight line produced by plotting the above data (Al-Obaidy, 1975). A unit of enzyme is defined as that amount of enzyme which produces a change in absorbance of 0·001 per second at 234 nm under the assay conditions. Linoleic acid, methyl linoleate and trilinolein were highly purified chemicals obtained from Bast of Copenhagen. A Beckman Model DB-Spectrophotometer was used in all measurements of absorbance.

(b) Modified thiocyanate method (Koch *et al.*, 1958). The modification involved the reduction in volume of the reactants used to effect the oxidation proper. To 0·5 ml of borate buffer adjusted to the desired pH was added 0·2 ml of the substrate and 10 ml of deionised water. The reaction

mixture was agitated by a magnetic stirrer and 0·2–0·5 ml of the enzyme was added at zero time. Two millilitre samples were withdrawn after four minutes and analysed in the usual manner. Proper controls were maintained.

Method of inhibition

Stock solutions of the inhibitors were prepared and a 0·1 ml aliquot added to 3 ml linoleate substrate in the spectrophotometric cell followed by the addition of 0·1 ml of the properly diluted enzyme. The activity was determined by the modified Surrey method. Proper controls were maintained.

Method of activation

Calculated amounts of calcium chloride were dissolved in deionised water and a measured volume pipetted into the reaction mixture before the addition of enzyme to make the final desired concentration of the activator. The enzyme was then added and the activity determined by the modified thiocyanate method. Proper controls were maintained.

RESULTS AND DISCUSSION

Purification studies

In order to eliminate interfering substances and to obtain information about the possible occurrence of lipoxygenase isoenzymes in broad beans, purification of lipoxygenase was tried using different techniques.

An ammonium sulphate fractionated enzyme was prepared essentially according to the method of Al-Obaidy (1975). It consisted of a 1:10 extraction of the 20 mesh broad bean powder with acetate buffer, pH 5·6 (0·05M), removal of inactive materials with barium acetate and acetone and collection of the precipitate obtained with ammonium sulphate between 10–40 % saturation. This was dissolved in acetate buffer, pH 5·6 (0·05M) and further fractionated with ammonium sulphate. The precipitate obtained between 15 and 30 % saturation was dissolved in tris buffer, pH 7·2 (0·05M) and designated enzyme preparation A. This was further purified by gel filtration on Sephadex G-150 resulting in one major peak with three shoulders and one minor peak. Enzyme activity was found to appear in one sharp peak (Fig. 1). The active fractions were pooled, frozen and stored in vials at −20 °C until used and designated enzyme preparation B. The specific activity determinations revealed that enzymes A and B showed five- and eleven-fold purification, respectively over that of acetate buffer extracts.

Enzyme preparation C was made from 1:10 water extracts of the broad bean powder and passage through Sephadex G-50. Two protein peaks were observed, the

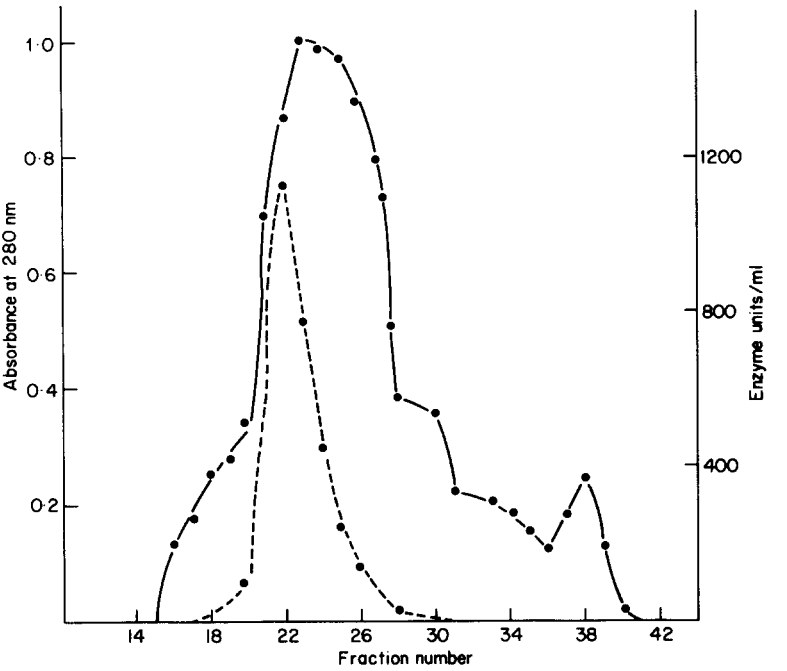

Fig. 1. Elution pattern of enzyme preparation A on Sephadex G-150 column (2·5 × 40 cm). ●—●, protein concentration; ●---●, enzyme concentration. Elution buffer was tris-HCl, pH 7·2 (0·05M), sample 88 mg protein, head pressure 30 cm and the flow rate 4·5 ml/5 min. The volume of each fraction was 4·5 ml.

enzyme peak appearing after the first protein peak (Fig. 2). The active fractions were pooled, concentrated by ultrafiltration and fractionated further on Sephadex G-150. This gave one major fast running protein peak followed by three minor peaks. Lipoxygenase activity was found in the fractions following the major protein peak (Fig. 3) and before the minor protein fractions. The active fractions were pooled, frozen in small vials, stored at −20 °C until used and designated enzyme preparation C. The enzyme showed an eight-fold purification over that of a water extract by specific activity determinations.

Further purification of the enzymes B and C was attempted using ion exchange resins—CM Sephadex, DEAE-Sephadex and DEAE-Cellulose. The elution was performed with different concentrations of linear NaCl gradients between 0·1 and 1M in the same buffer as used for equilibration. No elution of the enzyme could be effected from any of the above columns. It appears that the broad bean proteins are strongly adsorbed by the ion exchangers under the conditions of the above trials. Further work utilising different types of buffer, their respective ionic concentrations and pH values appears necessary to effect elution.

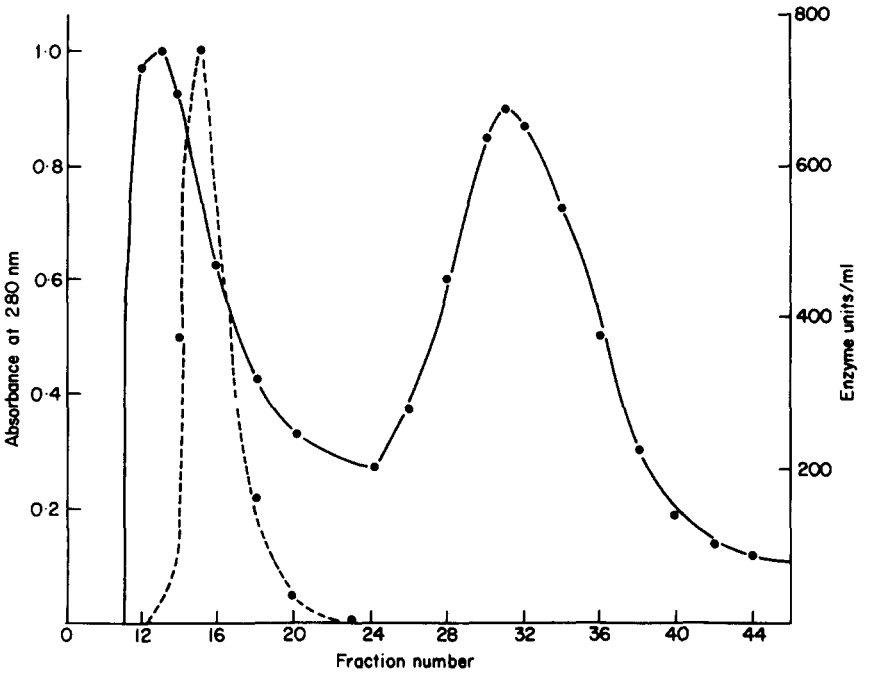

Fig. 2. Elution pattern of crude extract on Sephadex G-50 column (2·5 × 40 cm). ●—●, protein concentration; ●---●, enzyme concentration. Elution buffer was phosphate, pH 7 (0·05M), sample 471 mg protein, head pressure 50 cm and the flow rate 5 ml/5 min. The volume of each fraction was 5 ml.

Partial characterisation studies

(1) *Inhibition by metal binding agents:* Review of the literature shows that lipoxygenases from various sources and different preparations from the same source differ markedly in their inhibition by metal binding agents. The majority of preparations are not inhibited at all. Since metals such as calcium and iron have recently been shown to be involved in the catalysis of a few lipoxygenases, it became necessary to test the inhibition characteristics of the different lipoxygenase preparations.

In the preliminary experiment of this series, the inhibition of enzyme preparations A, B and C by $0{\cdot}02 \times 10^{-5}$M diethyldithiocarbamate (DEDTC) was investigated. This inhibitor was chosen since it had already been found to produce the greatest inhibition of the broad bean enzyme by Al-Obaidy (1975). All preparations were found to be almost completely inhibited.

To exclude the possibility of any non-specific inhibition, the effect of several other well known metal complexing agents was studied using enzyme preparation B at various stages of purification. Table 1 gives the results obtained. It can be observed that all the inhibitors tested produced inhibitions at low enough concentrations to be

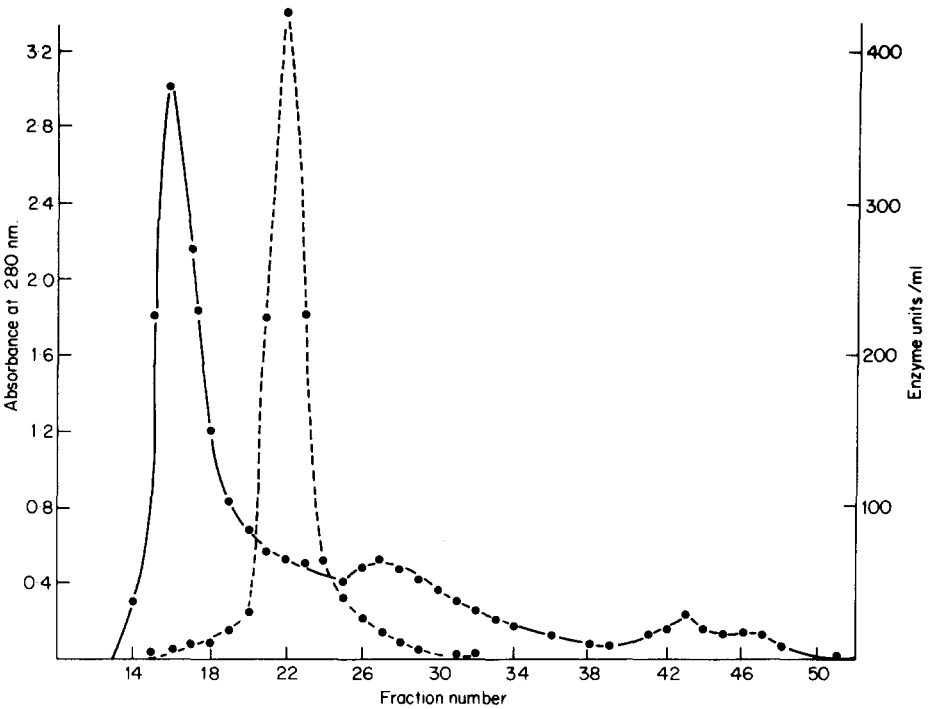

Fig. 3. Elution pattern of active G-50 eluates on Sephadex G-150 column (2·5 × 40 cm). ●—●, protein concentration; ●---●, enzyme concentration. Elution buffer was tris-HCl, pH 7·2 (0·05M), sample 50 mg protein, head pressure 30 cm and the flow rate 4·5 ml/5 min. The volume of each fraction was 4·5 ml.

significant. All enzyme preparations were inhibited and the greatest inhibition was observed in the most highly purified preparation. This gives further indication that the inhibitions observed resulted mainly from the interaction of the inhibitor tested with the enzyme and not from any impurities in the preparation.

