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# RAPID METHODS TO ASSESS FUNGAL CONTAMINATION IN TOMATO PRODUCTS

by

Maria Fernanda H. Pacheco Ferreira

#### A Master's Thesis

Submitted in partial fulfilment of the requirements for the award of Master of Philosophy of the Loughborough University of Technology

#### March, 1989

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### DEDICATION

To my husband and best friend Eduardo, and my dear daughter Maria Joao. Thanks for all their encouragment, understand i ng and moral support.

#### RAPID METHODS TO ASSESS FUNGAL CONTAMINATION IN TOMATO PRODUCTS

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#### Maria Fernanda H. Pacheco Ferreira

#### ABSTRACT

Some new, rapid methods were used in this study for tomato products as alternatives to the traditional Howard mould count. This method proved to be a fastidious but subjective method and therefore inaccurate for use in tomato guality control and/or for legislative purposes.

The Apizym system was used to assess fungal presence in tomatoes, tomato juice and crushed tomato, where different patterns of extracellular enzymes were obtained for the different mould species. Growth medium was also found to have an effect. This suggested the possibility of using this method to assess fungal presence in tomato products and tentatively identify them using their enzymic profile. Use of this method may not only apply to assessing raw material quality, but also as a possible monitoring test for assessing heat treatment adequacy or post process contamination.

ATP photometry was found to be suitable for assessing mould contamination in tomato juice, but erroneous results were obtained with crushed tomato. This may be related to various difficulties associated with the separation of microbial ATP from the food material, or with its extraction from the fungal hyphae, in some mould species.

The Direct Epifluorescent Filter Technique (DEFT) despite slightly overestimating the total viable count, possibly due to the effect of heat treatment on acridine orange staining characteristics, proved useful because of its rapidity in detecting fungal presence in tomato juice. Therefore, it could be used to assess quality during tomato juice production or to predict the shelflife of the product.

Impedance microbiology was used to detect mould presence in tomato juice. In this study it proved very useful in assessing tomato juice sterility using the relationship between impedance detection time and contamination levels. It also had the advantage of enabling rapid rejection of tomato juice contaminated with mould. This involved the use of a calibration curve based on the above relationship for which a cut-off point corresponding to the minimum detection time was determined.

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#### 1. INTRODUCTION

Tomatoes (*Lycopersicon esculentum*) are very perishable fruits whose consumption has largely increased in the last few years, either in the fresh state or as the various products that can be obtained from tomatoes.

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Because of their acidity, moulds are the principal spoilage microorganisms associated with tomatoes. Spoilage usually occurs after ripeningof or physical damage to the product. Presence of mould in processed tomato products indicates use of unacceptable raw fruit or poor sanitary practices.

In these products, moulds have traditionally been detected by the Howard Mould Count method since its introduction in 1911, not only for quality assessment but also for legislative purposes.

Samples are examined microscopically for mould filaments which are then counted. This method has been criticized because it is subjective and requires special training of the microscopist for mould fragment identification (Pettipher *et al.*, 1985).

The modern food industry produces an enormous variety and quantity of tomato products such as chilli sauce, tomato juice, pulp, paste, soup and ketchup. All these products are aimed to be safe and wholesome and great efforts are made to ensure that this objective is achieved. The most sensible way of doing this, is by preventing the entry of pathogenic or spoilage organisms through raw materials at the start of manufacture, and ensuring that all critical stages of the processing are monitored and controlled (Garrett, 1988).

As processing develops with greater demands for rapid microbiological testing, food processors can no longer afford to depend either on the microscopist's skill to identify mould fragments or to wait days for routine mould analyses of both raw material and final product samples. Recently rapid diagnostic methods for determining microbial counts have been developed such as enzymic and chemical methods, immunological, ATP photometry and impedance measurement. However, very little work has been carried out on mould detection on tomato products as alternative methods to the Howard Mould Count.

Pettipher *et al.*, (1985) have made attempts using DEFT, ATP and chemical assay for chitin.In this study, some of these rapid diagnostic methods such as DEFT, ATP photometry, impedance monitoring and the Apizym testing system together with diagnetic tablets were developed for estimating mould contamination in tomato products, and the potential of certain of these techniques as possible applications in determining processing efficiency evaluated.

#### 2. LITERATURE SURVEY

#### 2.1. History, distribution and economic importance

The tomato belongs to the Solanaceae family and to the genus *Lycopersicon* (Muller, 1940; Luckwill,1943). The genus consists of relatively few species of annual or short-lived perennial herbaceous plant. It is a warm season crop but despite its susceptibility to frost it can be grown successfully from the equator to latitudes as far as Canada (65<sup>0</sup>N), being native to the Andean region now forming parts of Chile, Columbia,Bolivia, Equador and Peru (Gould, 1983). It was recorded as growing in Italy in 1554 and in England in 1576 but only in 1835 the tomato became generally cultivated in the United States.

The tomato growing industry began making rapid strides in the latter half of the 19th century and still greater in first half of the 20th century. Almost all the commercially important varieties grown in the world belong to the species *Lycopersicon esculentum*.

The genus *Lycorpersicon* is divided into two subgenera: *Eulycopersicon* the red-fruited species, and *Ericopersicon*, the green-fruited species.

The economic importance of the tomato fruit is considerable and although its harvested area in the Mediterranean basin reached only 420 thousands HA, and 2,592 thousands HA all over the world in 1986 (Fig. 1),

the production of the fruit was 51 millions of metric tons in 1979/81 and increased to 59 millions of metric tons in 1986 (Fig. 2). It was only surpassed by grapes at 66 million of metric tons (FAO, Production Year Book 1986).

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# Fig. 1 TOMATO HARVESTED AREA

# Over a period of Five years



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Med.Basin

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Years

# FIG. 2 WUKLD TOMATO PRODUCTION

Over a Period of Five Years



World

Med.Basin

#### 2.2. Histology of the tomato

The tomato fruit is a fleshy berry, or in botanical terms, a swollen ovule. The body of the fruit, developed from the ovary wall, surrounds and encloses the seed and is known as the pericarp and consists of outer, radial and inner walls (Fig. 3).

The locular cavities occur as gaps in the pericarp and contain the seeds embedded in a jelly-like parenchymatous tissue originating from the placenta (Fig. 3). The number of locules in normal fruit varies from two upwards and is more or less characteristic for each variety.

The outer wall of the pericarp - epicarp - which is the tomato's skin does not have any pores - stomata or lenticles - and its cells are greenish-yellow in color and polygonal in shape (Eisenberg, 1952).

Although separated by the middle lamella (Fig. 4), the individual cells fit together closely and show definite cell wall outlines. The cell walls are rather thick and amber in colour.

Under the epicarp there is the mesocarp with flesh cells, which appear clear, thin walled and considerably larger than skin cells, with circular shape; the cells lying just beneath the skin and next to the edge of the seed cavity are smaller and narrower.

Fibrovascular bundles are the white, threadlike veins in the tomato flesh that carry moisture and nutrients throughout the fruit. Under the microscope they appear dark in colour and the vascular elements of the bundles resemble a series of tightly coiled springs.

The cells lining the seed cavity are smaller than flesh cells and are thin walled, with irregular shape.

# FIG. 3 CROSS SECTION OF TOMATO FRUIT



Source: Gould, 1983

Fig.3 Legend

- 1 Epicarp
- 2 Mesocarp
- 3 Endocarp
- 4 Seed cavities
  5 Fibrovascular bundles
  6 Core

- 7 Seeds
- 8 Septa

FIG. 4 PLANT CELL

Fig.4 Legend

- 1 Ribosomes
- 2 Mitochondria
- 3 Vacuolar membrane
- 4 Golgi apparatus
- 5 Plasma membrane6 Cell walls withmiddle lamella between
- 7 Chloroplasts with grana imbeded in stroma
- 8 Endoplasmic reticulum (ER)
- 9 Chloroplast
- 10 Plastid with starch grains



Source: Brooke, 1965

Core cells are smaller and round and have rather thick walls. The internal seed cells vary in shape from rectangular bricklike to almost ovoid shaped cells, and present considerable colour variation depending on the compactness of the cell structure and amount and size of the cell contents (Anon. 1968).

#### 2.3. Processing of tomato products

2.3.1. Tomato juice

#### 2.3.1.1. Definition and origin.

Tomato juice is one of the various products that can be obtained from raw tomato (Fig. 5) and is defined by the Federal Food, Drug and Cosmetic Act (1977), as "the unconcentrated liquid extracted from mature tomatoes of red or reddish varieties, with or without scalding followed by straining. In the extraction of such liquid, heat may be applied by any method which does not add water thereto. Such liquid is strained free from skins, seeds and other coarse or hard substances, but carries finely divided insoluble solids from the flesh of the tomato. Such liquid may be homogenized, and may be seasoned with salt. When sealed in a container it is processed by heat before or after sealing, so as to prevent spoilage" (Judge and Sons, 1971).

Tomato juice was introduced in the middle 1920s. The idea for a commercially canned tomato juice is attributed to Elliot O.Grosvenor of Tomato Products Company. In 1925 the first tomato juice was packed under factory conditions as a part of the regular manufacturing operations of the company (Cruess, 1958). It was first distributed in significant commercial quantities in 1928 (Tressler and Joslyn, 1971). Total packing of canned tomato juice and combination vegetable juices containing 70% or more tomato juice increased rapidly before World War II.

The attributes of quality in tomato juice, that is, flavour, colour, consistency and nutritive value, are greatly influenced by variety, climate, cultural practice in the field, harvest procedure, degree of ripeness at the time of harvest, length of storage before processing, washing and sorting, and each step of processing (Tressler and Joslyn, 1971).

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			Receiving			
			Dry Sort			
			Soak			
			1			
			Wash			•
	I.		Trim			
<del>_</del>			— <del>_</del> I			——
HOLE TOMATOES	CHILI SAUCE	JUICE	PULP	PASTE	CATSUP	SOUP
			1			1
el	Peel	Chop	Chop	Chop	Chop	Chop
Steam	Steam					
or	or	Hot Break	Hot Break	Hot Break	Hot Break	Hot Break
Chemical	Chemical			<u>     l</u>		
im & Core	Trim & Sort	Extract	Pulp	Pulp	Pulp	Pulp
t ort for Grade	Chop	Sterilize	Concentrate	Concentrate	Concentrate	Concentrate
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ose and Code	1				Fill	1
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0.01				,	Hold	
Natar or Air)	Water Cool				1 '	•
	114(0) 0001				Water Cool	

Source: Gould, 1983

5 PROCESSING OF TOMATOES

#### 2.3.1.2. Manufacture

At harvest time, the fruit must be picked every day, or every other day in order to ensure collecting it when it is at its prime - just ripe without green butts, and not overripe. It should be put in flat crates prior to deep ones or in baskets as the fruit will crush if piled in too many layers.

The tomatoes should be delivered to the factory promptly, as deterioration begins soon upon standing.

#### 2.3.1.2.1. Dry sort and washing

The first step in manufacture should be proper dry sorting to remove gross contamination and defective fruit followed by washing which is essential for the removal of soil, which is any substance foreign to the fruit, including spray residues, microorganisms, dirt, mould, *Drosophila* eggs etc.

The washing operation involves the soak period and the spray rinse period.

#### 2.3.1.2.2. Final sorting and trimming

The main role of these operations is the removal of off-colour and defective fruits or parts such as rotten areas, mouldy portions, because only large perfect fruit go to the scalder. The rotten fruit pass to the dump and the small and misshapen fruit to the pulping line together with unevenly ripened and overripe fruits.

Tomatoes with a small amount of rot or green area may often be trimmed and canned as extra or standard grade (Cruess, 1958).