It has been shown in the literature that the soybean (Holman & Bergstrom, 1951), pea (Siddiqi & Tappel, 1956*a*), alfalfa (Siddiqi & Tappel, 1956*b*), urd bean, mungbean, wheat and peanut (Siddiqi & Tappel, 1957), eggplant (Grossman *et al.*, 1972), potato tubers (Pinsky *et al.*, 1973), small fava bean (Eskin & Henderson, 1974) and barley (Lulai & Baker, 1976) lipoxygenases were not inhibited by metal binding inhibitors. Similarly, the lipoxygenase activity from rice bran was not affected by 2,2′-dipyridyl and EDTA but was partially affected by 1:10-phenanthroline (Shastry & Rao, 1975). However, Siddiqi (1962) and Al-Obaidy (1975) demonstrated that chick pea and broad bean lipoxygenases were inhibited by low concentrations of cyanide, azide and diethyldithiocarbamate. The acid optimum lipoxygenase isolated from peanut was inhibited by NaCN but no

TABLE 1
INHIBITION OF BROAD BEAN LIPOXYGENASE AT VARIOUS STAGES OF PURIFICATION BY METAL BINDING AGENTS

Enzyme preparation	*Specific activity*	*% Inhibition with*				
		$0{\cdot}02 \times 10^{-3}$M *DEDTC*	1×10^{-3}M *EDTA*	$0{\cdot}2 \times 10^{-3}$M *8-hydroxy-quinoline*	$0{\cdot}1 \times 10^{-3}$M *2,2-dipyridyl*	$0{\cdot}01 \times 10^{-3}$M *1:10-phe-nanthroline*
Crude broad bean acetate buffer extract	35	93	40	76	5·9	53
Enzyme preparation A	185	100	40	77	77	53
Enzyme preparation B	385	95	69	82	83	56

inhibition for alkaline optimum isoenzyme was observed (Sanders *et al.*, 1975). Flick *et al.* (1975) reported that the purple, green and white eggplant enzymes were completely inhibited by cyanide. Chan (1973) showed that soybean lipoxygenase was considerably inhibited by a variety of chelating agents such as diphenylthiocarbazone, 1:10-phenanthroline and 2,2′-dipyridyl. Roza & Francke (1973), however, failed to verify Chan's observations with the chelators, *O*-phenanthroline, EDTA, cyanide and 2,2′-dipyridyl. Similarly, Pistorius & Axelrod (1974) found that the chelating agents for Fe^{++} such as 2,2′-dipyridyl and *O*-phenanthroline did not inhibit soybean lipoxygenase activity.

The general pattern of preliminary results obtained and discussed above gives evidence for the possible participation of metals in broad bean lipoxygenase catalysis.

(2) *Effect of calcium ions:* Calcium ions have been found to have an activating effect on some lipoxygenases, an inhibiting effect on others whilst still other lipoxygenase preparations are not affected at all. Accordingly, experiments were planned to study the effect of Ca^{++} on crude broad bean water extracts and partially purified enzyme preparations B and C. Since Tween-20 has been found to affect calcium ion activation of soybean lipoxygenase in some studies (Zimmerman & Snyder, 1974), a modified thiocyanate method of assay was employed in the following studies.

Table 2 shows the results obtained with crude extracts. The enzyme possesses considerable activity even without the addition of calcium, suggesting the possible presence of bound calcium in the enzyme. Furthermore, the crude extract itself may contain some calcium. The addition of calcium to the reaction mixture is seen to have a definite activating effect, this effect being rapid until 0·6mM Ca^{++} concentration but diminishing thereafter. The above stimulating effect resembles the behaviour of crude soybean lipoxygenase (Restrepo *et al.*, 1973) wherein the preparations without calcium treatment possessed considerable activity, this activity being increased by about 70% with added calcium. Navybean crude

TABLE 2
EFFECT OF Ca^{++} CONCENTRATION ON THE ACTIVITY OF CRUDE BROAD BEAN LIPOXYGENASE

Ca^{++} concentration $\times 10^{-3}$M	*Absorbance at 480 nm*
0·00	0·32
0·20	0·46
0·40	0·61
0·60	0·72
0·80	0·78
1·00	0·83

extracts, on the other hand, possessed very little activity without added calcium. Addition of 0·49M Ca^{++} caused a several hundred-fold increase in activity (Koch *et al.*, 1971).

Results with the partially purified preparations are interesting since they throw light on the nature of these preparations. Figure 4 shows the responses of these preparations to increasing Ca^{++} concentrations. It will be noted that the two preparations differ markedly in their response. Preparation C is seen to be strongly activated by Ca^{++}, the increase in activity being rapid until 0·6mM and then slowing off. These results are in agreement with the recent findings of Zimmerman & Snyder

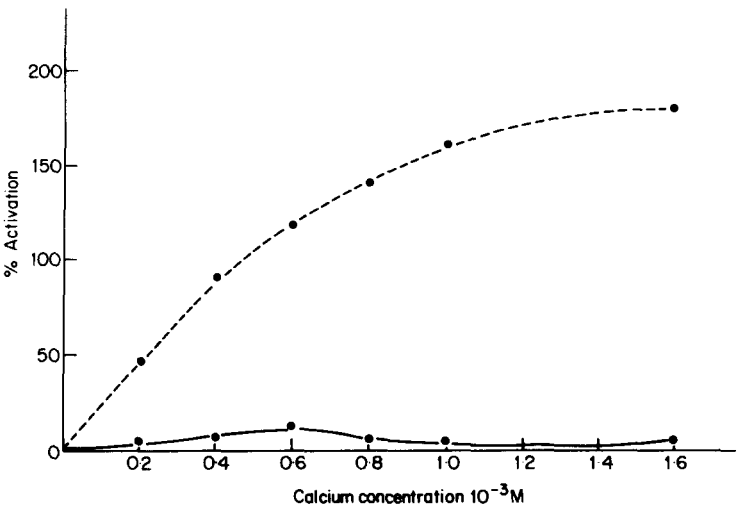

Fig. 4. Effect of calcium ion concentration on lipoxygenase activity of enzyme preparations B (●—●) and C (● --- ●). Enzyme activity was determined by the thiocyanate method and the reaction mixture consisted of 7·5–10 ml deionised water, 0·5 ml borate buffer, pH 7·0, 0·20 ml linoleate substrate, 0·0–2·5 ml calcium solution and 0·5 ml from the enzyme preparation.

(1974) who showed that purified soybean lipoxygenase preparation was activated by Ca^{++} over broad concentrations.

Results with preparation B (Fig. 4) are striking. Here no marked activation could be discerned over fairly broad Ca^{++} concentrations. The preparation may be similar to the isoenzymes from potato tubers (Pinsky *et al.*, 1973) or to the lipoxygenase preparation from soybeans (Yamamato *et al.*, 1970). The possibility that the preparation contains equal activities of two types of lipoxygenases, one being activated by Ca^{++} and the other being inhibited, could also not be ruled out. Recently, Eskin & Henderson (1974) were able to isolate these two activities from small fava beans by ammonium sulphate precipitations.

(3) *pH-activity profile of enzyme preparation C:* The Ca^{++} activated preparation C deserves special consideration in view of recent findings. Yamamato *et al.* (1970) isolated two lipoxygenases, a and b, from soybeans and found Ca^{++} to inhibit the former and activate the latter. Christopher *et al.* (1970) isolated two isoenzymes, designating them L_1 and L_2. Restrepo *et al.* (1973) showed that these were similar to the isoenzymes a and b of Yamamato with respect to their response to Ca^{++}. More recently, Zimmerman & Snyder (1974) separated lipoxygenase 2 from soybeans and found it to be activated by calcium ions. In short, the literature shows that the isoenzyme showing the phenomenon of calcium ion activation is the lipoxygenase 2 of Christopher *et al.* (1970) or the lipoxygenase b of Yamamato *et al.* (1970). Both these preparations have been interchangeably referred to in the literature and appear similar in properties.

On the basis of the above considerations, it was logical to assume that preparation C may contain lipoxygenase 2 as an essential component. To check this possibility further, the pH-activity profile of preparation C was determined. Figure 5 shows this profile. The enzyme shows an optimum pH value near 6. It is further observed that the enzyme preparation C, like lipoxygenase 2, is very sensitive to alkaline pH values and shows no activity at pH 9·0. The reported optimum pH values for pure isoenzymes 1 and 2 are 9·0 and 6·5, respectively. Earlier, Christopher *et al.* (1970) had differentiated isoenzyme 2 from isoenzyme 1 on the basis of elution profile from DEAE-Cellulose disc-gel electrophoresis, calcium ion activation, pH optimum and ester-acid preference.

(4) *Activity of enzyme preparation C towards esters and triglycerides:* Further evidence of the similarity of preparation C to the isoenzyme 2 came from the studies of the oxidation of methyl linoleate and trilinolein by preparation C. Table 3 gives the results obtained. It will be noticed that the preparation can oxidise both substrates although the ester oxidising activity appears much lower than the free acid oxidising activity. Such differences had been observed earlier in isoenzyme preparations from potato tubers (Pinsky *et al.*, 1973). Amongst the soybean lipoxygenase isoenzymes thoroughly studied, isoenzyme 1 is found to oxidise only linoleic acid and not methyl linoleate while isoenzyme 2 oxidises both the free acid and the ester (Christopher *et al.*, 1970; Zimmerman & Snyder, 1974). A comparative study of the free acid, ester

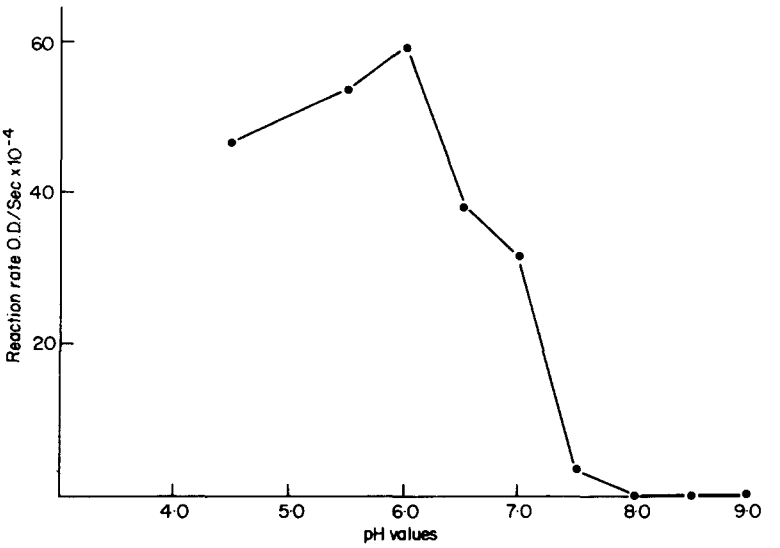

Fig. 5. pH-activity profile of preparation C. Enzyme activity was determined by Surrey's (1964) method.

and the triglyceride oxidising activity of isoenzyme 2 has not been reported in the literature. However, we find preparation C to be active on all three substrates and hence it should have an active 2-component.

(5) *Calcium ion activation of the ester and triglyceride oxidation by enzyme preparation C:* It is interesting to observe the activation of the oxidation of the ester and triglyceride by calcium ions (Table 3). Although activation was noted at all the pH values tested, that at pH 7·0 was the largest both with methyl linoleate and trilinolein as substrates. While no report exists in the literature regarding the effect of Ca^{++} on the ester and triglyceride oxidising activity of broad bean lipoxygenase, neither have such activations been observed with lipoxygenases from other sources (Koch *et al.*, 1971; Restrepo *et al.*, 1973; Zimmerman & Snyder, 1974). However, the lipid oxidising activity of legumes is quite diverse. With navybean extracts, Koch

TABLE 3
ACTIVITY OF ENZYME PREPARATION C ON METHYL LINOLEATE AND TRILINOLEIN

pH	*Absorbance at* 480 *nm*			
	Methyl linoleate		*Trilinolein*	
	No Ca^{++}	1×10^{-3}M Ca^{++}	*No* Ca^{++}	1×10^{-3}M Ca^{++}
5·5	0·037	0·097	0·082	0·117
7·0	0·031	0·175	0·047	0·087
8·0	0·020	0·097	0·031	0·047

(1968) could isolate three enzyme specificities—a lipoxygenase which is stimulated at pH 7·5 by calcium but not active on trilinolein, a lipoxygenase which, at pH 7·5, is not active on linoleic acid, is not stimulated by calcium but is active on trilinolein, and a lipoxygenase which is active on both linoleic acid and trilinolein but not stimulated by calcium ions. Using several crude preparations from soybean, Restrepo *et al.* (1973) found Ca^{++} activation of linoleic acid activity but not that of trilinolein. Using a purified isoenzyme 2 preparation from soybeans, Zimmerman & Snyder (1974) noted no stimulation of methyl linoleate activity by calcium although the free acid activity was found to be markedly stimulated. The broad bean enzyme studied is unique in that it oxidises linoleic acid, methyl linoleate and trilinolein and all three activities are markedly stimulated by calcium ions. The significance of these findings would be apparent when the role of calcium in the catalytic reaction is definitely established. Further work on these lines is in progress.

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THE ROLE OF NITRITE IN PREVENTING DEVELOPMENT OF WARMED-OVER FLAVOUR

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(Received: 16 August, 1978)

ABSTRACT

Development of warmed-over flavour (WOF) was followed in samples of beef, pork and chicken with and without added nitrite. Samples were evaluated by the 2-thiobarbituric acid (TBA) test and by sensory panel scores both before and after cooking at 0 days and again after storage for 48 h at 4°C. Added nitrite inhibited WOF development in cooked meat, resulting in a two fold reduction in TBA values for beef and chicken and a five fold reduction in pork. Sensory panel scores confirmed the protective effect of added nitrite in meat from all three species. Total lipid levels were not significantly related to WOF, but there was evidence for involvement of phospholipids.

INTRODUCTION

Tims & Watts (1958) first observed that cooked meat is rapidly oxidised at refrigeration temperatures, which is in marked contrast to the slow onset of rancidity commonly encountered in raw or frozen meat. The rapid onset of autoxidation occurring in cooked refrigerated meat has been called warmed-over flavour (WOF) as it is a serious flavour defect in cooked, refrigerated meat and becomes most apparent on rewarming the product (Tims & Watts, 1958; Sato & Hegarty, 1971). The problem has become even more important as precooked meats have assumed an increasingly larger proportion of the market, due to the rapid growth of fast food service facilities.

Zipser *et al.* (1964) first reported that TBA numbers are lower following storage of cooked cured pork than for uncured pork. Several investigators (Liu & Watts, 1970; Sato & Hegarty, 1971; Bailey & Swain, 1973) have reported that nitrite inhibits

Fd. Chem. 0308-8146/79/0004-0283/$02·25

development of WOF but have not systematically studied its role. Thus, the present investigation was undertaken to determine the role and function of nitrite in preventing development of WOF in cooked meat from beef, pork and chicken.

MATERIALS AND METHODS

Source of meat

Four 6 lb samples from each of three species (beef, pork and chicken) were removed from the carcasses at 24 h post mortem, then mixed and ground for 5 min in a Hobart chopper. The chicken consisted of breast and thigh meat from old hens, the pork was from *semitendinosus* and *biceps femoris* muscle from market weight (220 lb) hogs and the beef comprised flank steaks and hanging tender muscles from US Standard grade steer carcasses. All muscles were combined in the same proportion as they occur in the carcasses of the respective species.

Sample preparation

A total of 5 g of sugar and 12 g of NaCl were dissolved in 200 ml of deionised water and added to the sample which was then ground for 5 min. Following chopping, each sample was divided into two equal portions (3 lbs each). One portion was mixed with 75 ml of deionised water and chopped for an additional 5 min. This sample was used as the control without added nitrite. The remaining sample was mixed with sodium nitrite dissolved in 75 ml of deionised water to give a final concentration of 156 ppm of nitrite. Both the control and nitrite-treated samples were then divided into two portions, with one portion being analysed raw and the other being cooked.

Cooking

The samples to be cooked were packaged in Cry-O-Vac bags and sealed. After weighing, both the nitrite-treated and control samples were placed in a boiling water bath and heated to an internal temperature of 70 °C. Following cooling at room temperature for 20 min, the samples were reweighed. Cooking losses (drip) were then calculated by difference.

Samples of both the raw and cooked meat with and without added nitrite were analysed immediately by TBA numbers, taste panel evaluation and lipid analyses. The remaining raw and cooked samples, with and without added nitrite, were refrigerated at 4 °C for 48 h after which they were also analysed using the same tests.

TBA test

The distillation method of Tarladgis *et al.* (1960) was utilised for measuring the development of oxidative rancidity by the TBA test for all samples without nitrite. Several workers (Hougham & Watts, 1958; Younathan & Watts, 1959; Zipser & Watts, 1962) have shown that nitrite interferes with the distillation step by

nitrosation of malonaldehyde. Therefore, the modified TBA test of Zipser & Watts (1962) was used for all samples containing nitrite. The modified method adds sulphanilamide to bind the nitrite through diazonium salt formation and thus gives accurate TBA readings for samples containing nitrite (Zipser & Watts, 1962).

Taste panel evaluation

Sensory evaluation was carried out at 0 days and after 48 h storage at 4 °C by three trained panellists. All panellists were presented four different coded samples: raw without nitrite, cooked without nitrite, raw with nitrite and cooked with nitrite. All cooked samples were scored while hot, making it necessary to reheat the samples stored for 48 h at 4 °C for 20 min in Cry-O-Vac bags just prior to evaluation. Raw samples were scored only for aroma but cooked samples were scored after both smelling and tasting. The panel scoring system was as follows: 1 = very pronounced WOF, 2 = pronounced WOF, 3 = moderate WOF, 4 = slight WOF and 5 = no WOF.

Lipid analyses

Total lipids were extracted by the procedure of Folch *et al.* (1957) using the modification developed by Igene (1976). Separation of the neutral lipids and the phospholipids was achieved by the method described by Choudhury *et al.* (1960). The percentage of total lipids, neutral lipids and phospholipids in the raw and cooked samples was then calculated.

Statistical methods

Analysis of variance for TBA values and taste panel scores was calculated using a Michigan State University computer package program identified as MSU Stat System and was run on a Control Data Corporation (CDC) 6500 computer. Statistical significance of the difference between means was determined by the Least Significant Difference test as outlined by Snedecor & Cochran (1973). Correlation coefficients were calculated using a Texas Instruments' programmable calculator Model SR 52. Significance of the computed correlation coefficients was determined by the r distribution table from Snedecor & Cochran (1973).

RESULTS AND DISCUSSION

Changes in TBA values

Table 1 presents the mean squares for analysis of variance of TBA numbers. It reveals that there was a highly significant difference ($P < 0{\cdot}01$) in TBA values for the samples with and without added nitrite, which was true for all three species. In addition, TBA values for raw and cooked chicken differed significantly ($P < 0{\cdot}05$), but for pork and beef were significant at the 1 % level. Differences between TBA

TABLE 1
MEAN SQUARES FOR ANALYSIS OF VARIANCE OF TBA NUMBERS FROM CHICKEN, PORK AND BEEF

Source of variance	*df*	*Mean squares*		
		Chicken	*Pork*	*Beef*
Samples	3	5·43**	2·44	2·22**
Nitrite versus no nitrite	1	67·91**	40·92**	5·48**
Raw versus cooked	1	7·23*	16·71**	5·78**
Fresh versus stored	1	36·16**	35·84**	16·77**
Nitrite level × cooking	1	0·75	15·66**	1·04*
Nitrite level × storage	1	9·24**	15·08**	2·28**
Cooking × storage	1	2·57	14·62**	4·28**
Nitrite × cooking × storage	1	1·10	11·15**	0·87*
Residual error	21	0·89	0·89	0·15

* Significant at $P < 0·05$.
** Significant at $P < 0·01$.

numbers for fresh (0 days) and stored (48 h at 4 °C) samples were highly significant ($P < 0·01$) for all three species.

There were highly significant sample differences ($P < 0·01$) for both beef and chicken, while the TBA values for pork did not differ significantly between samples. Significant ($P < 0·05$) and highly significant ($P < 0·01$) interactions between the various variables and TBA values indicated that the variables did not consistently behave in the same manner (Table 1).

Table 2 shows the mean TBA numbers for the samples with and without added nitrite and gives mean differences with their significance. The data clearly

TABLE 2
TBA NUMBERS FOR CHICKEN, PORK AND BEEF WITH AND WITHOUT ADDED NITRITE

Treatment	*TBA numbers*		*Mean difference*
	Without nitrite	*With nitrite*	
Chicken samples			
Raw—0 day	2·52	1·36	1·16
Cooked —0 day	3·58	1·06	2·52**
Raw—48 h at 4 °C	5·52	1·47	4·05**
Cooked—48 h at 4 °C	6·98	3·05	3·93**
Pork samples			
Raw—0 day	1·52	0·85	0·67
Cooked—0 day	1·83	0·72	1·11
Raw—48 h at 4 °C	2·48	1·42	1·06
Cooked—48 h at 4 °C	7·85	1·64	6·21**
Beef samples			
Raw—0 day	0·92	0·66	0·26*
Cooked—0 day	1·07	0·75	0·32**
Raw—48 h at 4 °C	1·84	1·17	0·67**
Cooked—48 h at 4 °C	4·12	2·06	2·06**

* Significant at $P < 0·05$.
** Significant at $P < 0·01$.

demonstrate that the cooked samples from all three species were protected against autoxidation during storage for 48 h at 4 °C, with the TBA values for the nitrite-treated samples being two fold lower for beef and chicken and five fold lower for pork than those without nitrite ($P < 0.01$). Addition of nitrite also inhibited the development of rancidity in raw beef ($P < 0.01$) and raw chicken ($P < 0.01$) during storage at 4 °C for 48 h but had no significant influence on raw pork under the same conditions. Nitrite also protected against oxidative changes during cooking in both beef and chicken ($P < 0.01$) but did not significantly alter oxidative changes upon cooking pork. Added nitrite appeared to immediately (0 days) reduce autoxidation in raw beef but the differences between those raw samples with and without nitrite were not significant for either pork or chicken. Nevertheless, the trend was in the same direction and the differences may have been significant if a larger number of samples had been utilised.

The effect of nitrite in protecting against autoxidation—and thus in preventing development of WOF—is in agreement with the results of Sato & Hegarty (1971) and Bailey & Swain (1973). In addition, the present study indicates that oxidative changes also occur very rapidly in the raw meat, frequently being apparent at 0 days. Cooking was also shown to increase TBA values in the samples without nitrite, but it is probable that the oxidative processes occurring during cooking may contribute to desirable meat flavours and aromas (Herz & Chang, 1970).

Change in taste panel scores

The mean squares for analysis of the data on taste panel scores are summarised in Table 3. Nitrite significantly influenced taste panel scores for pork ($P < 0.01$) and beef ($P < 0.05$) but had no significant influence upon chicken. Storage of the samples at 4 °C for 48 h altered the taste panel scores for chicken ($P < 0.01$) but had no significant effect upon pork and beef. Cooking was found to affect the taste panel

TABLE 3
MEAN SQUARES FOR ANALYSIS OF VARIANCE OF TASTE PANEL SCORES FROM CHICKEN, PORK AND BEEF

Source of variance	*df*	*Mean squares*		
		Chicken	*Pork*	*Beef*
Samples	3	0·33	0·61	0·74
Nitrite versus no nitrite	1	0·29	1·53**	1·39*
Raw versus cooked	1	0·09	1·83*	10·89**
Fresh versus stored	1	5·80**	0·78	0·49
Nitrite level × cooking	1	4·72**	1·24*	2·33*
Nitrite × storage	1	0·16	0·41	0·01
Cooking × storage	1	0·16	0·27	0·12
Nitrite × cooking × storage	1	1·50	0·59	0·05
Residual error	21	0·68	0·27	0·31
Total	31			

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

scores for pork ($P < 0.05$) and beef ($P < 0.01$) but had no significant effect upon chicken. There was no significant difference between samples within a species for taste panel scores. The interactions were not significant except for nitrite level × cooking for all species.

Table 4 shows mean taste panel scores for samples with and without added nitrite for all three species and also presents mean differences with their significance. The

TABLE 4
TASTE PANEL SCORES FOR CHICKEN, PORK AND BEEF WITH AND WITHOUT ADDED NITRITE

Treatment	*Taste panel scores*		*Mean difference*
	Without nitrite	*With nitrite*	
Chicken samples			
Raw—0 day	4·74	4·74	0
Cooked—0 day	4·16	4·83	0·67
Raw—48 h at 4°C	4·33	3·17	−1·15*
Cooked—48 h at 4°C	3·16	4·41	1·25*
Pork samples			
Raw—0 day	4·66	4·75	0·09
Cooked—0 day	4·24	4·58	0·34
Raw—48 h at 4°C	4·58	4·58	0
Cooked—48 h at 4°C	3·24	4·58	1·34**
Beef samples			
Raw—0 day	4·91	4·74	−0·17
Cooked—0 day	2·99	4·07	1·08**
Raw—48 h at 4°C	4·49	4·41	−0·08
Cooked—48 h at 4°C	2·99	3·83	0·84**

*Significant at $P < 0.05$.
** Significant at $P < 0.01$.

results clearly demonstrate that the addition of nitrite retarded oxidation of cooked samples stored at 4°C for 48 h. The differences were significant ($P < 0.05$) for chicken and highly significant ($P < 0.01$) in the case of beef and pork. The addition of nitrite also inhibited the autoxidation of beef during cooking at 0 day ($P < 0.01$). The fact that the panel was generally unable to detect any oxidative changes between raw samples with and without nitrite before storage (0 days) is not surprising since odour changes are not easily detected in the raw state. Curiously enough, the raw chicken samples without nitrite after storage for 48 h at 4°C were preferred ($P < 0.05$) to similar samples with added nitrite. Neither the beef nor the pork samples were significantly different under the same treatments. Since the TBA values for raw chicken after 48 hours of storage at 4°C were higher for the sample without added nitrite, indicating a greater amount of oxidation, we can only conclude that results with the chicken samples were due to chance events that can occur when using small sample sizes, as was the case in this study.