#### 2.3.1.2.3. Peeling

The peeling of tomato can be done in different ways:steam peeling, lye peeling, infrared peeling, cryogenic peeling and gas peeling.

This operation must be done bearing in mind that its function is just to loosen the tomato's skin, leaving the inner flesh untouched, and its efficiency influences the quality of the finished product (Schulte, 1965).

#### 2.3.1.2.4. Crushing or chopping

At the end of the trimming belt, the tomatoes drop into the chopper. Following crushing or chopping of tomatoes for juice production, they may be heated. A high temperature at, or immediately after crushing produces a higher yield and a more viscous product that does not separate on standing (Goose and Binsted, 1964).

This operation can be done either by a hot-break process which involves preheating to 88  $^{0}$ C prior to extraction, to destroy the enzyme pectinmethylesterase and hence preserve the pectins, or by a cold-break process which is essentially a room temperature pulping (Holdsworth, 1983). It is generally agreed that hot break produces a better quality juice with respect to flavour, colour and improves the microbiological quality. A thicker, and more homogeneous juice is obtained by the hot break method because heat destroys the pectinmethylesterase and permits more efficient extraction of pectin. Luh and Daould (1970) reported that the breakdown of pectic materials in tomato juice by enzymatic action yields a product of low

The heat stability of pectic enzymes, when subjected to thermal treatment is therefore most important. Pectinmethylesterase is generally less heat stable than polygalacturonase (Tressler and Joslyn, 1971).

The activity of pectic enzymes is greaty accelerated as the temperature is increased to about 60 <sup>O</sup>C to 66<sup>O</sup>C. Beyond this point the activity is retarded until inactivation is reached at about 82 <sup>O</sup>C (Lopez, 1969).

The pectic enzymes cause a breakdown of pectin resulting in a thin bodied product that separates readily. The temperature should be raised to at least 82 <sup>O</sup>C for 15 sec to ensure their destruction(Lopez, 1969).

#### 2.3.1.2.5. Extraction and deaeration

The extraction of tomato juice may be accomplished by two main types of commercially available extractors: the screw type and the paddle type. Moyer <u>et al</u> .,(1959) observed that the yield of tomato juice extracted from fresh tomatoes ranged from 29.4 to 91.5 %, depending on the type of equipment used. However, it is commercially feasible to extract only 70% to 80% juice, a procedure that yields a very moist residue containing useful tomato materials that can be reextracted for use in other tomato products. In some cases, this low extraction yield (70%) is desirable because the extracted juice will have a high percentage of soluble solid components, which improve flavour, and at the same time a lower percentage of insoluble solids, which tend to reduce the quality of the finished juice (Tressler and Joslyn, 1971).

Since heating tomato juice containing dissolved or occluded air impairs the retention of vitamin C, deaerators may be used in which the product is vacuum deaerated (Tressler and Joslyn,1971). For practical reasons this operation takes place immediately after extraction of the juice.

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#### 2.3.1.2.6. Salting and filling

Sodium chloride may be added from 0.5% to 1.25% by weight to the extracted juice by direct dissolution in batch quantities, by use of tablets added to each can at the time of filling or by injecting concentrated brine made by dissolving salt (an average of 0.65% per weight) in tomato juice or serum (Tressler and Joslyn, 1971).

Filling machines are adjusted to give maximum fill which gives the best retention of quality and of vitamin C.

2.3.1.2.7. Homogenization

Tomato juice is sometimes homogenized before canning, as homogenization retards or prevents settling of the solids and produces a thicker juice. It is generally used for cold-break juice.

The juice is forced through narrow orifices at a pressure in the range of 1,000 psi to 1,500 psi and at a temperature of about 66 <sup>O</sup>C to break up the suspended solids (Goose and Binstead, 1964). This step is a usual one when juice is packed in glass (Tressler and Joslyn,1971). Tomato juice may also be milled by using a comminutor to assist in control of separation and product consistency.

#### 2.3.1.2.8. Containers and thermal processing

Tomato juice has been packed in cans made with plain,hot-dipped, or electrolytic tinplate bodies and enameled electrolytic tinplate ends.

The Codex Alimentarius appears to be directed toward a tolerance of 250 ppm of tin in canned products. At present, there is no official tolerance on tin in tomato products. However, more tomato juice will be processed in enameled cans or glass and carton containers, in the future.

Before filling, the cans should be sterilized with a relatively large volume of hot water at a minimum temperature of 82 <sup>O</sup>C to avoid thermophilic or flat sour spoilage, in particular by heat resistant strains of *Bacillus coagulans*, and then cooled rapidly in chlorinated water after holding for an appropriate length of time.

# 2.4. The composition of tomato fruits and products, and its significance to nutrition

#### 2.4.1. Major components

The yield and quality of tomato products depends in great measure upon the composition of the raw material. A discussion of some of the major components in tomatoes and their importance follows be low.

2.4.1.1. Moisture, Solids, Carbohydrates, Proteins and Amino acids, Organic acids, and Cell Wall Constituents

The moisture content of immature green tomatoes increases from about 91% to 93% as the fruit develops. Good quality ripe fruits have an average moisture content of 94% (Table 1).

Tomatoes contain usually from 7 to 8.5% of total solids, of which about 1% is skin and seeds. The percentage of solids in tomatoes is shown in Table 2 (Bolcato, 1936).

····	Тог	mato	Tomato Juice				Tomato		Chili	Tomato
	Fresh	Canned	Regular	Concentrated	Dehydrated	Cocktail	(Pulp)	Catsup	Sauce	Paste
Water (%)	93.5	93.7	93.6	75.0	1.0	93,0	87.0	68.6	68.0	75.0
Food energy (calories)	22	21	19	76	303	21	39	106	104	82
Protein, g	1.1	1.0	0.9	3.4	11.6	0.7	1.7	2.0	2.5	3.4
Fat. g	0.2	0.2	0.1	0.4	2.2	0.1	0.2	0.4	0.3	0.4
Carbohydrate:							-			
total, g	4.7	4.3	4.3	17.1	68.2	5.0	8.9	25.4	24.8	18.6
fiber, g	0.5	0.4	0.2	0.9	3.1	0.2	0.4	0.5	0.7	0.9
Ash, g	0.5	0.8	1.Í	4.1	17.0	1.2	2.2	3.6	4.4	2.6
Calcium, mg	13	6	7	27	85	10	13	22	20	27
Phosphorus, mg	<b>27</b>	19	18	70	279	18	<b>34</b>	50	52	70
Iron, mg	0.5	0.5	0.9	3.5	7.8	0.9	1.7	0.8	0.8	3.5
Sodium, mg	3	130	200	790	3934	200	399	1042	1338	38
Potassium, mg	244	217	227	888	3518 .	221	426	363	370	888
Vitamin A (I.U.)	900	900	800	3300	13100	800	1600	1400	1400	3300
Thiamin, mg	0.06	0.05	0.05	0.20	0.52	0.05	0.09	0.09	0.09	0.20
Riboflavin, mg	0.04	0,03	0.03	0.12	0.40	0.02	0.05	0.07	0.07	0.12
Niacin, mg	0.7	0.7	0.8	<b>3.1</b>	13.5	0.06	1.4	1.6	1.6	3.1
Ascorbic acid,										
mg	23	17	16	49	239	16	33	15	16	49

Source: Gould, 1983

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TABLE

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COMPOSITION OF TOMATO AND TOMATO PRODUCTS, 001

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Constituent	Percentage by w <sup>t</sup>
Total solids	7.0 - 8.5
Insoluble solids	1.0
Soluble solids	4.0 - 6.0
Sugar	2.0 - 3.0
Acid	0.3 - 0.5
Soluble protein and	
amino acid	0.8 - 1.2
Mineral constituents	0.3 - 0.6
Salt (NaCl)	0.05 - 0.1

#### Table 2. Solid composition of tomatoes

Source: Bolcato (1936)

The soluble carbohydrates of the fruits of commercial varieties of tomato are almost entirely reducing sugars (Lambeth <u>et al.</u>,1964). Since the sugars constitute 1.5-4.5% of the fresh weight (Table 1), equivalent to about 65% of the total soluble solids (Winsor,1966) they have an important effect on the taste of ripe fruit.

The quantity of sucrose found in tomatoes rarely exceeds 0.1% on a fresh weight basis (Goose and Binsted, 1964).

The reducing sugars, which usually make up from 50 to 65% of tomato solids are mainly glucose and fructose (Goose and Binsted 1964).

Davis (1968) reported that in general more fructose (1.34%) than glucose (1.12%) was present, but Widdowson and McCance (1935) found that the glucose content was higher than fructose (1.63% and 1.17%, respectively).

The sugar content increases progressively throughout maturation and ripening, a particularly pronounced rise occurring with the appearance of yellow pigmentation (Winsor *et al.*,1962).

The total nitrogen content of tomato fruit during ripening has been reported to decrease. According to Yu *et al.*,(1967) the total nitrogen content fell during development from an initially high value in small green fruit, to a minimum value near incipient ripeness, then rose again to a peak near the red stage and finally decreased towards overripeness.Subsequent work (Davis, and Cocking,1967) has indicated that the plastids are the major site of protein synthesis.

Reports on the amino acid composition of tomato fruit differ considerably but Miladi *et al.*,(1969) found 18 soluble amino acid in tomato juice (see Table 3). It is generally agreed that glutamic acid is the dominant amino acid of the ripe fruit and proline has the lowest measurable concentration (Table 3).

During ripening the total free amino acid content remains relatively constant, but processing of fresh tomato juice results in a substantial increase in the free amino acid as a result of denaturation and partial hydrolysis of protein .The greatest increase occurs in glutamic acid, followed by aspartic acid, alanine and threonine (Table 3). Asparagine and glutamine disappear during processing (Table 3), due to the loss of amide ammonia (NH<sub>3</sub>) to form glutamic and aspartic acids.

The organic acid in tomatoes is generally considered to be almost entirely citric acid and free acids are almost always determined as citric monohydrate. Nevertheless, the acids in tomato fruit have been subject of considerable investigation, not only because of their relevance to major taste components, but because total acidity plays an important part in the satisfactory processing of tomato products (Lambeth et al., 1964). The predominant acid of ripe abundant citric. with malic the most tomato fruit is next (Davis), 1964; Sakiyama, 1966). (Table 4).

## Table 3 AMINO ACID CONTENT OF FRESH AND PROCESSED

<u></u>	mg Amino Acids in 100 g Tomato Juice			
Amino Acid	Fresh	Processed		
Aspartic acid	.5 .5	51.6 .		
Threonine	1.0	• .9.0		
Serine	2.3	12.7		
Unknown	-	0.6		
Asparagine and glutamine	7.8			
Glutamic acid	21.9	212.5		
Proline	0.1	• 0.4		
Glycine	0.3	1.2		
Alanine	1.0	9.0 .		
Valine	0.4	1.7		
Methionine	0.2	0.9		
Isoleucine	0.6	3.8		
Leucine	0.6	3.0		
Tyrosine	0.5	34		
Phenylalanine	14	10.8		
Lysine	0.9	51		
Histidine	0.9	7.5		
Arginine	0.7	4 4		
Total	45.1	337.6		

#### TOMATO JUICE

Source: Gould, 1983

## Table 4 ORGANIC ACIDS IN FRESH AND PROCESSED

#### TOMATO JUICE

	mEq/Liter			
Acid	Fresh	Processed		
Acetic	1.06	1.56		
Lactic	1.37	1., <del>1</del> 6		
Succinic	0.60	0.49		
Alpha-ketoglutaric	1.10	0.53		
Pyrrolidone-carboxylic	0.81	8.10		
Unknown	0.17	0.25		
Malic	3.72	5.39		
Citric	60.92	66.92		

Source: Gould, 1983

Although pyrrolidone carboxylic acid is not a normal constituent of the fresh fruit it was found in processed tomato products. Chromatographic analysis reported by Miladi *et al.*,(1969) have separated eight organic acids from tomato juice and it has been pointed out that pyrrolidone carboxylic acid was the second major organic acid in the processed juice, whereas malic acid occupies equivalent position in fresh juice (Table 4).

During the ripening of whole fruit from mature green to red, acidity increases initially from 7.47 meq/100 ml to a maximum value 9.50 meq/100 ml coinciding, approximately, with the first appearance of yellow pigment, followed by a progressive decrease in acidity (7.14 meq/100 ml) with red pigment formation (Winsor *et al.*, 1962).

The main constituents of the cell walls of tomato fruit are pectic substances, hemicelluloses (xylans, gluco-mannans, arabo-galactans,*etc*), cellulose (unbranched long chain polymer of ß-glucose) and some protein.

The hydroxyl groups of cellulose protrude from the sides of the chain in regular order (Fig. 6) and the chains readily become hydrogen-bonded together to form crystalline structures(Fig. 7). Around 70% of the cellulose in plant cell wall is in the crystalline condition (Fig. 8)

The progressive loss of firmness with ripening is the result of a gradual solubilization of protopectin in the cell walls to form pectin and other products. Between the walls of adjacent cells there is a very thin layer of material, referred to as the middle lamella (Fig.9). This layer is largely pectin in nature and appears to act as an intercellular cement, determining the firmness with which adjacent cells adhere to each other.

Pectins are polymers of B-D-galacturonic acid linked 1-4 which vary in chain length and molecular weight; they are also esterified to varying degrees with methyl groups (Crean, 1969) to give pectic acids (low ester pectins) and pectinic acids (high ester pectins)(Fig.10 a,b,c,). The growing plant forms first an insoluble compound - protopectin (Fig. 10 d) - which binds the cells firmly

21





Source: Proudlove, 1985





Legend

Fig. 7 Model to explain the arrangement of cellulose molecules in a microfibril. Each molecule in such a sheetlike pair is held to its partner and to similar molecules above and below by hydrogen bonds (heavy lines). For simplicity no H atoms are shown. About 40 such sheetlike arrays occur in each microfibril (lower right)

Fig. 8 DIAGRAMS ILLUSTRATING THE MICELLAR STRUCTURE

OF CELL WALLS



Legend:

- A linear macromolecules are grouped to form mecillae or crystallites
- B possible formation of crystalline regions among very long macromolecules (the length of the cellulose molecule is c. 1.5  $\mu$ ). The individual micelles are separated by amorphous regions. Water molecules are indicated by o



## FIG. 9 LOCALISATION OF MIDDLE LAMELLA BETWEEN

#### ADJACENT CELLS

Source: Harder et al., 1965

Fig. 10 The basic molecular structure of pectic substances



(d) Suggested model for the structure of protopectin

Source: Goodmough, 1980

Legend:

- 1/2 pectin chains
  - 3 hydrogen bonding
together. As the fruit ripens to full maturity this protopectin is changed into pectin, which still holds the cells in place but less rigidly, so that the fruit is no longer hard.

Further growth of the tomato allows the pectin itself to be broken down into soluble compounds with little binding power, so that the overripe fruit is soft and mushy.

These transformations of the pectinic materials within the tomato, are brought about by the action of pectic enzymes formed within the cells of the plant as it grows.

These enzymes present can be divided into pectinmethylesterases (PME), exo and endo polygalacturo-

nases (PG) and exo and endo pectin *trans*-eliminases (Goodenough, 1980). The first of these enzymes PME, removes off the ester group of methyl polygalacturonate making the chains into polygalacturonic acid chains (Fig. 11). These chains are then very susceptible to cleavage by PG. An exo PG cleaves a single uronic acid unit from the end of the chain, whilst an endo PG cleaves bonds between units anywhere along the chain (Fig. 12).

The importance of these enzymes is very large in fruit softening as well as in soft rot diseases (Hulme, 1970).

The *trans*-eliminases enzymes do not occur in tomato fruit although they are common in fungal and bacterial rots, and the majority of wall softening in tomato fruit seems to be due to the action of PME and PG (Goodenough, 1980).

27 Fig11 Pectin methyl esterase removes the methyl group in each monomeric unit of the polymer



Source: Goodenough, 1980





#### 2.4.2. Other components

These are components present in tomatoes in smaller quantities than those discussed above but which none the less are of some significance. A discussion of these follows below.

bywt

#### 2.4.2.1. Minerals, volatile components and vitamins

The quantity of total minerals varies between 0.3 and 0.6% (Gould,1983). It appears that potassium has a dominant role in tomato fruit composition (Table 1) and quality, together with nitrate and phosphate it constitutes some 93% of the total mineral matter (Ansiaux,1960). The specific role of potassium is still unknown, but there is little doubt that, in addition to being required to maintain cell organization and permeability, it can act as an activator for various systems such as pyruvic kinase, and is associated with protein metabolism (Evans and Sorger,1966).Tomatoes are considered a good source of minerals such as manganese, copper and iron (Tressler and Joslyn,1971).

Iron is the most important in terms of providing adequate nutrition. An 8-oz serving of tomato juice provides 2.0 mg of iron in the reduced ferrous state which is about 10/20% of the RDA of iron. This is important because it is consumed in a product that also provides ascorbic acid, which helps retain the iron in its reduced state and is necessary for iron absorption (Natl. Acad. SCi-Natl. Res. Counc. 1968).

A large number of organic susbstances are present in tiny amounts in the volatile fraction. These include hydrocarbons, alcohols, phenols, ethers, aldehydes, ketones, esters, lactones, sulphur compounds, amines and a wide variety of heterocyclic molecules.

Ron Buttery and his colleagues at the U.S. Department of Agriculture's laboratories at Albany, California considered that cis-3-hexenal, trans-2-hexenal, hexanal,  $\beta$ -ionone, 1-pentan-3-one, 3-methylbutanal, cis-3-hexanol and 3-methylbutanol contributed most to the odour of tomato juice (cited by Hobson, 1988).

Fresh tomato, tomato juice and other processed tomato products make a significant contribution to human nutrition due to the concentration and availability of several nutrients in these products and to their widespread consumption (Table 1).

Vitamin C, the antiscorbutic vitamin necessary for normal metabolism, wound healing and collagen synthesis, is usually associated with tomato juice and other tomato products. Whole red-ripe tomatoes contain nearly all the vitamin C activity in the reduced ascorbic acid form. The ascorbic acid concentration in fresh ripe tomatoes is about 25 mg per 100 g which supplies about 40% of the adult Recommended Daily Allowance (RDA) of 60 mg and about twothirds of the RDA of 40 mg for children. The nutritive value of processed tomato products depends both on the initial nutrient concentration in the fresh tomato, and on the effects of processing and storage of the finished In the manufacture of tomato juice, ascorbic acid is destroyed, product. mainly by oxidation. Ascorbic acid is oxidized to dehydroascorbic acid (Fig. 13), which is further oxidized to degradation products (Fig. 14). The rate of oxidation is dependent on the dissolved air, enzyme content, contamination by iron and copper and temperature of the juice. The longer the tomato juice is held at optimum conditions for oxidation, the lower will be the retention of ascorbic acid after processing (Clifcor and Peterson, 1947). The temperature to which tomato products such as tomato juice are heated in the presence of the air, is the most important factor in the rate of ascorbic acid destruction; it has been found that this destruction increases with the increase of the temperature in the presence of air (Gould, 1983). Guerrant (1945) showed





CH<sub>2</sub>OH

dehydroascorbic acid (oxidised form)

Source: Froudlove, 1985

Fig 14 THE BREAKDOWN PRODUCTS OF ASCORBIC ACID



Source: Counsell and Hornig, 1981

Fig 13 STRUCTURE OF ASCORBIC ACID

that retention of ascorbic acid was greater (92%) after 15 seconds preheating before extraction at 57 <sup>O</sup>C ; retention decreased to 54% after 35 minutes preheating at 88<sup>O</sup>C . Oliver (1967) found that the addition of sulphite is frequently used to protect the vitamin against oxidation during heat treatment involved in pasteurization of fruit juices. Temperature of storage is extremely important to retention of nutrients in tomato products. Since tomato products are usually held in warehouses that are not refrigerated, the vitamin concentration will be less than in the fresh product or immediately after processing. To maintain vitamin levels at or above that of fresh tomatoes, fortification of tomato products has been proposed by Bauerfeind and Pinkert (1970) and Siemers (1971). Nutritional labelling of tomato products requires the processor to take into account the conditions under which the product is to be stored and the length of storage. When tomato juice is fortified to 50 mg per 100 g, 60% (30 mg per 100 g) may be expected to remain after one year under normal storage conditions.

Tomatoes are also a good source of vit.A (Fig.15) present in the form of βcarotene (Fig. 16). Fresh ripe tomatoes and tomato juice contain 1,150 International Units (IU) of vit.A per 100g (Booker *et al.*,1940). Thus a small tomato or a glass of tomato juice should supply 20% or more of the adult RDA of 5,000 IU.

In relation to the average consumption of tomatoes and the RDA for vitamins, tomatoes provide significant amounts of vit. C and vit. A (four times the vit.A content of orange juice) (Gould, 1971). In addition they provide small amounts of the B complex vitamins: thiamin, niacin and riboflavin (Table 1).



## FIG.15 STRUCTURE OF RETINOL

2.5. Causes of mould spoilage of tomato destined for processing

In the present state of growing and the conveyance of the tomatoes to the factories, it cannot be avoided that part of the fruits are more or less liable to attract quantities of moulds. The infection of the tomato in the field by moulds often comes from the ground on which it is grown: remarkable quantities of fungal spores can survive a long time in the ground and in favourable climatic and seasonal conditions can be very difficult to remove in the manufacturing process (Gould,1983). The behaviour pattern of the moulds show various forms: some limit their action to the epidermis, some extend to the tissue of the mesocarp and endocarp; the former group are agents of spottedness, the latter of rotten matter.

The following circumstances can assist their development:

#### 2.5.1. Use of unsound, poor quality raw stock

be

Only sound tomatoes of high quality should allowed in the processing line, as there are some moulds that develop better in the fruits with a cracked or damaged skin. The affected tomatoes present more or less wide zones invaded by hyphae gathered in a mycelium that can assume several different appearances by forming interlaced felts similar to tissues.

#### 2.5.2. The state of maturity

Some moulds attack unripe fruit more easily whereas some begin their activity only at an advanced stage of maturity (Pederson, 1929).

2.5.3. Excessive filling of the crates at harvest

At harvest, the fruits are placed in wooden crates for conveyance to the factory; these crates even if rigid do not always prevent the consequences of travelling on bumpy roads or of the weight of the upper layers of the fruits on the lower ones and of badly stacked crates leading to crushing and consequent loss of juice. In this state the tomatoes can get spoiled (Pederson, 1929).

# 2.5.4. Length of time and climatic conditions from the harvest to the processing line

Tomato spoilage may be significant if there is storage under adverse climatic conditions for too long before getting to the factory or whilst at the factory before processing (Pearce, 1940).

2.5.5. Rough handling of raw stock during handling and before or during washing operations

Care must be taken to avoid crushing or breaking of the tomatoes in bulk handling, particularly during washing operations, as localised moulds can develop in small creases and in places where the raw material breaks down (Natl. Canners Assoc., 1968).

¥

#### 2.5.6. Insufficient heat treatment

An important factor in the prevention of flat-sour of tomato juice is the use of a thermal process adequate to destroy the heat resistant flat-sour spores present in the juice, and the inactivation of pectic enzymes which is reached at a temperature of about 82<sup>o</sup>C (Lopez,1969). However, fungi producing ascospores are capable of surviving pasteurisation and causing spoilage.