The taste panel data are in essential agreement with those from the TBA test and indicate that nitrite inhibited development of WOF in all three species for the

cooked samples held at 4 °C for 48 h. This is in conformity with results presented by a number of workers (Zipser *et al.*, 1964; Sato & Hegarty, 1971; Bailey & Swain, 1973) and verifies the inhibitory action of nitrite against autoxidation.

Relationship between TBA numbers and panel scores

Table 5 presents the correlation coefficients between TBA values and sensory panel scores by species. The data clearly demonstrate that TBA numbers and taste panel scores for WOF were closely related with r values of $-0{\cdot}71$ for chicken ($P < 0{\cdot}01$), $-0{\cdot}57$ for pork ($P < 0{\cdot}01$) and $-0{\cdot}36$ for beef ($P < 0{\cdot}05$). Thus, the results

TABLE 5
CORRELATION COEFFICIENTS BETWEEN TBA VALUES AND SENSORY EVALUATION SCORES

Sample	*Number of samples*	r *values*
Chicken	32	$-0{\cdot}71$**
Pork	32	$-0{\cdot}57$**
Beef	32	$-0{\cdot}36$*

*Significant at $P < 0{\cdot}05$.
**Significant at $P < 0{\cdot}01$.

confirm the existence of the relationship between WOF and TBA numbers. These results are in essential agreement with those of Zipser *et al.* (1964) who observed a close relationship between TBA values and development of oxidative rancidity in cooked meat.

The significance of the differences between species for the correlation coefficients between TBA numbers and sensory evaluation scores was tested for significance by using the Z-test as described by Snedecor & Cochran (1973). It was found that the correlation coefficients for chicken and beef were significantly different ($P < 0{\cdot}05$) whereas those for pork and beef were not significant. It is possible that TBA values are a more sensitive measure of oxidative deterioration than WOF scores, but it is difficult to prove this to be the case.

Relationship of total lipids and phospholipids to WOF

The levels of total and neutral lipids as a percentage of tissue and the levels of phospholipids as both percentage of tissue and as percentage of total lipids are given in Table 6. Beef was found to be considerably higher in both total and neutral lipids than pork or chicken, which bears out the greater amount of intramuscular fat in beef (Pearson *et al.*, 1977). Phospholipids were quite constant in all three species, when expressed on the basis of percentage in the tissue, and agreed closely with the values given by Dugan (1971). However, when expressed as percentage of total lipids the mean values varied from a low of 10·40% for beef to a high of 23·57% for

TABLE 6
TOTAL AND NEUTRAL LIPIDS AND PHOSPHOLIPID LEVELS FOR CHICKEN, PORK AND BEEF

Sample	*Total lipids* % *tissue*	*Neutral lipids* % *tissue*	*Phospholipids* % *Lipid*	% *Tissue*
Chicken	2·85	2·14	23·57	0·66
Pork	3·51	2·77	19·66	0·66
Beef	7·22	6·09	10·40	0·73

chicken. These values agree reasonably well with those reported by Wilson *et al.* (1976).

Table 7 presents correlation coefficients between total lipid levels and TBA values. The low and non-significant correlation coefficients suggest that total lipids do not make a major contribution to TBA values.

TABLE 7
CORRELATION COEFFICIENTS BETWEEN TBA VALUES AND TOTAL LIPID LEVELS

Sample	*Without nitrite*		*With nitrite*	
	Number of samples	r *value*[a]	*Number of samples*	r *value*
Chicken	14	0·35	16	0·34
Pork	14	0·06	12	0·30
Beef	14	0·07	10	0·23

[a] None of the *r* values were significant at $P < 0·05$.

The correlation coefficients between TBA numbers and phospholipids, both as a percentage of tissue and as a percentage of total lipids, are given in Table 8. Although the data are not conclusive in the samples without nitrite, the relationships between TBA values and phospholipids as a percentage of total tissue are considerably higher than those for TBA values and phospholipids as a percentage of

TABLE 8
CORRELATION COEFFICIENTS BETWEEN TBA NUMBERS AND PHOSPHOLIPIDS AS A PERCENTAGE OF TISSUE AND OF TOTAL LIPIDS

Sample	*Without nitrite*		*With nitrite*	
	Number of samples	r *value*	*Number of samples*	r *value*
	TBA values versus phospholipids as % *total tissue*			
Chicken	14	0·41	16	−0·04
Pork	14	0·63*	12	0·14
Beef	13	0·17	10	0·30
	TBA values versus phospholipids as % *total lipids*			
Chicken	14	0·24	16	−0·49*
Pork	14	0·43	12	−0·38
Beef	13	0·21	9	0·52

* Significant at $P < 0·05$.

total lipids. Thus, the results suggest that the level of phospholipids is more closely related to TBA values than was true for total lipids. Further proof for this is found in Table 9 which shows WOF to be more highly correlated with phospholipids as a percentage of tissue in all samples without nitrite than with total lipids in the same samples. Thus, the data generally support the idea that phospholipids are involved in development of WOF, which is in agreement with results obtained in our laboratory in other studies (Wilson *et al.*, 1976; Pearson *et al.*, 1977). Table 9 also reveals that the relationships between WOF and either lipids or phospholipids were always much lower in the samples containing added nitrite than in those without nitrite. The low relationships for the samples with added nitrite appear to be due to its protective action against the development of WOF.

TABLE 9

CORRELATION COEFFICIENT BETWEEN WARMED-OVER FLAVOUR SCORES AND TOTAL LIPIDS AND PHOSPHOLIPIDS AS PERCENTAGE OF TISSUE AND AS PERCENTAGE OF TOTAL LIPIDS FOR SAMPLES WITH AND WITHOUT NITRITE

Sample	*Without nitrite*		*With nitrite*	
	Number of samples	r *value*	*Number of samples*	r *value*
		WOF scores × total lipids		
Chicken	14	−0·67**	16	−0·01
Beef	15	0·51*	10	−0·60
Pork	14	−0·12	12	0·07
		WOF scores × phospholipids (% lipid)		
Chicken	14	0·14	16	0·27
Beef	13	−0·29	9	0·17
Pork	14	−0·19	12	0·37
		WOF scores × phospholipids (% tissue)		
Chicken	14	−0·81**	16	0·33
Beef	13	−0·66*	10	−0·14
Pork	14	−0·42	12	−0·05

* Significant at $P < 0·05$.
** Significant at $P < 0·01$.

Standard partial regression coefficients were calculated between TBA values and total phospholipids (% of tissue) while total fat was held constant. Only in the case of the samples without nitrite was there a clear effect, with standard partial regression coefficients of 0·40, 0·77 and 0·18 for chicken, pork and beef, respectively. The magnitude of the standard partial regression coefficients demonstrates the direct effects of phospholipids upon TBA numbers independent of fat content. Thus, the present study verifies the fact that phospholipids seem to be involved in the rapid autoxidation that occurs during development of WOF. The results further suggest that nitrite prevents development of such oxidative changes, perhaps by protecting against oxidation of the phospholipids.

ACKNOWLEDGEMENT

Michigan Agricultural Experiment Station Journal Article No. 8417.

The authors acknowledge the assistance of W. T. Magee of the Department of Animal Husbandry for assistance with the statistical analysis and interpretation of the results.

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PROPERTIES OF *LENTINUS EDODES* AMYLASES AND AMINO ACID CONTENT OF THE MYCELIUM

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(Received: 29 September, 1978)

ABSTRACT

Ethanol proved to be better than acetone for amylase fractionation from the culture filtrate of Lentinus edodes. *The total yield amounted to* 84% *and* 77% *of the initial activity in the case of ethanol and acetone, respectively. Two alcohol precipitates were fractionated at* 25–50% *and at* 50–85% *alcohol concentration. The optimum activities for the first and the second alcohol precipitates were attained at pH* 6·6 *and* 7·1, *respectively and the optimum temperatures were* 40 °C *and* 35 °C, *respectively. The preparations were completely inactivated when heated at* 70 °C *for* 20 *min and at* 60 °C *for* 25 *min, respectively.*

Sixteen amino acids were identified in Lentinus edodes *mycelium propagated on a modified malt sprouts extract medium. All the essential amino acids were detected and determined except methionine which was not identified. The amino acids, in descending order, were leucine + isoleucine > phenylalanine > lysine > tyrosine > tryptophan > cysteine > valine.*

INTRODUCTION

Microbial amylases have been purified by many procedures. Beckham *et al.* (1965) reported that where liquid mash was employed, the desired enzymes were usually extracellular and were present in the liquor; hence no extraction steps were required. The properties of the isolated enzyme, mainly the pH and temperature relationships, are of great importance in the control of enzymic reactions in food processing, as reported by Reed (1966). Microbial enzymes and microbial proteins are now used in many aspects of food processing. Successive studies were carried out by Labaniah (1975) on the utilisation of citrus and vegetable wastes for the production of

Fd. Chem. 0308-8146/79/0004-0293/$02·25

microbial protein by the submerged cultivation of five edible mushrooms. Pomeranz (1976) reported the advantages of using micro-organisms as producers of protein from by-products of malting and brewing with special reference to malt sprouts and spent grain. Accordingly, the present work was aimed at isolating the amylase of *Lentinus edodes* propagated on a modified malt sprouts extract medium and at studying the properties of the isolated enzyme preparation as well as assessing the amino acid content of the mycelium propagated on the same medium.

MATERIALS AND METHODS

All chemicals used were of BDH AR grade except for the maltose and yeast extracts, which were Difco products, and the dextrin was for microbiological purposes.

Source of materials

Lentinus edodes IFRI 2571 c, furnished by the Indian Forest Research Institute, was used in the present work. Malt sprouts were obtained from the El-Ahram Beer Factory, Giza, Egypt.

Inoculum

A spore suspension of *Lentinus edodes* containing 26×10^8 spores per millilitre was prepared and used as the inoculum. The number of spores in the suspension was determined by the indirect cell count technique of De Moss and Bard (1957).

Modified malt sprouts medium

This medium was recommended by El-Zalaki & Hamza (1979) for amylase production by *Lentinus edodes*. Malt sprouts (120 g) were boiled with distilled water (500 ml) for one hour and filtered through cheese-cloth. Dextrin (10 g), yeast extract (5 g), potassium dihydrogen phosphate (1 g) and calcium carbonate (1 g) were added to the extract and diluted to one litre. Portions of the extract (100 ml) were transferred to Erlenmeyer flasks (*ca.* 500 ml), sterilised at 121 °C for 20 min and cooled.

Propagation of Lentinus edodes

Each flask, containing 100 ml of sterilised medium, was inoculated with 1 ml of the spore suspension and incubated at 30 °C for three days. The mycelium was separated from the growing medium by filtration.

Mycelium yield

The fungal growth was removed by filtration through cheese-cloth, washed twice with 100–150 ml of distilled water and dried on a filter paper at 60 °C for 12 h. The dry weight of the mycelium was obtained.

Amino acids

The method of Block *et al.* (1958) for acid and alkaline hydrolysis of the dry fungus mycelium was followed. The uni-dimensional descending multiple development technique of paper chromatography was employed for the resolution of amino acids. Whatman No. 1 filter paper was used. The solvent used for the separation was *n*-butanol:acetic acid:water (144:13:34). For colour development, the chromatograms were dipped into a solution of 0·25% ninhydrin in acetone containing 1% acetic acid, then dried to room temperature and placed in the dark for 12 h over sulphuric acid for maximum colour development. The amino acids spots of the unknowns were first identified by comparison with those of standards. The individual spots were cut out and eluted separately using 5 ml of 75% ethyl alcohol containing 0·25 mg of copper sulphate for 30 min. The colour intensities were measured using the Spekol spectrophotometer at a wavelength of 514 nm.

Tryptophan was determined colorimetrically in the alkaline protein hydrolysate according to the method of Miller (1967) using *p*-dimethyl aminobenzaldehyde.

Amylolytic activity

The amylolytic activity of the culture filtrate was determined colorimetrically according to the method described by Pmston (1964).

Portions (1 ml) of buffered starch solution of pH 7 were pipetted into large test tubes marked at 50 ml. One tube was labelled 'test' and the other 'control'. Both tubes were placed in a water bath at 37 °C. After 5 min 0·1 ml of the enzyme solution was added to the 'test' tube. The enzymic action was stopped after 30 min with 2 ml of 5% (v/v) sulphuric acid solution. Enzyme solution was then added to the control followed by 2 ml of sulphuric acid solution. After diluting both to about 30 ml, 1 ml of 0·01 N iodine solution was added to each tube and the mixture was made up to 50 ml with distilled water. After shaking well, the tubes were left for 15 min so that the colour could be developed fully.

An iodine blank was prepared with water, 0·1 ml enzyme solution, iodine and sulphuric acid. The tubes were read against this blank using a red filter (660 nm).