The list of species that have been isolated from fruit products after a heat process is headed by *Byssochlamys fulva* and *B. nivea* which have been recorded as causing spoilage in strawberries in cans or bottles (Put and Kruiswijk, 1964; Richardson, 1965), blended juices with a passion fruit content and fruit gel baby foods (Hocking and Pitt, 1984).

Harper *et al.*,(1972) reported that pectic enzymes of *Rhizopus stolonifer* survive the canning process normally appplied to fruit and if even a small proportion of infected fruit is processed, these enzymes can cause softening and so spoilage of canned apricots.

#### 2.5.7. Contaminated plant equipment - plant sanitation

The design and accessibility for cleaning of metal equipment are of primary importance. The plant must be regularly washed and disinfected with a germicidal solution. In large-scale commercial production, even if all precautions have been taken, some spoiled fruit can get through. The incidence of spoiled fruits on the mould content in the finished product varies appreciably (Bohrer and Reed, 1948).

2.6. Genera of moulds frequently encountered in tomato amd tomato products

Moulds are small, generally microscopic organisms lacking chorophyll and conductive tissue. Most of the 100,000 species known are strictly saprophytic, living on organic matter. The fungal body typically consists of microscopic filaments that branch in all directions, spreading over or within the substratum utilized for food. Each of these filaments is known as a hyphae and is a thin, transparent tubular wall, filled or lined with a layer of protoplasm varying in thickness. The mass of hyphae is called a mycelium (Alexopoulos and Minns, 1979).

Fungal reproduction presents two general types: sexual and assexual. Asexual reproduction, sometimes called somatic or vegetative, does not involve the union of nuclei, sex cells or sex organs, whereas sexual reproduction is characterized by the union of two nuclei. Fungal classification depends on the kind of spores formed which sometimes are very difficult to identify (Fig 17 a,b,c and Fig. 18 a,b,c,d). Most of the plant pathogens which infect tomato fruit belong to the Class Deuteromycetes or Fungi Imperfecti and to the Class Zygomycetes. Some of the more important mould genera causing spoilage of tomato fruits, are represented in Fig 19.

2.6.1. Genus Alternaria

Alternaria species are among the commonest saprophytes on decaying vegetation of all kinds. Many species of Alternaria are pathogens on plants, including a number of kinds used for foods. A. citri produces rots in lemons; A.solani infects potatoes and tomato fruit, where it produces a disease known as 'nailhead spot' (Ellis, 1971).

# FIG.17 Spores produced by Fungi Asexual

37



Section through sporangium showing sporangiospores

C)





Source: Alexopoulos, 1979



#### FRUIT AND VEGETABLES

FIG.19 SOME OF THE MOULD GENERA THAT CAUSES SPOILAGE OF



Rhizopus

Fusarium

Alternaria

Legend: i - conida (asexual spores) ii - conidiophores iii - sterigmata iv - vesicle v - sporangium vi - sporangiospores vii - sporangiophore viii - columella ix - rhizoids (root-like hypha) x - macroconida

xi - microconida

xii - compact stromatic

Source: Duckworth, 1979

A. tenuis is the most common saprophytic species. This species has been reported to cause spoilage of fresh tomatoes (Harwig *et al*.,1979).

#### 2.6.2. Genus Botrytis

This is a common genus in temperate zones, where it occurs mainly as a pathogen on a variety of plant crops. Vegetables and small berry fruits are particularly susceptible. Invasion may occur before maturity or postharvest, both in transport and in storage. They are characterised by the production of conidia on pegs from spherical swellings. The most commonly encountered species in foods is known as *B. cinerea*, although it is probable that this name includes a group of related species rather than a single well defined taxon (Coley Smith *et al.*, 1980).

*B. cinerea* is a virulent cause of rots in many kind of fresh fruits including grapes, strawberries and tomatoes (Harwig *et al.*,1979). This species can also cause mechanically small whitish rings often with dark centres. Rot can spread rapidly at higher temperatures during packing and transport (Ryall and Lypton, 1979).

#### 2.6.3. Genus Penicillium

This genus is very diverse in terms of numbers of species and range of habitats.Most species are simply described as ubiquitous, opportunistic saprophytes. Nutritionally they are very undemanding being able to grow in almost any environment with a small quantity of mineral salts, and any but the most complex forms of organic carbon, and a wide range of physico-chemical environments, i.e. a<sub>w</sub>, temperature and redox potential.

A few species are destructive pathogens on fruit such as *P. digitatum* and *P. italicum*. *P.aurantiogriseum* is among the most commonly encountered fungi

on earth. It can cause spoilage of a variety of stored fruits and vegetables including apples, pears, grapes and tomatoes (Barkai-Golan, 1974). *P.italicum* has been reported as a destructive species on fruit juices and tomato fruit (Barkai-Golan, 1974).

#### 2.6.4. Genus Aspergillus

This genus has the ability to grow at high temperatures and low  $a_W$ . It usually grows more rapidly than penicillia, but takes longer to sporulate.

*A. niger* is distinguished from all fungal species by its spherical black conidia, derived from colonies which show little or no other colouring. It is among the fungi most commonly reported from foods, and can be described as ubiquitous. It competes with *A. flavus* as the most common fungus in spoiling nuts, especially peanuts and pecans (Doupnik and Bell, 1971). Barkai-Golan (1980) reported it to be the most common fungus causing post harvest decay of cold stored fresh fruit, isolating it from apples, grapes and tomatoes.

2.6.5. Genus Rhizopus

It is distinguished from other genera in the order *Mucorales* by the formation of rhizoids, which are conspicuous at the base of the conidiophores, by columellae which often collapse into umbrella shapes in age and by dry sporangiospores with striate walls. *Rhizopus* species appear to be able to attack almost any kind of fruit and vegetables and the tomato is no exception. In severe cases of *Rhizopus* rot, the fruit resembles a red, water filled balloon (Ryall and Lypton, 1979). 2.7. Existing and potential methods of mould assessment in tomato products2.7.1. Howard mould count

2.7.1.1. History and nature

In 1911, B.J.Howard of the Food Division, Bureau of Chemistry and Soils United States Department of Agriculture first introduced the technique of mould counting in tomato products.The method was intended as a means of combating the insanitary conditions then existing in many American canning factories where, at the time, tomato juices and purees were largely byproducts from the canning of whole tomatoes (Smith,1952).

Basically, the principles of mould counting have remained unchanged since their introduction in 1911. The method (A.O.A.C., 1984) is strictly empirical and consists of examining a well mixed sample of the fruit product microscopically under rigidly defined conditions. The number of microscopic fields in which mould filaments in excess of a defined minimum size and condition are observed and counted. This is then expressed as a percentage of the total number of fields examined (usually 50). This figure is the Howard mould count for that particular fruit sample and it represents an indication of the percentage distribution of non viable mould within the product. Since the mould observed during the count derives from that originally growing on and spoiling the fruit from which the product was prepared, or from mould growing in the processing machinery, clearly the Howard mould count provides some indication of the microbiological condition of the fruit used in the product, or of the conditions of the factory.

There is no doubt that mould growth in significant quantities is not found in sound tomatoes and, as mould in the course of its development breaks down the tomato tissue to form rot its presence in tomato products is a fairly reliable indication of rotten fruit having been used in manufacture. The amount of mould found in canned tomato paste and other products, can therefore be an index of the care taken by the packer in selecting, sorting and trimming the tomatoes. The possibility does exist, however, of good fruit becoming contaminated with moulds which have grown on dirty factory equipment and this may give rise to high mould counts in the product(Anon, 1968).

#### 2.7.1.2. Levels of acceptance for mould in tomato products

In 1916,the Howard Mould Count was first employed in legislation by the Food Drug Administration and from that time until May 1931 a maximum limit of 66% positive fields for all comminuted tomato products was enforced.

This applied to all products packed, sold and shipped in the United States.

From 1931 until June 1940, this limit was reduced to 50% positive fields and from 1940 until the present time the American standards have applied in respect to tomato juice which today stands at 20% positive fields. Some other countries have followed this standard but Canada for example, still maintains 50% for all tomato products except juice which is set at 25% positive fields. The actual acceptance level for mould for tomato products has been established by Food and Drug Administration as shown in Table 5.

Product	Tolerance (%)
Tomatoes (in juice)	. 12
Tomatoes (in puree)	25
Tomato juice	20
Tomato ketchup	30
Tomato puree	40
Tomato paste	40
Tomato soup	40
Tomato sauce (undiluted)	40
Other products	40

Table 5. Acceptance level for mould in tomato products

Source: Gould, 1983

2.7.1.3. Principal differences between mould and tomato tissue

The identification of mould fragments and their differentiation from particles of tomato tissue (Fig. 20) is the most difficult aspect of the Howard mould count (Goose and Binsted, 1964). The following table (Table 6) may prove helpful in deciding whether a filament is, in fact, mould or tomato tissue.

Table 6. Characteristics of mould filament and tomato tissue

#### MOULD FILAMENT

# TOMATO TISSUE

- segmented
- branching
- granular appearance tubular structures
- with parallel walls

- never segmented
- branching indefinite
- clear, vitrous, fibrous
- large with thin walls
- fibrovascular tissue as small coilded springs

Source: Gould, 1983

FIG.20 FILAMENTS FOUND IN TOMATO PRODUCTS





Source: Gould, 1983



Plate 1 Tomato skin cells found in tomato juice (x400).

Plate 2 Disrupted tomato tissue present in tomato juice (x400).





Plate 3 Tomato fibrovascular bundles found in tomato juice (x400).

Plate 4 Mould hypha found in tomato juice during Howard mould count (x400).



#### 2.7.2. Other methods of assessing mould contamination

During the past few years, use of new processing techniques for fruit and vegetables has resulted in different degrees of comminution which ultimately affect the count. Therefore more objective methods for fungal detection that do not rely on filament counting are needed (Notermans *et al.*, 1986).

Apart from the traditional methods to assess fungal contamination several methods have been developed for mould detection in food but most of them have one or more drawbacks (Notermans *et al.*,1986).

### 2.7.2.1. Methods which assess the presence of viable cells

#### 2.7.2.1.1. Plating technique

Over many years of practice spread techniques have been found to give significantly higher counts of moulds than

have pour plate methods (Jarvis et al., 1983).

Two major assumptions of the plate technique are that:

1. microorganisms are in suspension as dissociated single cell units so that each colony on the plate arises from an individual cell, and ,

2. all cells that are planted in the culture medium, will multiply to produce a visible colony (Banwart, 1979).

Neither assumption is correct especially in the case of mould as the inoculum contains clumps of spores and mycelium, and single spores. Jarvis *et al.*, (1983) reported that dilution counts on mould propagules, do not follow a strict decimal progression, and explained it either (due to fragmentation of mycelium and breaking of spore clumps during dilution, or competitive inhibition when large numbers of colonies are present on the plates.

Also, this technique is generally inappropriate to canned tomatoes as far as determining the "mould count" pre-processing, which is something Howard mould count can do.

#### 2.7.2.1.2. Most probable number (MPN)

Most probable number methods are rarely used for mould estimations, although studies by Koburger and Norden (1975) demonstrated consistently higher counts than using serial dilutions methods.

The precision of MPN techniques will be influenced considerably by homogeneity. Since the theory of MPN calculation is dependent upon Poissonian distribution and linear dilution factors the MPN method can rarely be precise for mould dilutions which arise from mycelium, conidial clumps and free conidia (Jarvis et al., 1983).

#### 2.7.2.1.3. ATP bioluminescence

AMP

1

All viable cells contain Adenosine-triphosphate (ATP) which is the primary source of energy in all living organisms.