Units are defined as the number of milligrammes of soluble starch digested by 10 ml of enzyme solution in 30 min at 37 °C. As 1 ml of buffered substrate contains 6 mg starch:

$$\text{U/10 ml} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 6 \times \text{dilution factor}$$

Total nitrogen

Total nitrogen was determined by the semi-micro Kjeldahl method as described by the AOAC (1970).

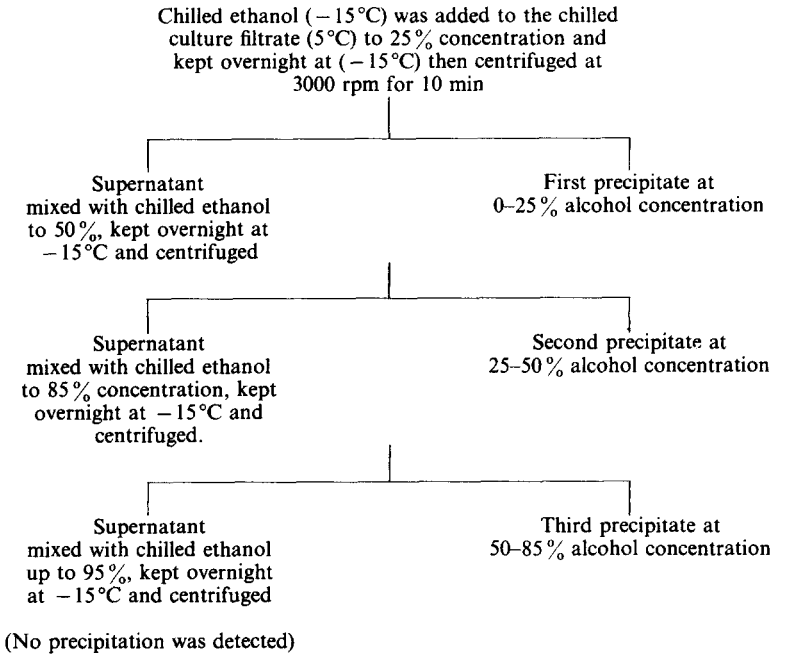

Fig. 1. Diagrammatic representation of alcoholic precipitation of amylase from the culture filtrate of *Lentinus edodes*.

Isolation of amylase

Solvent fractionation was followed according to the procedure shown in Fig. 1.

Effect of pH

Buffered soluble starch solutions of 0·1 % adjusted to various pH values covering the range 4·0–8·0 were used.

Effect of temperature

Portions of soluble starch of 0·1 % buffered at an optimum pH of 6·6 and 7·0 were used to demonstrate the effect of temperature over the range 30–60 °C on the activity.

Heat stability

Aqueous enzyme solutions (5 ml) of 0·1 % were heated in test tubes to temperatures of 50, 60 and 70 °C for different intervals, i.e. 5, 10, 15, 20, 25 and 30 min. The tubes were immediately cooled by dipping in tap water. The heat treated enzyme solutions were used for amylolytic activity determination.

RESULTS AND DISCUSSION

Isolation of Lentinus edodes *amylases*

Two precipitants—ethyl alcohol and acetone—were tried for the fractionation of the amylases from the culture filtrate of *Lentinus edodes*. The (0–25%) alcohol precipitate contained no amylolytic activity, therefore it was neglected. The recovery of amylase activity in the other two successive alcohol fractions represented 84·08% of the enzyme units as given in Table 1. The results reveal that the 25–50% alcohol

TABLE 1
RECOVERY OF *Lentinus edodes* AMYLASE UPON FRACTIONATION WITH ETHYL ALCOHOL AND ACETONE

Enzyme solution	*Volume of solution (ml)*	*Amylolytic activity (U/ml)*	*Total units*	*Recovery of enzyme units (%)*	*Enzyme units per mg protein*
Culture filtrate	1700	745·5	5960×10^2	100·00	74·54
25–50% alcohol precipitate	400	914·7	3656×10^2	61·23	130·57
50–85% alcohol precipitate	400	313·7	1252×10^2	22·85	62·70
				Total 84·08	
Culture filtrate	1700	745·0	5960×10^2	100·00	74·54
0–25% acetone precipitate	400	241·1	964×10^2	16·19	24·10
25–50% acetone precipitate	400	738·3	2732×10^2	49·53	105·43
50–85% acetone precipitate	400	178·1	712×10^2	11·94	35·60
				Total 77·66	

precipitate contained the major part of the activity. The recovery of the enzyme units amounted to 61·23% and the specific activity of the fractions was 130·6. The results of the acetone fractionation given in Table 1 show the recovery of the enzyme units distributed in the three fractions. The recovery represented 16·19, 49·53 and 11·94% of the enzyme units in the first, second and third fractions, respectively with the second fraction being the most active, as indicated by the specific activity. Generally, the results given in Table 1 reveal that ethanol and acetone fractionation recovered about 84% and 77% of the initial amylolytic activity. Accordingly, ethanol was preferred to acetone for the precipitation of *Lentinus edodes* amylase.

pH–activity relationship

The results, shown in Fig. 2, indicate that the optimum activity of the 25–50% alcohol fraction was attained at pH 6·6 while the 50–85% alcohol precipitate showed optimum amylolytic activity around the neutral pH at approximately pH 7·0. The results indicate that the crude enzyme preparation precipitated from the culture

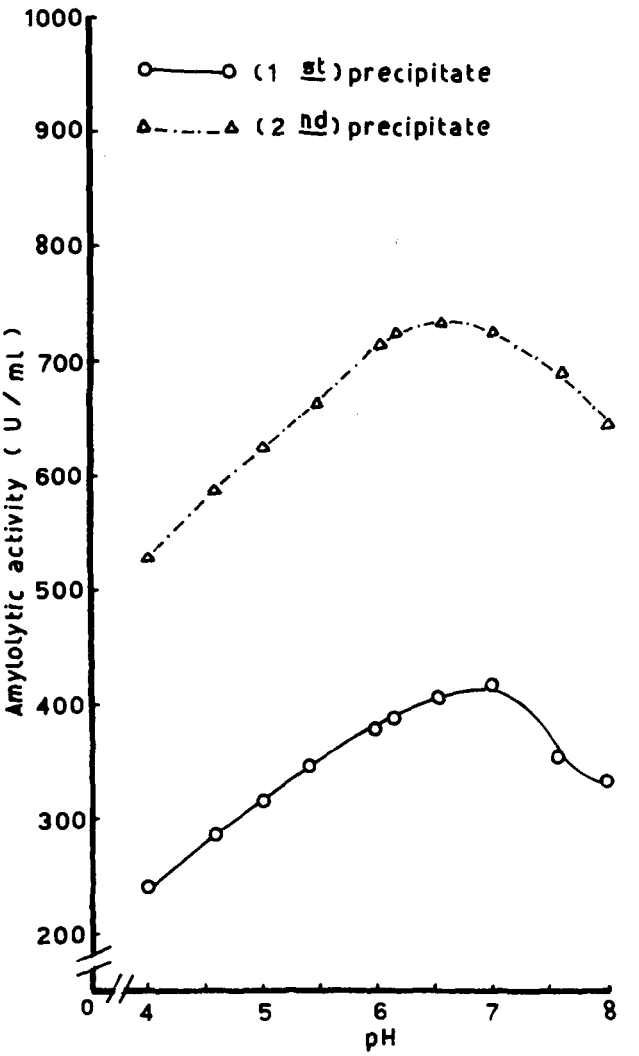

Fig. 2. pH–activity relationship of *Lentinus edodes* amylase of 25–50% (first) and 50–85% (second) alcohol precipitates.

filtrate of *Lentinus edodes* may contain two amylases or one amylase of two pH maxima.

Temperature–activity relationship

The optimum amylolytic activity of the (25–50%) alcohol precipitate was attained at 35–40°C while the optimum temperature for the amylolytic activity of the (50–85%) alcohol fraction was in the temperature range 30 to 40°C. Also, the

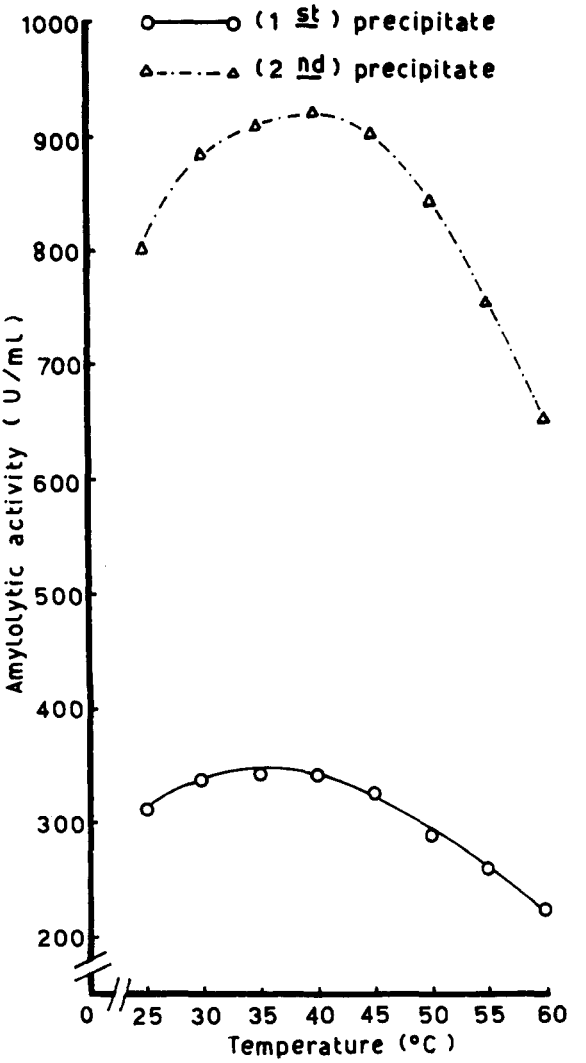

Fig. 3. Effect of temperature on *Lentinus edodes* amylases of 25–50% (first) and 50–85% (second) alcohol precipitates.

activity of this fraction was not markedly affected by the wide temperature range of 25–45 °C, as shown in Fig. 3.

Heat stability

The heat stability of the two alcohol precipitates was investigated at 50, 60 and 70 °C. The amylolytic activity of the two alcohol precipitates decreased upon heating. This decrease in the activity increased with the increment of the heating

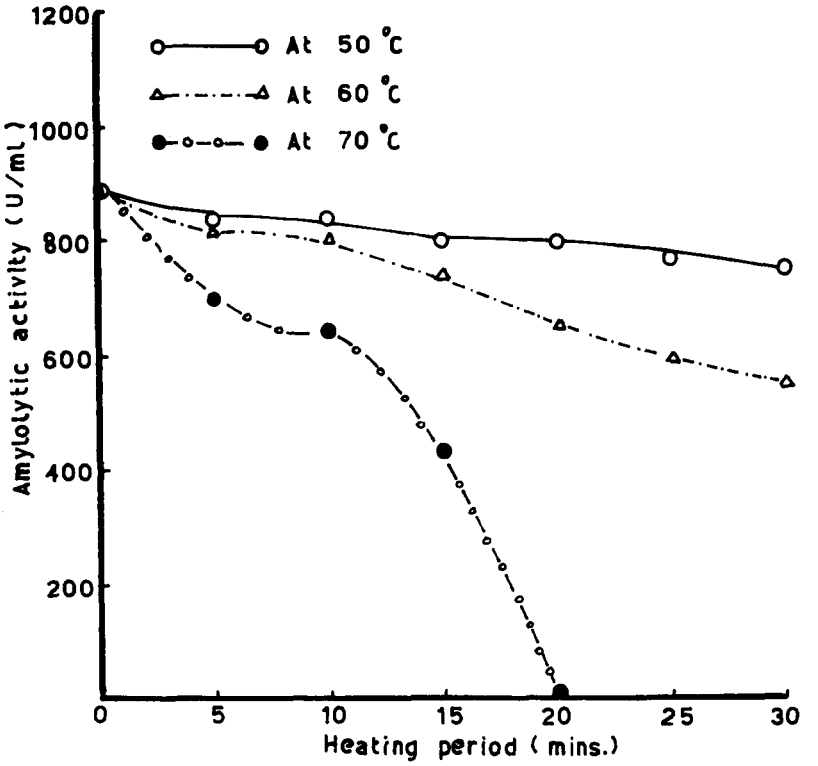

Fig. 4. Activity of *Lentinus edodes* amylases of 25–50 % alcohol precipitates as affected by heating to different periods.

period at a given temperature, as can be seen from Figs. 4 and 5. The amylolytic activity of the 25–50 % alcohol fraction was completely removed when it was heated at 70 °C for 20 min, while heating at the same temperature for 15 min caused a 50 % reduction of the activity. The amylolytic activity of the 50–85 % alcohol fraction greatly diminished upon heating at 70 °C and the enzyme was completely inactivated upon heating at 70 °C for 25 min.