The flashing of firefly tails is due to bursts of ATP reacting aerobically with a substrate, luciferin under the influence of the luciferase enzyme (McElroy et al., 1969) as follows:

ATP = adenosine triphosphate PP = phosphate = luciferin 2 = luciferase Ma++ 1-2-AMP + PP 1 + 2 + ATPO2 1-2-AMP oxiluciferin + 2 + CO<sub>2</sub> + AMP

= adenosine monophosphate

+ LIGHT

In this reaction under optimal conditions, one photon of light is produced for each molecule of ATP utilized.

Since light emission is proportional to the concentration of ATP, a linear relationship can also be established between ATP concentration and the number of viable cells in a sample, as microbial cells contain relatively constant levels of ATP (D'Eustachio and Levin, 1967).

Sharpe *et al.*, (1970) were among the first investigators to correlate bacterial ATP levels with bacterial numbers in a variety of foods.Other investigators have since used the ATP assay as an index for estimating bacterial numbers in meat (Stannard and Wood, 1983;Cook *et al.*, 1984; Littel *et al.*, 1984) and milk (Bossuyt, 1981,1982) and yeast levels in beer (Miller *et al.*,1978) and fruit juices (Stannard and Wood,1983).Pettipher *et al.*, (1985) tried to use this method to assess mould contamination in tomato concentrates.

2.7.2.1.4. Impedimetry

Although the concept of electrical impedance measurement of microbial growth was advanced by G.N.Stewart in 1899, it was only in the 1970s that the method was first employed for the estimation of bacteria in foods (Wood,Lach & Jarvis, 1977).

Impedance is defined as opposition to the flow of alternating current through a conducting medium. The impedance of a particular system is determined by various factors such as temperature, ionic content and molecular size.

When microorganisms grow in culture media, they metabolize substrates of low conductivity into products of higher conductivity, and thereby decrease the impedance of the media, as is generally associated with bacteria, whereas mould cultures are charaterised by an increase of impedance ("left-hand signal") which means that they metabolize substracts of high conductivity. These impedance changes can be measured by electrodes placed in the growth media

The time taken for a culture to reach a threshold is known as Detection Time which is inversely proportional to the number of organisms in the initial inoculum. A linear relationship between total viable count and impedance/conductance detection time was shown for a variety of foods (Wood,Lach & Jarvis, 1978).

Cady (1975) and Ur and Brown, (1975) have been evaluating impedance as a means of monitoring the overall microbial quality of various foods. Williams and Wood (1982) used impedance to detect fungi in foods; Schaertel, Tsang and Firstenberg-Eden (1987) tried to use impedance to detect yeasts and moulds in various foods.

#### 2.7.2.1.5. Apizym system

Enzymic activities are widely used in the characterization of microorganisms (Bascomb,1980). This method is a semi-quantitative micromethod enzyme system (APIZYM) designed for detection of 19 individual enzymes which can be further tested by spectrophotometric and/or electrophoretic procedures.

It has been used by Townsend and Blankenship (1988) to assess the heat treatment given to semi-commercially and commercially prepared meat and poultry products; Bridge and Hawksworth (1984) used the Apizym system as an aid to the rapid identification of *Penicillium* isolates. Ngabirano (1987) used this method for screening extracellular enzymes produced by fungi in decayed citrus fruits.

#### 2.7.2.2. Methods which assess the presence of viable and non viable cells

#### 2.7.2.2.1. Direct microscope count

With this method the liquid food may be determined directly using a counting chamber. In this study the haemocytometer has been used and the procedure will be exlained in the experimental section (3.2.1.2.).

#### 2.7.2.2.2. Fluorescent antibody technique

The presence of fungal mycelium can be detected microscopically following the application of fluorescent antibodies (Preece, 1971).

Immunofluorescent techniques using either direct or indirect staining procedures have been described for a number of fungi (Preece,1971). Such techniques permit detection of reactive antigens in mycelium and have been used to study fungal invasion of barley grains (Warnock,1971) and pistachio nuts (Denizel,1974). However the technique is limited by the non-availability of specific antisera.

#### 2.7.2.2.3. Direct epifluorescent filter technique (DEFT)

The direct epifluorescent filter technique was developed at the Institute of Food Research (formerly the National Institute for Research on Dairying), as an alternative to the resazurin dye reduction test for the assessment of the bacteriological quality of milk in incoming tankers and in silos. The technique is very rapid, taking 20/30 minutes, and involves filtering a sample pretreated with enzyme and surfactant, staining the retained bacteria with the fluorescent dye, acridine orange, and counting the number of bacteria fluorescing orange using epifluorescence microscopy (Pettipher *et al.*, 1980).

However there are some disagreements concerning the relation between cell viability and the orange colour of staining. Rodrigues and Kroll (1986) using DEFT for the enumeration of yeast in beverages, found that this method was unsuitable for heat treated samples because non-viable cells fluoresced orange which did not follow the general staining pattern of DEFT.

Pettipher *et al.*, (1985) attempted to use DEFT to detect mould contamination in tomato concentrates.

#### 2.7.2.2.4. Immunological assay

In a study by Notermans and Henvelman (1985) an enzyme linked immunosorbent assay (ELISA) for detection of moulds was described. The ELISA was used based on detection of heat stable, water extractable and genus specific antigen(s) produced by moulds.

Further work carried on by Notermans *et al.*, (1986) using the same technique revealed that the production of antigen(s) was correlated with mycelium weight, showing that this method is suitable for a quantitative detection of moulds in food products. However, for more reliable results standardized antigen(s) have to be produced and used as internal standards in the ELISA.

#### 2.7.2.2.5. Chitin

Chitin is a polymer of N-acetyl-D-glucosamine, and is a major constituent of the walls of fungal spores and mycelium. It also occurs in the exoskeleton of insects, but is not present in bacteria or in foods. So the chitin content of a food or raw material can provide an estimation of fungal contamination.

A number of studies have indicated that the chitin assay is a valuable technique for estimating the extent of fungal invasion in foods such as corn or soybeans (Donald and Mirocha, 1977), wheat (Nandi, 1978) and barley (Whipps and Lewis, 1980).

However, according to Cousin *et al.*, (1984) products from rot-free tomatoes gave positive glucosamine assays even after acetone extraction. Moreover, chitin content does not increase proportionally with fungal growth and insect contamination of samples can cause grossly misleading results (Sharma *et al.*, 1977). Nevertheless, the chitin assay merits continued study.

#### 2.7.2.2.6. High performance liquid chromatography (H.P.L.C.)

This method was developed to analyse for the degree of mould contamination in processed fruit and vegetable products (Lin and Cousin, 1985). The method was based on detection of glucosamine, a breakdown product of chitin which is one of the major constituents of fungal cell walls. Although the results were reasonable, further work is needed to increase its precision before it can be used by the food industry for routine analytical quality control.

#### 2.7.2.2.7. Ergosterol

Ergosterol is the major steroid produced by fungi, but at most is a minor component of plant sterol(Weete, 1974).So,

like chitin, ergosterol can be used as a measure of fungal invasion in foods and raw materials. A methodology for estimating ergosterol in cereals has been provided by Seitz *et al.*,(1977,1979). Ergosterol is detected by ultraviolet absorption,optimally at 282 nm, a wavelength at which other sterols exhibit little or no absorbance.

The ergosterol assay is reported to have a high sensitivity to early fungal growth (Seitz et al., 1979).

It appears to be a useful indicator of fungal invasion of foods, and holds promise as a routine technique for quality control purposes.

#### 2.7.2.2.8. Tenuazoic acid

Tenuazoic acid (TA) is known as a mammalian toxin (Harvan and Pero, 1976), and has been implicated in onyalai, a hematologic disorder of humans in Africa (Steyn and Rabie, 1976).

When Mislivec *et al.*, (1987) investigated the mould flora of fresh tomatoes used in the production of ketchup they also tested for the presence of TA on an overall as well as on a geographical basis. This was because earlier reports (Ramsay *et al.*,1952: Ayres *et al.*,1964) indicated that one of the principal genera to invade tomatoes is *Alternaria*, which produces TA as a toxic metabolite in fresh tomato tissue.

The regular occurrence of the *Alternaria* metabolite TA in the mouldy tomatoes may eventually prove to be valuable as an indicator that mouldy tomatoes were used during the processing of tomato products.However, further work must be done on this area, to fully evaluate its potential.

#### 2.7.2.2.9. Gas chromatography

Offen and Dart (1983) reported a rapid method for detecting spoilage fungi. GC was used to determine the amount of methanol released from pectin by the fungal enzyme pectinesterase .Preliminary studies were reported on pure and mixed spore suspensions of *Aspergillus* and *Penicillium* species.

Eyles and Adams (1986) used GC to analyse spoiled food and unspoiled control samples for the presence of microbial metabolites. The metabolites

sought, using simple, rapid GC techniques were volatile fatty acids and, in some cases, alcohols. The results of their study showed that this method can provide useful information during the investigation of spoilage of canned foods and related products, and probably will be applicable in the future to detect mould contamination.

#### 2.8. Aims of present work

1. To use of the Apizym system as a means of screening mould contamination in tomato products.

2. To attempt to profile some moulds in terms of their enzymic activity during growth, and use of diagnostic tablets as a possible application in determining processing efficiency.

3. To use ATP photometry, DEFT and impedance as rapid diagnostic methods to assess fungal contamination in tomato products in the food industry.

4. To use the Howard Mould Count as the traditional method for quality assessment of tomato products and compare it with the new rapid diagnostic methods.

#### 3. EXPERIMENTAL

#### 3.1.Materials

#### 3.1.1. Fruit and fruit juices

Tomato fruit (*Lycopersicon esculentum*), tomato juice and crushed tomato were used throughout this study. Fruits were supplied by wholesale fruit merchants and the juice and crushed tomato from local retail outlets.

#### 3.1.2. Moulds

The following moulds were used in this investigation. They were obtained from CAB IMI (Kew) as freeze dried cultures which were reconstituted with sterile distilled water followed by subsquent purification on malt extract agar MEA (LABM Code LAB37).

Alternaria species (CMI 56271) Aspergillus niger (CMI 17454) Cladosporium herbarum (CMI 49630) Fusarium culmorum (CMI 159025) Mucor hiemalis (CMI 21217) Penicillium digitatum (CMI 143627) Rhizopus stolonifer (CMI 17314)

3.2. Investigations into extracellular enzymic activity in tomato fruit, tomato juice and crushed tomato

3.2.1. Standardisation of the inoculum

It has been suggested that the single most important factor to consider in influencing reproducibility is that of a standardised initial inoculum size (Thompson and Watling, 1983).

Reproducibility was achieved by the careful standardisa- tion of parameters such as the volume of agar used, incubation conditions, inoculum size and age of culture

used for inoculation.

#### 3.2.1.1. Spore suspension preparation

A spore suspension of the different moulds was prepared by washing 8/10 days malt extract agar slopes using 10 ml of sterile distilled water, followed by filtration through sterile filter paper (Whatman No.1) to remove fungal hyphae.

3.2.1.2. Haemocytometer

The Haemocytometer slide mod. B.S.748 from HAWKSLEY CRISTALLITE, and coverslip were carefully cleaned. The edges of the grooves in slide were wet and pushed down hard with the thumbs and the coverslip put into position over the chamber grids. When the slide and coverslip were in close contact a rainbow effect (Newton's rings) should be seen from a shallow angle.