Amino acids content

The amino acids of the mycelium obtained after the propagation of *Lentinus edodes* on a modified malt sprouts extract medium were identified. Chromatography indicated the presence of fifteen amino acids—cysteine, lysine, histidine, arginine, aspartic acid, serine, glutamic acid, threonine, alanine, proline, valine, phenylalanine, leucine, isoleucine and tryptophan. All the essential amino acids were identified except methionine.

The essential amino acids were determined quantitatively and the results obtained

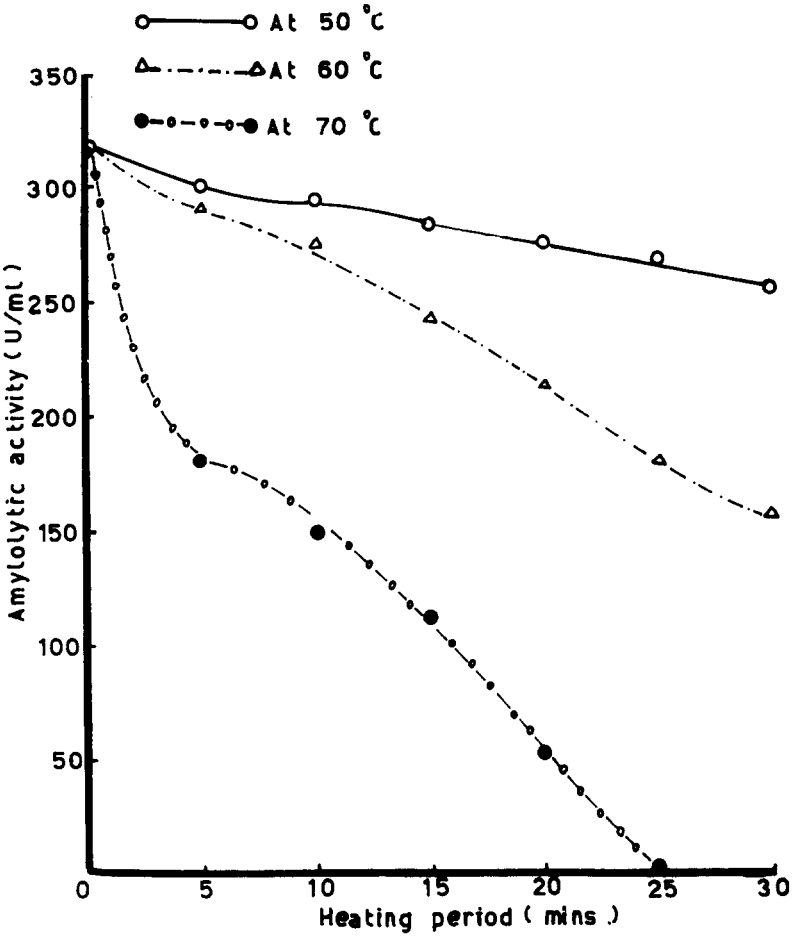

Fig. 5. Activity of *Lentinus edodes* amylases of 50–85% alcohol precipitates as affected by heating to different periods.

in the present study, as compared with those reported by Labaniah (1975), are given in Table 2. The results revealed that the amino acids of the mycelium obtained in the present work, in descending order, were as follows: leucine + isoleucine, phenylalanine, lysine, tyrosine, tryptophan, threonine, cysteine and valine. In the present results methionine was not identified while cysteine and tyrosine showed a relatively high content. No marked difference was noted in the contents of phenylalanine and lysine in mycelia grown on the two media. The threonine, valine and leucine + isoleucine contents in the case of propagation on vegetable wastes exceeded those when the mycelium was grown on a malt sprouts medium (Table 2).

TABLE 2
ESSENTIAL AMINO ACIDS CONTENT OF *Lentinus edodes* MYCELIUM PROPAGATED ON TWO DIFFERENT MEDIA

Amino acids	*Propagated on malt sprouts medium*	*Propagated on vegetable wastes**
	(*g*/16 *g nitrogen*)	
Cysteine	2·13	—
Lysine	6·95	7·45
Methionine	—	0·78
Phenylalanine	8·87	8·72
Threonine	2·28	3·20
Tyrosine	3·63	—
Valine	1·90	4·42
Leucine + Isoleucine	9·28	17·03
Tryptophan	2·47	1·20

* Labaniah (1975).

The reverse was detected with tryptophan. It is clear that the variation in the growth medium resulted in obvious differences in the amino acid contents of the mycelium of the same mushroom.

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EFFECT OF TRADITIONAL COOKING METHODS ON THE ASCORBIC ACID CONTENT OF SOME NIGERIAN LEAFY AND FRUIT VEGETABLES

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(Received: 26 October, 1978)

ABSTRACT

Representative samples of vegetables purchased from a local market, without the age and time of harvest being known, were analysed for ascorbic acid content. The ascorbic acid content in fresh samples was highest in Hibiscus esculentus *L. with* 203 mg/100 *g* ± 51·38 *and lowest in* Vernonia amygdalina *with* 30·95 *mg*/100 *g* ± 4·85.

After laboratory preparation and cooking of the vegetables, the loss of this vitamin was significant in all the samples. The use of firewood as the source of heat and of mud-pot utensils caused a higher loss of ascorbic acid in all the vegetables than cooking with gas using aluminium pots.

INTRODUCTION

L-ascorbic acid is usually considered as synonymous with vitamin C because it is the main substance in plants with vitamin C activity.

Fruits are major sources of vitamin C (Boqert *et al.*, 1966), and it is found that quite high amounts of vitamin C are contained in green and red peppers, parsley and turnip greens and that some green leafy vegetables are excellent sources of ascorbic acid, even exceeding the amounts in most fruits. There is also abundant evidence in the literature to show that ascorbic acid consumption by Nigerians has been quite good, with fruits being the major source (Kotnis & Houssain, 1964; Nicol, 1958; Oke, 1966).

Oke (1968) reported that since Nigeria is rich in fruits, symptoms of vitamin C deficiency have been rare. However, fruits are now sold by producers who are mainly from the low-income group and the cost of fruit is becoming high. Therefore Nigerians depend on vegetables as their source of vitamins, especially vitamin C. The high cost of fruits in Nigeria, particularly in the urban areas, is causing their consumption to fall.

Fd. Chem. 0308-8146/79/0004-0303/$02·25

In a survey carried out in Igbo Ora in Western Nigeria (Kotnis & Houssain, 1964) it was found that the intake of ascorbic acid by the villagers varied from 280 % of the recommended allowance in children 3–5 years old to about 400 % in pregnant women. Nicol (1958) found that lack of fresh fruits and leaves in the Northern Region of Nigeria resulted in varying degrees of dietary deficiencies of ascorbic acid. The consumption of fruits is therefore declining amongst the low-income stratum of the population which forms the greatest percentage of the community. Very little is known about the effect of cooking on some Nigerian foodstuffs, although it is universally believed that ascorbic acid is destroyed by the native way of cooking (Oke, 1967) and almost all vegetables are consumed in a cooked state. Ascorbic acid is very easily oxidised and if this oxidative process continues beyond the stage of dehydroascorbic acid it becomes irreversible; therefore the vitamin loses its activity. The enzyme oxidase facilitates rapid oxidation of the vitamin in the presence of air since it is in direct contact with ascorbic acid. Heat inactivates the oxidase enzyme but it also destroys the vitamin even in the absence of the enzyme (Davidson & Passmore, 1975).

Even though some vegetables contain higher amounts of ascorbic acid than fruits (Boqert *et al.*, 1966), Mapson (1970) points out that fruits possess an advantage over many vegetables because of the acidic media of the fruit juices compared with the nearly neutral vegetables. The fact that many fruits are eaten raw is an advantage because some loss of ascorbic acid is usually incurred during cooking (Mapson, 1970; Davidson & Passmore, 1975). Therefore it would be helpful to know the amount of this vitamin which is derived from vegetables cooked in the traditional way. It might be valuable to suggest how cooking procedures could be modified so as to retain more of the vitamin without sacrifice of economy, palatability and appearance. In order to get the body tissues well saturated with vitamin C which may help to reduce the severity of stress, heart diseases, cancer and other diseases (Anderson, 1977) the ascorbic acid loss from foods must be curtailed.

In most food composition tables (e.g. Food Composition Table for Use in Africa, 1968) records of ascorbic acid composition of vegetables were given only for raw or dried samples. The various traditional treatments and cooking methods to which these vegetables are subjected differ widely from one community to another. Hewston *et al.* (1948) and Oser *et al.* (1943) reported that little information is available on the retention of vitamins and minerals by common foods cooked in family quantities.

MATERIALS AND METHODS

Materials

Ten of the most popular vegetables were chosen for the study: *Celesia argentea* (green and red types), *Amaranthus chlorostachys*, *Crassocephalum crepidivides*, *Basella alba*, *Tallinum triangulare*, *Cochorus oliturius*, *Hibiscus esculentus* L., *Solanum gilo Raddi* and *Vernonia amygdalina*.

In all cases vegetables were purchased although with no knowledge of their age or the time since harvesting. Thus, it cannot be stated that the vegetables were fresh but on physical examination they were neither wilted nor damaged. This was purposely done so as to make the samples representative of what an average person would buy from the market, without any prior knowledge of the history of the vegetables.

Maturity affects the ascorbic acid content of some vegetables. It was reported by Oke (1968) that the vitamin C content of the vegetable sample *Cochorus oliturius* varied considerably with age, i.e. an approximate 233 % increase, whereas, in some others, the vitamin C content was more or less constant during maturation.

The vegetables were divided into three groups according to the differences in the treatment and cooking methods (see Table 1).

Group 1: The leaves and the soft stems of the samples were plucked off the tough stem. They were cut into tiny pieces and washed several times to get rid of the sand and dirt. They were then parboiled for about five minutes with enough water to just cover the tops of the vegetables. The samples were drained and washed again. The traditional reason for this action is that the natural juice that comes from the vegetables, if not parboiled, has three effects:

(i) it gives a sour taste to the stew made from it shortly after cooking;
(ii) it gives a dark coloration to the stew;
(iii) the dark colour is culturally unattractive for stews and therefore psychologically unacceptable.

The vegetables are subjected next to a further cooking for about 10 min in a previously prepared sauce which is boiling on the fire.

Group 2: This group requires no parboiling. The edible parts are washed and cut into tiny pieces. Then the samples are added to either an estimated amount of water, as in the case of *Cochorus oliturius* and *Hibiscus esculentus*, or into pre-prepared sauces, as in the case of *Basella alba* and *Tallinum triangulare*. In this group no water is discarded, the whole product being consumed.

Group 3: This group requires a special squeeze-washing process several times because of the tough nature of the vegetable fibre and to remove their bitter taste. After this process, the vegetables are parboiled as in group 1. They are then rinsed again and subjected to further cooking in pre-prepared sauce.

All the experimental cookings of groups 1–3 were done in the laboratory using a gas-fire as the source of heat and various modern cooking pans. It should be noted that only a small fraction of the community has such facilities. Therefore, to make the results more meaningful, all ten vegetables were analysed after cooking in mud pots using firewood, which is used by a large percentage of the population.

Analytical method

This procedure is an adaptation of the method described by Roe *et al.* (1966) and is based on the oxidation of ascorbic acid to dehydroascorbic acid, subsequent transformation to diketogulonic acid followed by coupling with 2,4-dinitrophenyl

TABLE 1
ASCORBIC ACID AND MOISTURE CONTENT OF SOME COMMON NIGERIAN LEAFY AND FRUIT VEGETABLES[a]

Local name of vegetable	*Botanical name of vegetable*	*% Moisture content*	*Mean ascorbic acid content of raw sample (mg/100 g ± SE)*	*Parboiled for approx. 5 min.* *Mean ascorbic acid content of vegetable (mg/100 g ± SE)*	*Mean ascorbic acid content in the discarded water (mg/100 g)*	*Final cooking for 10 min* *Mean ascorbic acid content of vegetable (mg/100 g ± SE)*	*% of total loss of ascorbic acid*
			Group 1				
1. Sokoyokoto (green)	*Celesia argentea*	89·74	32·95 ± 1·76	8·7 ± 1·50	4·84 ± 0	1·1 ± 0	96·67
2. Sokoyokoto (red)	*Celesia argentea*	89·80	38·40 ± 3·00	11·2 ± 1·15	3·33 ± 0	2·7 ± 0·86	92·97
3. Tete	*Amaranthus chlorostachys*	85·63	41·14 ± 1·73	16·00 ± 1·20	7·00 ± 0	8·1 ± 0·90	80·32
4. Ebolo	*Crassocephalum crepidivides*	81·70	45·91 ± 2·83	3·80 ± 0·81	0	1·0 ± 0	97·83
			Group 2				
5. Amunututu (sejesoro)	*Basella alba*	92·50	62·60 ± 10·85	54·23 ± 7·23	Not applicable	50·35 ± 7·53	19·57
6. Gbure	*Tallinum triangulare*	92·93	63·10 ± 11·37	29·20 ± 1·61	Not applicable	24·15 ± 0·08	61·73
7. Ewedu	*Cochorus oliturius*	81·61	54·40 ± 8·08	9·90 ± 2·02	Not applicable	7·6 ± 1·84	86·03
8. Ila (Lady's finger) or Okra	*Hibiscus esculentus* L.	90·18	203·00 ± 50·38	35·50 ± 0·23	Not applicable	18·0 ± 0	91·14
			Group 3				
9. Igbagba (Igbo)	*Solanum*[b] *gilo Raddi*	88·87	36·75 ± 4·05 (10·9 ± 0·05)	0	0	0	100
10. Ewuro (Bitterleaf)	*Vernonia*[b] *amygdalina*	85·80	30·95 ± 4·85 (3·2 ± 0·14)	0	0	0	100

[a] Cooking done under laboratory conditions (i.e. use of gas fire and aluminium pots).
[b] The ascorbic acid contents after the traditional squeeze-wash process are recorded in parentheses.

hydrazine under carefully controlled conditions to give red coloured osazones which are then compared with standard ascorbic acid solutions. This method is known for its accuracy and can be used for fresh food samples. The means and standard errors were calculated from four different vegetable samples, each treated once, and the ascorbic acid content was determined.