The culture was shaken thoroughly and a drop of it was introduced at the edge of the cover glass. The chamber then filled by capillarity. The slide was placed on the microscope stage and the central area of counting grid focused using the high power objective (x40). All the cells in each of five randomly selected 0.04 mm<sup>2</sup> areas were counted. The number of cell/cm<sup>3</sup> was calculated as follows:

Depth of well = 0.1 mm Area of small square =  $0.0025 \text{ mm}^2$ Volume of small square =  $0.1 \times 0.0025 = 2.5 \times 10^{-4} \text{ mm}^3$ Area of square =  $0.04 \text{ mm}^2$ Volume of square =  $0.04 \text{ mm}^2 \times 0.1 \text{ mm} = 0.004 \text{ mm}^3$  $4 \times 10^{-3} \text{ mm}^3/103 \text{ mm}^3 = 4 \times 10^{-6} \text{ cm}^3$ 

Dividing the average number of spores counted per square by  $4 \times 10^{-6}$  cm<sup>3</sup>, the number of spores existing in a spore suspension was then obtained.

3.2.1.3. Calibration curve

A serial dilution for each mould spore suspension was prepared using sterile distilled water. The following dilutions were prepared: 100%, 50%, 12.5% and 6.25%. The % transmittance of each dilution was recorded by using a Colorimeter (JENWAY PC01 filter No.2-470 nm).

The calibration curve was obtained by plotting the logarithm of spore concentration per cm<sup>3</sup> using the haemocytometer value on the X axis, against the logarithm of % transmittance on the Y axis.

3.2.1.4. DEFT count

The direct epifluorescent filter technique can reliably and accurately assess numbers of active vegetative bacteria and yeasts (Pettipher et al., 1980;

Rodrigues and Kroll, 1986) and bacterial spores (Kelly and Kroll, 1987).

The procedure of this technique is as follows:
From a 8/10 days malt extract agar slopes, a spore suspension was prepared from *P.digitatum* as described in section 3.2.1.1. The filtration, staining and membrane mounting were as procedure described in the section 3.7.

The spores fluorescing orange were counted in 25 fields by the image analyser and microcomputer. The spores that stained green or black were visually counted, and the DEFT count/ml was obtained by multiplying the average number of clumps/microscope field by the microscope factor. The microscope factor was calculated as follows:

#### M.F.= Area membrane through which sample was filt. (mm<sup>2</sup>)

Microscope field area (mm<sup>2</sup>) x Sample vol. (ml)

The area of the filter used was calculated from the internal radius of the filter tower ( $\mathbf{r} \times \text{radius}^2$ ). The area of the microscope field of view was calculated from the radius of the field which is measured using a stage micrometer. Calculations of membrane area and microscope field area are given below:

#### Membrane area

diameter = 15 mm

radius = 7.5 mm

Membrane area= 3.1415927 x (7.5)<sup>2</sup> = 176.71459 mm<sup>2</sup>

Microscope field area

diameter = 0.36 mm radius =0.18 mm Mic. field area =  $3.1415927 \times (0.18)^2 = 0.1017876 \text{ mm}^2$ 

### Microscope Factor = <u>176.71459</u> = 1736.11 0.1017876

#### 3.2.2. Inoculation of the fruit and fruit juice

Whole tomatoes at different maturation stages were surface sterilized with sodium hypochlorite solution (1% available chlorine) for 2 minutes, swabbed with 70% alcohol, washed with sterile distilled water and dried with absorbent paper.

The fruits were punctured with a sterile scalpel in the form of a cross (+) of about 2 x 2 cm and 4 mm deep into the tomato. Mycelial discs (diameter 0.7 cm) from the edge of 7 days cultures maintained on MEA, were cut and inoculated into the wounds. The inoculated fruits were incubated at 25  $^{\circ}$ C for 5 days, in plastic trays covered with cling film (plasticised PVC).

5g of tomato juice and crushed tomato were transferred to sterile MacCartney bottles and were inoculated by adding 0.1 ml of a spore suspension prepared as described in section 3.2.1.1. and incubated at 25 <sup>0</sup>C for 48 hours.

#### 3.2.3. Extraction of extracellular enzymes

The decayed rind and adhering tissue were removed, cut into small pieces of weight 5g and suspended (1:2 w/v) in a solution containing 2% NaCl (w/v) in 0.01M cysteine hydrochloride solution . The tissue was homogenised for 2 minutes using an Ystral GmbH D-7801 type X1020 homogeniser. The homogenate was filtered under suction, through sterile filter paper (Whatman No.1-5.5cm diameter). The filtrate was centrifuged at 2000 rpm for 10 minutes, using a MSE Super Minor centrifuge and the supernatant was collected with a Pasteur pipette. Any supernatant which was not to be used immediately was dispensed into 5 cm<sup>3</sup> aliquots and stored at -60  $^{0}$ C.

Several problems were associated with the use of whole tomatoes such as difficulty in producing extracts and latent infection of tomatoes giving erroneous results. It was decided to use tomato juice and crushed tomato.

#### 3.2.4. Apizym system

The Apizym system (API Laboratory Products Ltd) consists of a plastic gallery of cupules; in the bottom of each there is a fabric support carrying various substrates and buffer.For the preparation of the specimens, galleries, incubation, reading and recording the reactions, the manufacturer's instructions were followed throughout.

Samples tested included purified extracts from infected whole tomatoes, tomato juice and crushed tomato. 50  $\mu$ I of rot extract were inoculated into each cupule of the gallery by digital micro pipette (VOLAC Ref. R.880/B volume range 10-100  $\mu$ I). The gallery was placed in an incubator tray containing a little water to provide a humid atmosphere during incubation. The incubation tray was covered with a plastic lid and incubated at 37°C for 4 hours.

After incubation, the reaction was terminated by addition of 20  $\mu$ I of reagent ZYM A (tris-HCI,lauryl sulphate and distilled water) and colour was developed by addition of 20  $\mu$ I of reagent ZYM B (fast blue BB and 2-methoxy ethanol). The gallery was exposed to daylight for 10 minutes. Activity was obtained by comparing the colour developed in 10 minutes to the colour chart provided , and was expressed on the scale of 0 no activity, <1 trace, 1-2 low activity (5-10 nM substrate hydrolised), 2-4 medium activity (10-30 nM substrate hydrolised). The enzymes detected by the Apizym system are specified in Table 7.

#### 3.2.5. Diagnostic tablets

Diagnostic tablets containing chromogenic and modified conventional substrates which are able to detect preformed enzymes may be useful in the rapid differentiation/identification of fungi. The following diagnostic tablets were used:  $\beta$ -N-acetyl glucosaminidase (Ref. 500-21),  $\beta$ -galactosidase (ONPG) (Ref. 580-21) and  $\checkmark$ -glutamyl aminopeptidase/Caprylate Esterase (C-8 Est) (Ref.486-21) from Rosco Diagnostica.

0.25 ml of appropriate extract were dispensed into a test tube and a diagnostic tablet was added to each tube. The tube was covered with " Parafilm" and incubated at 37 <sup>0</sup>C for 4 hours.

After the incubation period the tests were read as follows: the  $\delta$ -glutamyl aminopeptidase test was considered to give a positive reaction, when after adding 20 µl of fast blue BB solution (Zym B) a red violet colour developed after 10 minutes and a negative reaction corresponded to yellow (to light brown). The β-galactosidase (ONPG) test was read as follows : A yellow colour (even slight) corresponded to a positive reaction, whereas colourless corresponded to a negative one.

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### TABLE 7. APIZYM SYSTEM - SUMMARY OF RESULTS

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		CUPCTDATE	-14	RESULT			
140	ENZTINE ASSATED FOR	SUBSTRATE	рп	Positive	Negative		
1	Control			No colour or colour of the sample if it has an important colouration			
2	Phosphatase alcaline	2-naphthyl phosphate	8.5	Violet			
3	Esterase (C 4)	2-naphthyl butyrate	6.5	·Violet	}		
4	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet			
5	Lipase (C 14)	2-naphthyl myristate		Violet	to an light.		
6	Leucine arylamidase	L-leucyl-2-naphthylamide	•	Orange	oosed itense		
7	Valine arylamidase	L-valyl-2-naphthylamide	•	Orange	en exf		
8	Cystine arylamidase	L-cystyl-2-naphthylamide		Orange	as be s. sed to		
9	Trypsin	N-benzoyi-DL-arginine-2-naphthylamide	8.5	Orange	llery h agent		
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	the ga the re theer		
<b>.</b> 11	Phosphatase acid	2-naphthyl phosphate	5.4	Violet	trol if on of as no		
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate		Blue	e con additi Iarv h		
13	a galactosidase	6-Br-2-naphthyl-2D-galactopyranoside	-	Violet	n of th alter than a		
14	β galactosidase	2-naphthyl-8D-galactopyranoside	*	Violet	coloui source		
15	ß glucuronidase	Naphthol-AS-BI-pD-glucuronide	-	Blue	ur or light s		
16	a glucosidas <del>o</del>	2-naphthyl-xD-glucopyranoside	-	Violet	o colc tense		
17	β glucosidas <b>e</b>	6-Br-2-naphthyl-βD-glucopyranoside	-	Violet	Z.S.>		
18	N-acetyl-β glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	*	Brown			
19	a mannosidase	6-Br-2-naphthyl-2D-mannopyranoside	-	Violet	]		
20	a fucosidase	2-naphthyl-xL-fucopyranoside	-	Violet	1		
		1		1	1		

Source: API System SA

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The B-N-acetyl glucosaminidase test was read immediately after the incubation period. Only a strong yellow colour was read as a positive reaction.

#### 3.3. Howard mould count

3.3.I. Single plan

The Howard cell *model* 44.002(m) (A.O.A.C. 1984) supplied by Graticules Ltd., was cleaned so that Newton's rings were produced between slide and cover glass (33 x 33 mm). Using a pasteur pipette, a portion of well mixed sample was placed upon the central disc, spread evenly and covered with a coverslip so as to give an uniform distribution. The slide was placed under a light microscope and examined using the x10 objective such that each field of view covered 1.5 mm.

For each sample, 75 fields were examined in such a manner as to be representative of all the sample, and a field was scored as positive if any of the following lengths exceeded one-sixth of field diameter, using a mould count record form (Fig. 21):

-length of single unbranched filament;

-length of single filament plus length of branches (aggregate length);

-aggregate length of two moulds;

-aggregate length of three moulds (no more than aggregate length of three mould filament could be counted);

-aggregate length of all filaments in a clump of mould (a clump of mould was considered a single piece, and aggregate lengths of all filaments were counted).





Source: Gould, 1983

The proportion of positive fields was calculated from the results of examination of all observed fields, and reported as percentage of positive fields containing mould filaments.

When crushed tomato was examined water was added to make a mixture having a total solids content 8.5 to 9.5%, (refractive index 1.3440 to 1.3454 corrected to 25 <sup>0</sup>C) as assessed by Abbé refractometer.

#### 3.3.2. Multiple plan

In this method increments of ten fields were counted according to the standard Howard mould count technique.

Depending on the product being tested, a selection was made of the appropriate mould count acceptance level (Table 5). The Howard mould count slide was prepared as previously described and counts in increments of ten fields were made. After counting the first ten fields:

a)the sample unit was accepted if the number of positive fields did not exceed the first value under the column C, (20% for tomato juice and 40% for crushed tomato from Table 8);

b)the sample unit was failed if the number of positive fields equaled or exceeded the first value under the column R (Table 8);

c)subsequent following counts were made on an additional ten fields if a decision to accept or fail the sample unit could not be reached on the first ten fields, i.e., if the number of positive fields fell between column C and column R. The process continued until the number of positive fields fell into the conditions referred in a) or b) and the sample was subsequently accepted or rejected.

	10%		20%		30%		40%		50%		60%		70%		80%	
N <sub>c</sub>	-c =	= r	C	r	c	r	С	r	с	r	с	r	с	r	C	r
10	1	4	1	6	0	7	0	8	1	9	2	10	4	10	5	2
$\tilde{2}\tilde{0}$	1	5	0	8	2	10	4	13	6	15	8	17	10	19	13	20
$\overline{30}$	· 1	Ĝ	3	10	5	13	7	17	11	19	14	23	17	26	20	28
40	ĩ	7	5	12	8	16	11	21	16	25	20	29	<b>24</b>	32	28	36
50	$\overline{2}$	8	7	14	11	19	· 15	25	21	30	26	35	31	40	36	44
60	3	9	9	16	14	22	20	29	<b>26</b>	34	32	41	38	48	44	52
70	4	10	11	18	17	25	24	33	31	40	38	46	45	54	52	60
80	5	- 11	13	20	<b>20</b>	28	28	36	36	45	44	52	52	62	61	68
90	6	11	15	22	23	31	32	40	42	49	50	58	60	68	70	76
100	8	12	17	23	26	- 35	37	43	47	54	57	64	66	75	78	83
110	10	13	20	23	. 30	- 37	42	47	53	58	63	69	74	80	86	90
120	12	13	24	25	36	37	48	49	60	61	72	73	84	85	96	97

<sup>o</sup>N<sub>c</sub>, the cumulative number of fields to count; c, the maximum cumulative number of positive fields permitted to accept the sample unit for the appropriate percent mold level; r, the minimum cumulative number of positive fields necessary to fail the sample unit for the appropriate percent mold level. <sup>b</sup>The sample unit cannot be accepted at this level. <sup>c</sup>The sample unit cannot be rejected at this level.

Gould, 1983

The total number of fields tested and the number of positive fields found were cumulative. The results were recorded as number of positive fields per number of fields counted and not as percentage of positive fields.

3.4. Total viable count

3.4.1. Plate count - spread plate technique

This technique has been used as a standard method for mould counting for many years. Throughout this study, it was used as a reference point of comparison with the results obtained with the other methods.

The autoclaved sterilized, molten, cooled oxytetracycline glucose yeast extract agar (OGYEA) was poured into sterile petri plates. After solidification , the plates were dried slightly before use using a plate dryer (Unitemp Drying Cabinet N0.2 Laboratory Thermal Equipment). Aliquots of serial dilutions (0.1 ml) from 10<sup>-1</sup> to 10<sup>-6</sup> in quarter strength Ringer solution (Oxoid Code BR52) were added to the dried surface of each plate and uniformly spread over the agar by means of a flame- sterilized glass rod, bent in the shape of a hockey stick.

During incubation at 25  $^{0}$ C for 48 hours, growth and multiplication of cells occurred until a visible colony was formed. Counting was performed on the plates that contained between 30 and 300 colonies. The number of colonies was multiplied by the dilution factor, and divided by the quantity of inoculum (0.1 ml) and reported as the number of colony forming units per g (c.f.u./g).

#### 3.5. Bioluminescence assay

This method checks the sterility of fruit juices. For this reason, they must first be incubated at a specific temperature, so that even a single contaminating microorganism can multiply to the point where its presence can be detected using the reagents described in sectin 3.5.1 and measured with LUMAC 2010 A Biocounter.

#### 3.5.1. Reagents

Reagents used in Bioluminescence ATP assay were LUMIT-PM, purified luciferin-luciferase containing purified firefly (*Photinus pyralis*) luciferase,D-luciferin, bovine serum albumin, dithiotreitol in freeze dried form for the sensitive measurement of ATP ; LUMIT-buffer pH 7.75 containing 21 ml 0.025M Hepes buffer, MgSO4, EDTA and sodium azide which suppresses microbial growth: L-NRB nucleotide releasing reagent for microbial cells containing 11.2 ml of an ionic surfactant enabling the ATP release from microbial cells; F-NRS nucleotide releasing reagent for somatic ATP in fruit juices containing 10.2ml.of a non-ionic surfactant, 0.250M Hepes buffer; F-SOMASE, calcium activated ATPase, containing potato-apyrase in freeze dried form, ensuring a quantitative release of intracellular ATP from somatic cells.

#### 3.5.2. Sample preparation

The sample was prepared according to the manufacturer's instructions (Lumac's Method for Rapid Sterility Testing of Fruit Juices).

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Initially, the fruit juice kits supplied one set of instructions (Method 1) but these were superceded when the composition of the kits changed to enable automatic assays to be carried out (Method 2). Both methods are quoted below, but of necessity Method 2 has been used recently.

Method 1 (Cat. N0. 9289): from each of both samples, a control sample (non pre-incubated) and another one incubated at 25  $^{0}$ C for 48 hours, 500  $\mu$ I were removed into a cuvette using a digital micro pipette (VOLAC Ref. 880-E volume range 100-1,000  $\mu$ I), and added to 500  $\mu$ I of F-NRS and 20  $\mu$ I of SOMASE. After incubation at room temperature for 45 minutes, two portions of 50  $\mu$ Iwere separated into different cuvettes and followed with normal procedure for ATP measurement.

Method 2 (Cat. 9289-3): 50  $\mu$ l of each sample was taken (under the same initial conditions) and 100  $\mu$ l of F-NRS and 50  $\mu$ l of SOMASE added. The assay then proceeded as above.

No instructions were available in either test as to the sample concentration to be used, and after several assays a 1:10 dilution proved to be the most suitable for the ATP extraction and subsequent assay.

#### 3.5.3. ATP measurement

The cuvette was transferred into the counting chamber of the biocounter and was injected with 100  $\mu$ I of L-NRB.After 30 seconds the same quantity of LUMIT-PM was injected into the cuvette and the result was recorded after 10 seconds as RLU (relative light units).

The sample was considered non-contaminated if the RLU for the incubated sample was less than three times the RLU reading for the control sample, and contaminated if it was more. In the case where the sample RLU was less then three times the RLU for the control sample, but was more than two times that value, the result was confirmed by extending the incubation period by another 24 hours.

A total viable count (TVC) on OGYEA (Oxytetracycline glucose yeast extract agar, Oxoid Code CM 545 and Oxoid supplement Code SR 73) was carried out for all the samples, in parallel as the ATP assay, and colonies enumerated after incubation at 25 <sup>0</sup>C for 48 hours. Details of the TVC method were as described in section 3.4.

#### 3.6. Direct epifluorescence filter technique

The DEFT technique is a direct microscopic method which enables the rapid assessment of bacteria and bacterial spores by using acridine orange solution; this dye causes living organisms to fluoresce orange under UV light. The following reagents and equipment were used in this study:

Acridine Orange (0.025% w/v) in 0.1M citric acid-NaOH buffer pH 6.6; 0.1M citric acid -NaOH buffer pH 3.0; Iso-propyl alcohol and non fluorescent immersion oil.

Epifluorescence microscope (NIKON) with appropriate UV light source; filters for blue /violet fluorescence at 470 nm and eyepiece graticule.

NIKON mercury 1Amp power supply (HBO-100W/2)

AMS image analyser with a combination of high speed video processing circuitry and an 8 bit microcomputer

NEC JB-902M monochrome character display optimized for use as a computer video display terminal

Filter DEFT MANIFOLD 4015 and towers for 25 mm membrane;

Vacuum pump (AEI A.C. Motor type BS.2406 B B56-A-1.425 rpm);

Polycarbonate membrane filters (PC MEB. 25 mm diameter 0.6 mm pore size

).

Minisart (NML SM 16534K) cellulose acetate disposable filter (0.2 m pore size) (for purification of reagents)

35 µm pore size nylon filter for pre-filtration of tomato juice

#### 3.6.1. Sample preparation

A sample of tomato juice "spiked" with a mould culture and another one without artificial contamination were incubated at 25  $^{0}$ C for 48 hours, diluted 1:10 in sterile distilled water and pre-filtered through a pre-sterilized 35 $^{\mu}$ m pore size nylon filter.

#### 3.6.2. Filtration

A nucleopore polycarbonate membrane filter (0.6µm pore -size)was mounted with the shiny side uppermost onto the sintered glass base of the filter manifold, connected to a vacuum pump, and the filter tower replaced. One ml of the pre-filtered diluted sample was filtered. The membrane filter was rinsed by filtering 2.5 ml of Isopropanol through it.

#### 3.6.3. <u>Staining the membrane</u>

The membrane filter was overlayed with 2.5 ml of buffered acridine orange solution, previously filtered through a cellulose ester membrane filter ( $0.2\mu$ m pore size) to remove bacteria and particulate matter, and allowed to stain for 2 minutes. The membrane was washed by filtering with 2.5 ml of 0.1M citric acid-NaOH buffer solution pH 3.0, filtered through the same type of filter as the acridine orange solution and rinsed again by filtering with 2.5 ml of Isopropanol.

#### 3.6.4. Mounting the membrane

The filter tower was removed then the filter membrane,

using a sterile scalpel to lift it and a pair of wide tipped forceps to hold and transfer it. It was thoroughly air dried . A small drop of DEFT immersion oil was placed on a clean microscope slide and the membrane filter laid on top, shiny side uppermost. Another drop of immersion oil was placed on top of the filter with a coverslip on top of this and everything was pressed down on a firm surface to reduce the thickness of the oil layers.

#### 3.6.5. Counting the moulds

Single spores, clumps or fungal hyphae were counted in 25 random fields and the final count per ml was obtained from the microcomputer.

A TVC on oxytetracycline glucose yeast extract agar (OGYEA) was done in parallel with every DEFT assay.

#### 3.7. Impedimetric detection

As microorganisms grow in a suitable medium their metabolic activity breaks down substrates into simpler compounds. This changes the electrical characteristics of the medium and may be detected by applying an alternating voltage across two electrodes immersed in the culture.

A unique feature of impedimetry is that the greater the concentration of cells, the faster a result is obtained.

In this study Bactometer model 32 (Fig. 22), Bactometer incubator and Honeywell thermal strip chart recorder (Electronic 196) (Fig. 22) and Bactometer disposable modules (Fig. 23) were used throughout.

#### 3.7.1. Sample inoculation

A spore suspension was prepared as described in the section 3.2.1.1., from slopes of moulds listed in section 3.1.2. From each spore suspension 1 ml was removed into a separate vial and mixed together. 5 g of tomato juice were "spiked" with 0.01 ml of the previous mixture and incubated at 25 <sup>0</sup>C for seven days.

#### 3.7.2. Disposable modules - filling procedure

A sterile disposable Bactometer module was removed from its bag and positioned on the bench such that the metal connector faces away from the operator. The tape from both the front and the rear wells of ,the module were removed as eptically and discarded. Four modules were used in all: the wells of two modules had 1.5 ml of sterile malt extract agar (MEA) added to each and allowed to cool; the other two modules were filled in the same way with potato dextrose agar (PDA) and all of them were aseptically covered with sterile tape. Previous tests showed that these two media gave the best (shortest) detection times.

The test wells (Fig. 23 Nos. 9,10,11,12,13,14,15,16) were inoculated in duplicate with 0.1 ml of a serial dilution  $(100/10^{-7})$  in sterile distilled water, prepared from the contaminated sample. The reference wells (Fig. 23 Nos. 1,2,3,4,5,6,7,8) were inoculated with the same volume as the inoculum (0.1 ml) and the same diluent (sterile distilled water), to make the volume of both the sample and reference wells identical and to minimize the amount of drift occurring in the strip chart channel. The modules were incubated at 25  $^{0}$ C in the Bactometer incubator.

FIG. 22 BACTOMETER MODEL 32 (FRONT VIEW)



Source: Bactomactic

FIG.23 BACTOMETER DISPOSABLE MODULES



Source: Bactomatic

3.7.3. Calibration curve

This procedure was carried out immediately after the sample inoculation, and after 2,4 and 7 days of incubation at 25 <sup>0</sup>C, in order to obtain various detection times corresponding to different levels of mould growth within the sample.

The calibration curve was obtained by plotting the bactometer detection time in hours on the X axis, against the logarithm of the TVC counted on PDA (Oxoid Code CM139) and MEA for the same concentrations assessed at 2 days, 4 days and 7 days, on the Y axis. This made it possible to relate impedance detection time to viable count.