RESULTS AND DISCUSSION

Results are given in Tables 1 and 2. Apart from the *Vernonia amygdalina* and *Solanum gilo Raddi*, which had to undergo special treatment before cooking (see group 3), all other vegetables in the raw state were good sources of ascorbic acid. The *Hibiscus esculentus* L. (Lady's finger or *Okra*) is the richest source of ascorbic acid with about $203 \pm 50{\cdot}38$ mg/100 g. Group 3 in Table 1 has very little ascorbic acid content left after the traditional squeeze-washing process and this is further destroyed at the end of the initial parboiling. The total loss of ascorbic acid ranged from 19·57% in *Basella alba* to 100% in *Solanum gilo Raddi* and *Vernonia amygdalina* under laboratory conditions. The percentage loss of ascorbic acid in samples cooked by traditional methods is higher than that from samples cooked by laboratory methods. The loss ranged from 73·54% in *Tallinum triangulare* to 100% in *Solanum gilo Raddi*, *Vernonia amygdalina*, and green and red types of *Celesia argentea*. The reasons for this are that the intensity of the fire applied under local conditions is higher than under laboratory conditions and that the mud pot retains heat for a much longer time than the aluminium pot. Secondly, the traditional method of parboiling in enough water to cover the vegetables causes more loss of vitamin C, as can be seen in groups 1 and 2 of the Tables 1 and 2. These findings correspond with those of Krehl & Winters (1950) who reported that the greatest losses of both minerals and vitamins occur as a result of cooking by the 'old fashioned' method of just sufficient water to cover. They also reported that vegetables cooked in little water, i.e. half a cupful (125 ml), or in the more rapid pressure cooker with half a cupful of water showed quite similar losses. Krehl & Winters (1950) further reported that the pressure cooker method and the waterless methods retained more of the ascorbic acid contents, probably due to the much shorter cooking time which allows for less possibility of losses due to oxidation in the case of this vitamin. Oke (1967) reported that in Nigeria the average woman is not particular about the amount of water used. She boils the vegetables in a slight excess of water and discards the water; hence the percentage of vitamin retention by this method is lowest compared with other methods, such as pressure cooking with little water or the waterless method. The discarded water also contains an appreciable amount of ascorbic acid (see Tables 1 and 2). Krehl & Winters (1950) again suggested that the factors governing the serious loss of vitamins and minerals were the leaching effect of cooking water and the influence of oxidation, particularly of

TABLE 2

ASCORBIC ACID CONTENT OF SOME COMMON NIGERIAN LEAFY AND FRUIT VEGETABLES PREPARED TRADITIONALLY[a]

Local name of vegetable	*Botanical name of vegetable*	*Parboiled for 5 min*		*Boiled for 10 min*	*% of total loss of ascorbic acid from raw sample in Table 1*
		Mean ascorbic acid content of vegetable (mg/100 g ± SE)	*Mean ascorbic acid content of the discarded water (mg/100 g ± SE)*	*Mean ascorbic acid content of vegetable (mg/100 g ± SE)*	
		Group 1			
1. Sokoyokoto (green)	*Celesia argentea*	7·55 ± 1·12	1·76 ± 0·13	0	100
2. Sokoyokoto (red)	*Celesia argentea*	9·65 ± 1·21	3·85 ± 0·16	0	100
3. Tete	*Amaranthus chlorostachys*	13·00 ± 1·50	5·35 ± 0·49	3·5 ± 0	91·50
4. Ebolo	*Crassocephalum crepidivides*	3·00 ± 0·28	0·95 ± 0·08	1·2 ± 0	97·29
		Group 2			
5. Amunututu (sejesoro)	*Basella alba*	23·3 ± 0·37	Not applicable	15·75 ± 0·14	74·83
6. Gbure	*Tallinum triangulare*	27·0 ± 1·27	Not applicable	16·70 ± 0·51	73·54
7. Ewedu	*Cochorus oliturius*	3·60 ± 0	Not applicable	0	100
8. Ila (Lady's finger)	*Hibiscus esculentus* L.	8·45 ± 1·18	Not applicable	5·30 ± 0·75	97·29
		Group 3			
9. Igbagba (Ígbo)	*Solanum gilo Raddi*	0	0	0	100
10. Ewuro (Bitterleaf)	*Vernonia amygdalina*	0	0	0	100

[a]Cooking done in mud pots on local wood fire.

ascorbic acid which is sensitive to oxidation, and showed that a portion of the loss was accounted for in the drained liquid. Davidson & Passmore (1969) mentioned that a helping of good garden vegetables a day will improve health, but this can only be true if the methods of preparation are improved. Even the ascorbic acid content of fresh plants can be improved by the environment. Richardson (1954) and Boqert *et al.* (1966) have both suggested that light was the main environmental factor influencing the ascorbic acid content of fruits and vegetables. They found that there was an average increase of 33 % in ascorbic acid within seven days as a result of light intensity compared with plants under heavier shading. In this respect Nigeria, being a less industrial country, enjoys less pollution of the atmosphere and therefore abundant solar light.

In conclusion, since there is a great difference in the ascorbic acid content of plants from the time they are plucked on the farm to the time they arrive at the table ready for consumption, and since Nigerians have to depend on vegetables for a large proportion of their ascorbic acid requirements, their methods of preparation need to be improved.

ACKNOWLEDGEMENT

I should like to seize this opportunity to thank the Director and the laboratory staff of this Department who have contributed in no small measure to the execution of this project.

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EFFECT OF THERMAL PROCESSING ON THE STEREOISOMERISATION OF MAJOR CAROTENOIDS AND VITAMIN A VALUE OF CARROTS†

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(Received: 16 November, 1978)

ABSTRACT

The effects of thermal processing on the vitamin A value of carrots were studied. A loss of soluble solids of up to 35% *of the total solids was observed. The major carotenoid fraction was found to contain nine pigments, with α- and β-carotenes accounting for about* 83%. *The* cis *isomers of α- and β-carotenes increased with a consequent decrease in the* all-trans *isomers for the canned carrots. This resulted in a* 15% *decrease in the total effective carotenes, which translates to a similar decrease in vitamin A value.*

INTRODUCTION

Vitamin A is a derivative of certain compounds, one group being the carotenoid pigments. Among the more than 100 naturally occurring carotenoids only about ten have been reported as having provitamin A activity. The most important provitamin A carotenoids found in plants are α-, β-, γ-carotenes and cryptoxanthin (Bickoff, 1957; Roels, 1967). Moreover, the provitamin A carotenoids are found only in certain red, yellow and green fruits and vegetables. These serve as our major dietary source of vitamin A, accounting for approximately half of our dietary vitamin A intake (Food and Nutrition Board, 1974).

Comparatively, the provitamin A cartenoids are more stable to light and oxidation than retinol. This may be due to the location of the carotenoids within the plant tissues. However, heat treatment which disintegrates tissue, if coupled with

† Approved by the Director of the New York State Agricultural Experiment Station for publication as journal paper No. 3183.

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Fd. Chem. 0308-8146/79/0004-0311/$02·25

exposure to oxygen, light and acid, can result in the destruction of the provitamin A carotenoids. In addition, heat, acid and light have been reported to cause isomerisation of vitamin A and carotenoids (Zechmeister, 1962). This isomerisation is due to the conversion of the *all-trans* isomers to the *cis* isomers. The *all-trans* isomers of the carotenoids predominate in the fresh tissues because they have the most stable configuration. The *cis* isomers have been reported to have lower biological potencies than the *all-trans* isomers (Zechmeister, 1949); thus isomerisation can lead to a reduction in available vitamin A.

Most analytical methods for vitamin A in plant products either measure the total carotenoids or determine β-carotene. The validity of such methods depends on the assumption that β-carotene is the major carotenoid present (Bickoff, 1957; Borenstein & Burnell, 1966) and does not allow for differences in activities of other provitamin A carotenoids which may be present. Provitamin A carotenoids other than β-carotene have been reported and, moreover, these exist under different conditions as isomers with different biological potencies (Zechmeister, 1949; Goodwin, 1955).

Apparent increases in carotenoid content of processed carrots compared with fresh carrots have been reported as cited by Baloch *et al.* (1977). This increase in carotenoid would mean an increase in vitamin A content of the processed carrots, as shown by Watt & Merrill (1963). Some studies have tried to explain such increases. Perhaps the best explanation is the leaching of soluble solids (Baloch *et al.*, 1977).

This study was undertaken to look into the changes in carotenoid content and the isomerisation of major carotenes in carrots during thermal processing and their effect on vitamin A value.

MATERIALS AND METHODS

Materials

Carrots (*Daucus carota*; variety, Chantenay) grown during the 1977 growing season were used. They were processed in the pilot plant of the New York State Agricultural Experiment Station. Retorting conditions of 115·6 °C for 30 min for No. 303 cans were used (Lopez, 1969). During the entire process the carrots were not exposed to high temperatures except during retorting.

Methods

In order to prevent photo-induced isomerisation of the carotenoids, sample preparation and all analyses were carried out under subdued light conditions. All solutions and chromatographic columns were covered with aluminium foil to protect them from direct exposure to light. Sampling was carried out before and after retorting. The solid contents of two cans were mixed together after the liquid was completely drained. The water-soluble solids of the liquid portion were

determined using the refractometer methods (AOAC, 1975). A 100 g sample of the mixed diced carrots was blended in a Waring Blender with 100 g of distilled water for 4 min at high speed. The blended homogenate was stored in airtight containers, protected against direct exposure to light and frozen at −10 °C. Moisture content was determined by drying a 20 g sample of the blended homogenate in a vacuum oven at 65 °C for 40 h.

Extraction of carotenoid pigments: A 20 g sample of the blended homogenate was mixed thoroughly with an extractant (1:1 v/v, acetone and light petrol, boiling point (BP) 36·9 °C–55·9 °C) and filtered through a fritted glass funnel under suction. This was repeated at least five times until the residue was colourless. The filtrates were combined and allowed to separate out into two phases. The acetone layer (lower phase) was withdrawn, mixed with an equal volume of light petrol and allowed to separate out again. This procedure was repeated until the subsequent light petrol did not pick up further colour from the acetone layer. This extract was then saponified to prevent interference from lipids, chlorophylls or esters of hydroxylated carotenoids. A solution of 40 % (w/v) potassium hydroxide in methanol was added to the light petrol extract (1:4 v/v), mixed for 1 min and allowed to stand in the dark for 1 h. The lower phase (methanolic potassium hydroxide) was withdrawn and the light petrol layer was washed free of any traces of potassium hydroxide and methanol with distilled water. It was then dried by passing through anhydrous sodium sulphate and made up to a known volume with light petrol. The total carotenoids was determined from this sample.

Chromatography: The light petrol extract was concentrated to 20 ml at room temperature and fractionated on a Silica Gel 60-Methanol column according to Purcell (1958) and three fractions—hydrocarbon, monohydroxy and polyhydroxy carotenoids—were obtained. Each fraction was collected, evaporated to dryness under vacuum at room temperature and made up to a known volume with light petrol in the case of the hydrocarbon and monohydroxy carotenoid fractions. The polyhydroxy carotenoid fraction was made up with methanol.

The hydrocarbon fraction was concentrated at room temperature and applied to a glass column (30 cm × 18 mm inside diameter), tightly packed to a 15-cm height with MgO: Hyflo-Super Cel (1:1). The column was developed into nine fractions by the use of light petrol (for the first) followed by increasing concentrations of acetone in light petrol, stepwise up to 10 % (v/v). This allowed for the elution of phytoene, phytofluene, α-carotene, β-carotene, ζ-carotene, β-zeacarotene and γ-carotene. Neurosperene and lycopene, corresponding to the eighth and ninth fractions, respectively, were eluted with 2 % methanol in the 10 % acetone/light petrol mixture. Upon collection of the individual fractions in the order stated, they were evaporated to dryness and made up to known volumes with light petrol.

The α- and β-carotenes were also concentrated at room temperature and chromatographed according to Sweeney & Marsh (1970) into their individual isomers. α-carotene fractionated into three bands corresponding to *neo*-α-carotene

B, *all-trans* α-carotene and *neo*-α-carotene U. The β-carotene in turn fractionated into four bands corresponding to *neo*-β-carotene D, *neo*-β-carotene B, *all-trans* β-carotene and *neo*-β-carotene U, respectively. They were all individually collected and made up to known volumes with light petrol.

Identification and quantitative estimation: For identification, the relative positions of the carotenoid pigments on the chromatographic column and the wavelengths at maximum absorption obtained from their spectra (260–520 nm) were used (Davies, 1965). Each individual pigment concentration was determined using the known absorption coefficient specific for that pigment (Davies, 1965; Sweeney & Marsh, 1970; Foppen, 1971). The absorbance at maximum (λ max) was measured and used for calculating the amount of each pigment, using the following formula (Davies, 1965):

$$\mu g/g \text{ dry wt} = \frac{A \times \text{Vol.}}{E^{1\%}_{1\text{ cm}}} \times \frac{10^6}{W} \times \frac{1}{\%\text{TS}}$$

where A = absorbance at λ max, Vol. = volume of carotenoid sample (ml), 10^6 = conversion factor from grammes to microgrammes, $E^{1\%}_{1\text{ cm}}$ = the absorption coefficient, W = weight of blended carrot homogenate, %TS = percentage total solids of raw material.

RESULTS AND DISCUSSION

During preliminary investigations on the effect of canning on the carotenoid content of carrots, an increase in total carotenoids was observed after processing. However, when the leaching of soluble solids into the canning liquid medium was taken into consideration, a decrease was observed, as shown in Table 1. The liquid medium turned out to contain 4% soluble solids, which represents 35% of the total solids. This causes a decrease in the amount of dry matter and increases the relative concentration of carotenoids calculated on the dry weight basis. The neglect of the leached soluble solids in previous studies is responsible for reported increases in the carotenoid content of processed carrots and other plant products.

TABLE 1

TOTAL, HYDROCARBON, MONOHYDROXY AND POLYHYDROXY CAROTENOIDS CONTENT OF FRESH AND PROCESSED CARROTS

Sample	*Total carotenoids (μg/g, DW)*	*Hydrocarbon carotenoids (μg/g, DW)*	*Monohydroxy carotenoids (μg/g, DW)*	*Polyhydroxy carotenoids (μg/g, DW)*
Fresh	582·0	569·9	2·0	0·9
Canned	580·9	563·2	1·8	1·0

Represents average of four determinations.

When analysing for vitamin A of a plant product it is necessary to separate out and only measure those carotenoids with provitamin A activity. The initial fractionation of the total carotenoids into hydrocarbon, monohydroxy and polyhydroxy carotenoids clearly shows (Table 1) the hydrocarbon carotenoids as comprising the major portion of carrot carotenoids. Also, as shown in Table 1, there is a difference between total carotenoid value and the sum of the values of the individual fractions. This discrepancy is probably due to the use of mixtures of carotenoids for quantitative determination, causing an overestimation.

The hydrocarbon fraction, upon further analysis, was separated into nine individual pigments as shown in Table 2. Some of these carrot pigments have been

TABLE 2
HYDROCARBON CAROTENOID PIGMENTS ISOLATED FROM FRESH CARROTS

Fraction	*Carotenoid*	*Proportion of total pigments (%)*
1	Phytoene	7·9
2	Phytofluene	4·2
3	α-carotene	25·7
4	β-carotene	56·9
5	ζ-carotene	3·1
6	β-zeacarotene	0·8
7	γ-carotene	0·9
8	Neurosperene	0·4
9	Lycopene	0·1

found previously (Baloch *et al.*, 1977). The presence of phytoene, phytofluene and β-zeacarotene has not been reported in carrots and this study has identified the first two as colourless carotenoids, using the method reported by Tomes (1963). Of all the nine pigments only α-, β- and γ-carotenes have provitamin A activity. The others, accounting for about 16% of the total pigments, do not contribute to the vitamin A value of carrots. Therefore, they were removed to prevent interference in subsequent quantitative determinations. α- and β-carotenes account for 83% of the total pigments and this makes them the major carotenoids as far as vitamin A in carrots is concerned. The nine pigments were found in both fresh and canned carrot samples.

In the absorption spectra of some of the pigments—α-, β-, ζ- and γ-carotenes, β-zeacarotene, neurosperene and lycopene—a shift was noticed in their wavelengths of maximum absorption to shorter wavelengths: 1–2 nm for the fresh carrots and 2–5 nm for the canned carrots were observed when compared with published values (Davies, 1965; Foppen, 1971). This shift, coupled with the observation of diffuse bands of the fractions on the chromatographic column, indicates the presence of *cis* isomers along with the *all-trans* isomer (Zechmeister *et al.*, 1943).

Since the *cis* isomers are known to have lower biological activities than the *all-trans* isomer (Zechmeister, 1949), further separation of the major provitamin A

TABLE 3
STEREOISOMERS OF α- AND β-CAROTENES ISOLATED FROM FRESH AND CANNED CARROTS

Isomer	*Fresh*	*Canned*
	(μg/g, *DW*)	
Neo-α-carotene B	1·8	65·0
All-trans-α-carotene	145·8	108·0
Neo-α-carotene U	0·7	3·8
Neo-β-carotene D	—	—
Neo-β-carotene B	9·2	98·2
All-trans-β-carotene	291·8	191·0
Neo-β-carotene U	2·5	18·4

Represents an average of four determinations.

carotenoid isomers is necessitated. These isomers are shown in Table 3. A significant increase in the *cis* isomers of both α- and β-carotenes with a simultaneous decrease in the *all-trans* isomers can be observed in the canned carrot samples. Weckel *et al.* (1962) and Sweeney & Marsh (1971) have reported similar results. The increase in *cis* isomers is attributed to the thermal processing which also favours the formation of more of the *neo* B isomer, a *di-cis* isomer relative to the *neo* U isomer and a *mono-cis* isomer, from the *all-trans* isomer.

TABLE 4
EFFECTIVE CAROTENE VALUES FOR FRESH AND PROCESSED CARROT SAMPLES

Isomer	*Fresh*	*Canned*
	(μg/g, *DW*)	
Neo-α-carotene B	0·3	10·4
All-trans-α-carotene	77·3	57·2
Neo-α-carotene U	0·1	0·5
Neo-β-carotene B	4·9	52·1
All-trans-β-carotene	291·8	191·0
Neo-β-carotene U	1·0	7·0
Total	375·4	318·2

The vitamin A value of foods is usually reported in International Units (IU). 0·6 μg of *all-trans* β-carotene is equivalent to 1 IU of vitamin A (Food and Nutrition Board, 1974). Since the *cis* and *all-trans* isomers have different biological activities, it is necessary to calculate the effective value of each isomer. This is done by multiplying the amount of each isomer by its biopotency value based on the *all-trans* β-carotene (Sweeney & Marsh, 1971; Gerbhardt *et al.*, 1977) having 100. These effective carotene values are shown in Table 4. The total effective carotene value, equivalent to microgrammes of *all-trans* β-carotene, was then used to calculate the

TABLE 5
VITAMIN A VALUE AND % US RECOMMENDED DIETARY ALLOWANCE (RDA) OF FRESH AND CANNED CARROTS

Sample	*Vitamin A (IU/g)*	*% RDA*
Fresh	625·7	11·1
Canned	530·3	9·4

vitamin A value of each carrot sample (Table 5). A decrease of 15% in the total effective carotenes resulted from the thermal processing.

CONCLUSION

The leaching loss of soluble solids (35% of the total solids) in the canned carrots results in a decrease in the relative amount of dry matter. This leads to an increase in the relative concentration of the provitamin A carotenoids, causing an increase in the reported vitamin A value of processed carrots over fresh carrots when reported on a dry matter basis.

The major carotenoid fractions (hydrocarbon) found in carrots contained phytoene, phytofluene, α-, β-, ζ- and γ-carotenes, β-zeacarotene, neurosperene and lycopene. The composition of the major provitamin A carotenoids in carrots—that is, α- and β-carotene isomers—is affected by thermal processing. A substantial increase in the concentration of the *cis* isomers and a 25–35% decrease in that of the *all-trans* isomers were observed. Consequently, this is reflected in a 15% decrease in the vitamin A value of the processed carrots.

Vitamin A analysis, as opposed to total carotenoid analysis, requires the separation of individual carotenoids and the further isolation of the major provitamin A carotenoids into their stereoisomers.

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Technical Note

The Hydration of Nootkatone in Aqueous Acids

ABSTRACT

Nootkatone **1** *undergoes facile hydration to the* 13*-hydroxy derivative* **2** *in aqueous citric acid (pH* 2·4*). The non-detection of* **2** *in grapefruit juice is attributed to the protective effect of the cloud particles. The odour potency of* **2** *is about* 1/60 *of* **1**.

INTRODUCTION

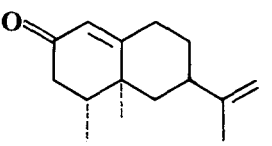
In the course of studies of the acid-catalysed transformations of terpenoids, Baxter *et al.* (1978) have noted that compounds possessing an isopropenyl group undergo facile hydration in dilute aqueous acids. Nootkatone **1**, which has such a function, is allegedly the character-impact component of the aroma of grapefruit (MacLeod & Buigues, 1964; MacLeod, 1966) and as such its stability in dilute citric acid is of intrinsic importance.

The characteristic musty-grapefruit odour of a dilute solution of nootkatone in aqueous citric acid (pH 2·4) was found to diminish in intensity over a period of several weeks. Solvent extraction of the solution after four weeks afforded a mixture of nootkatone and a more polar product (2:3) which was separated by preparative thin layer chromatography. The latter product was an oil, $[\alpha]_D^{20}$ + 133° (*c* 0·99, $CHCl_3$), which was assigned the molecular formula $C_{15}H_{24}O_2$ on the basis of elemental analysis and mass spectroscopy. Absorptions at 239 nm (ε17,100 EtOH) in the ultra-violet and at 1665 cm^{-1} in the infra-red spectra indicated that the product retained the α,β-unsaturated ketone structure. An absorption at 3460 cm^{-1} in the infra-red spectrum, coupled with the observation of the base peak in the mass spectrum at *m/e* 59, were indicative of the presence of a hydroxyisopropyl group. Confirmation that this product was the expected 13-hydroxy derivative **2** was

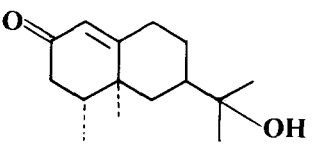
Fd. Chem. 0308-8146/79/0004-0319/$02·25

obtained by comparison of its ir and nmr spectra with previously reported values (Hikino *et al.*, 1968).

Similarly, a solution of nootkatone in 2M H_2SO_4 afforded a 2:3 mixture of starting material and **2** after 30 h. The ready hydration of nootkatone in aqueous acids contrasts with an earlier report of its acid stability under anhydrous conditions (MacLeod & Buigues, 1964).



1



2

The compound **2**, purified by repeated preparative thin-layer chromatography on silica until no significant odoriferous impurities were apparent by gas–liquid chromatography–odour port analysis, had a burnt floral-woody odour. The odour potency of **2** in aqueous solution was found to be about 1/60 of that of nootkatone. This is significantly lower than the odour potencies reported by Teranishi (1971) for other nootkatone related compounds and reflects the introduction of the hydroxyl group into the molecule.

While nootkatone readily affords the hydration product **2** in homogenous dilute acid, analysis (by successive column and thin layer chromatography) of extracts of grapefruit juices which had been stored at various temperatures showed that no significant amounts of **2** had been formed. However, thin layer chromatography analysis of extracts from cloud particles and supernatant liquor established that the nootkatone is mainly associated with the former and as such is not exposed to the action of aqueous citric acid.

ACKNOWLEDGEMENTS

We thank Firmenich et Cie for a gift of (+)-nootkatone, Mr R. Rosen for technical assistance and the Directors of Cadbury Schweppes Ltd for permission to publish this work.

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(Received: 7 July, 1978)

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