#### 3.7.4. Use of the calibration curve to assess fruit juice sterility

Several different samples of tomato juice were purchased at the local retail outlets, and were tested for their sterility using the calibration curves previously obtained. Knowing the detection time for each sample the TVC and therefore the nunber of c.f.u./g was easily obtained from the calibration curve. The opposite situation has also been tested as follows: 5g of each one of the different samples were separately contaminated with 0.1 ml of a spore suspension of *Aspergillus niger* and *Rhizopus stolonifer* prepared as described in section 3.2.1.1., and incubated at 25 <sup>0</sup>C for 48 hours. After the incubation period, the bactometer detection times were obtained as described in the previous section and again correlated with the number of c.f.u./g of mould present in the tested samples.

A TVC on PDA was carried out in parallel for each sample after the incubation period.

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#### 4. RESULTS AND DISCUSSION

#### 4.1 Howard mould count

The Howard mould count (HMC) method has been used since the early 1900s to detect rot caused by mould in comminuted tomato products. However, some criticism has been directed against this method with regard to its declared objective. Among these objections the first one covers the validity of relationship between the count and the percentage of unsound fruit or unsatisfactory processing conditions and the second one refers to the accuracy or reliability of the technique itself (Dakin, 1964).

An attempt was made to investigate and assess the potential of this method, using it to determine the percentage of positive fields in tomato juice and crushed tomato in three different situations.

#### 4.1.1 Mould assessment on tomato juice

From three samples of tomato juice, one was inoculated with *A.niger* spore suspension as described in section 3.2.2. and incubated at 25 <sup>0</sup>C. Another sample was simply incubated at 25 <sup>0</sup>C, and the third one was kept in the refrigerator with no inoculation. The three samples were daily assessed using HMC and the results are represented in Fig. 24.

A general relationship normally exists between the HMC and the quantity of spoiled, rotten or mouldy fruit used in preparing a product, as shown in Fig. 24, where sample C, inoculated with *A.niger*, and incubated at 25  $^{0}$ C for 48 hours, gave the highest percentage of positive fields (60%), as expected.

However, some questions regarding the validity of HMC arise from these results: for instance on day 0, the samples A and B with no inoculation, show a big difference between the counts obtained with HMC, with 28% (sample A) and 9% (sample B), respectively. Moreover, HMC gives a higher count

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### Fig.24 Mould Assessment

on Tomato Juice samples





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reading on sample A with no inoculation, rather than the sample C (26%) inoculated with *A.niger*. In addition, the number of positive fields progressively increases in both the samples (A and B) with no inoculation, over the 3 days period, where sample A almost reached the same HMC (52%) as the inoculated sample (C) with 60%, which shows some inaccuracy or inconsistency of HMC in assessing mould fragment in these products.

#### 4.1.2 Mould levels in tomato juice and crushed tomato

Three different batches of tomato juice were examined for the mould acceptance levels over a 15 days period; the data obtained using Howard mould count is represented in Fig. 25. This figure shows a wide range of values corresponding to the three different samples, with initial values of 33.3% in sample A (greater than the acceptance level which is 20%) , 20% in sample B and 16% in sample C and termination values (day 15) of 29.3% (Sample A), 64% (Sample B) and 13% (Sample C) being observed. The coefficient of variation corresponding to these samples of tomato juice was 13.4% for sample A, 31.6% for sample B and 47.4% for sample C, which clearly shows the unreliability of the HMC in providing consistent results.

The same assessment was done using crushed tomato and another tomato juice sample, over 20 days; mean values are presented in Fig. 26. Howard mould counts obtained on crushed tomato show a variation between 44% (day 0) which is slightly higher than the 40% of acceptance level referred in Table 5 , and 12% (day 20), whereas in tomato juice they vary between 24% (day 0) and 30.6% (day 20), with coefficients of variation of 39.9% for tomato juice and 17.8% for crushed tomato.

Once more these results show that HMC is not a very accurate method to assess mould presence in tomato products. Comparing the coefficient of variation of the last sample of tomato juice (39.9%) with the above samples

# Fig.25 Mould levels in three different Tomato Juice samples



Howard Mould Count Results are mean values of 3 experiments performed in triplicate

# Fig. 26 Comparison of mould levels bet. Tomato Juice & Crushed Tomato



Howard Mould Count

Results are mean values of three experiments performed in triplicate

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(13.4% sample A, 31.6% sample B,and 47.4% sample C), presented in the Fig. 25, there is a difference in the results given by HMC. The coefficient of variation of tomato juice (39.9%) and crushed tomato (17.8%) are also very different. According to Bandler *et al.*, (1987) the number and size of mould filaments present, depends not only on the amount of rot in the raw stock, but also the method of processing.

The process involved in tomato juice production is longer than the one used to produce crushed tomato, and the longer the processing line, the higher is the possibility of mould being fragmented and distributed throughout the mass, thus giving a relatively high count reading, or the product to become contaminated if there is a poor plant sanitation.

#### 4.1.3 Mould contamination in tomato juice and crushed

#### tomato inoculated with a wide range of moulds

Four samples of tomato juice (C, D, E, F) were inoculated with *C.herbarum*, *F.culmorum*, *R.stolonifer and P.digitatum* respectively and incubated at 25  $^{0}$ C. One sample with no inoculation was also incubated at 25 $^{0}$ C - Sample A (control incubated) - and the last sample - B (control) - was kept in the refrigerator and assessed by HMC at the same time as the others. All the samples were assessed by HMC for mould presence after 48h, 72h and 96h of incubation. The results obtained are presented in Fig. 27. With exception of sample F (inoculated with *P.digitatum*) which after 96h of incubation showed a fall to 64%, there is a continuous increase of the HMC values over the 96h incubation period, as expected. Even sample B (with no incubation), which was kept in the refrigerator, increases from 20% to 28% (after 72h) rising to 30% after 96h, which may show the ability of moulds to grow in a product, despite the temperature not being their optimum; at 25  $^{0}$ C, which is the optimum temperature, they can give different values, as moulds vary between

### Fig.27 Mould levels in Tomato Juice

inoculated with a range of moulds



species in the quantity and quality of mycelium they produce in relation to weight of product spoiled. Some are capable of causing a large amount of spoilage with little mycelium present, whilst others produce masses of hyphae in fragmented form (Dakin, 1964). This, when assessed by the HMC will give different count readings, depending on the species, as shown on Fig. 27.

Fig. 28 presents the results obtained with HMC on crushed tomato, under the same experimental conditions as tomato juice, i.e. four samples were inoculated with the same species of mould and incubated at 25 <sup>0</sup>C; one sample was just incubated at 25 <sup>0</sup>C and another one was kept in the refrigerator.

The results show some differences in mould growth in crushed tomato; for instance, the control sample B-1 with no incubation or inoculation, maintains the same value of 28%, after 48h and 72h, reaching 36% after 96h, suggesting that crushed tomato is possibly a more stable product than tomato juice, perhaps regarding its enzymic content or other constituents that possibly are more efficient in "protecting" this product from mould action.

All the other samples of crushed tomato show a lower value at 48h of incubation period, comparing with the values obtained with tomato juice (Fig. 27). Furthermore the percentage of positive fields obtained after 72h in crushed tomato, are generally lower than the ones reache in tomato juice. However after 96h of incubation, all the readings follow the same increase noted in tomato juice, with exception of samples D-1 and E-1, inoculated with *R.stolonifer* and *F.culmorum* which gave 92% and 96% of positive fields, respectively.

#### 4.1.4 Howard mould count count - general discussion

As has been discussed before, the results obtained with HMC show the fluctuation of values between wide limits, in the different situations that were

### Fig.28 Mould levels in Crushed Tomato

inoculated with a range of moulds



assessed. Thus a badly spoiled sample can on occasion give low counts as happened in crushed tomato (Fig. 28), inoculated with *C.herbarum* (C-1) and *P.digitatum* (F-1) with values of 32% and 36%, respectively, after 48h incubation which is lower than the acceptable level for crushed tomato (40%) meaning that the finding of a low count is not necessarily a reliable indication of the use of sound fruit and hygienic conditions.

Moreover, the reliability of the Howard mould count, and its ability to provide consistent results is a question upon which, opinions differ widely. Dakin (1964) claimed that "in the hands of properly trained analysts the Howard mould count is a satisfactory method and the results obtained by following the official procedures are reasonably consistent".

It is clear that the value of the Howard mould count is extremely suspect and that its use for quality assessment of samples especially for legislative purposes cannot be defended. (Dakin, 1964).

Current efforts have been made to use other methods such as Apizym system, ATP photometry, direct epifluorescent filter technique and impedance monitoring, which are based on other principles, rather than the visual identification and counting of mould filaments within a product. Therefore some attempts were made to check their viability in substituting Howard mould count.

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#### 4.2 Apizym system

As an alternative to Howard mould count, the Apizym system was used to assess mould presence in several samples of tomato fruit, and tomato products such as tomato juice and crushed tomato.

This is a semi-quantitative micromethod enzyme system designed for the detection of 19 individual enzymes, which can be very helpful in identifying moulds through their enzymic profile (Bridge and Hawksworth, 1984).

In this study, tomato fruits were inoculated with *A. niger*, *P. digitatum* and *R. stolonifer* as described in section 3.2.2., and stored at room temperature for 4 days. A control sample was also tested during the same period of time.

The enzymic activity of the above moulds with *C. herbarum* and *F. culmorum* inoculated in tomato juice and crushed tomato, was also assessed using Apizym system after incubation at 25  $^{0}$ C for 48 h.

It was decided to change the length of time of the experiment to 48h, in order to get an assessment at the same time as ATP assay which was being used in parallel (section 4.3.).

#### 4.2.1. Enzyme profiles of fungal rot extracts obtained from tomato fruit

Fig. 29 shows enzyme activity of *A.niger* rot extract obtained as described in section 3.2.3., from tomato fruit after four days. The following classification was used throughout this study: <1 trace amounts, 1-2 low activity (5-10 nM substrate hydrolysed), 2-4 medium activity (10-30 nM substrate hydrolysed), 4-5 high activity (30-40 nM substrate hydrolysed); a fold cut table (Table 7) is given to assist in interpretation of the data, at the end of this section. The following enzymes (the figure in parenthesis refers to well number in Table 7) were detected with medium activity on day 0: acid phosphatase (11),  $\beta$ galactosidase (14) and N-acetyl-glucosaminidase (18). Enzymes glucosidase

# Fig.29 Aspergillus niger Enzymic Activity in Tomato Fruit



Results represent mean values of three experiments performed in triplicate

(16) and ß glucosidase (17) were not detected on day 0. The enzymes lipase (C14) (5), trypsin (9), chymotrypsin (10), ß glucuronidase (15), mannosidase (19) and fucosidase (20) were not detected in any of the sampling days. The lack of enzyme activity in wells (9), (10), (15), (19) and (20) was in agreement with Ngabirano(1987) except for lipase (C14) (5) which the author found to be active, when using extracts of *A.niger* in decayed citrus fruit.

The remaining enzymes were detected with low activity (2), (3), (4), (6), (7), (8), (12) and (13).

On day 2, medium activity was detected in enzymes alkaline phosphatase (2), acid phosphatase (11), and high activity in naphthol-AS-BI-phosphohydrolase and N-acetyl-B glucosaminidase (18). On day 4, high activity was (12), detected in enzymes acid phosphatase (11), naphthol-As-Blphosphohydrolase (12) and ß galactosidase (14), and medium activity in cystine arylamidase (8), galactosidase (13) and N-acetyl-B glucosaminidase (18). Extracellular enzymes produced by *P.digitatum* in tomato fruit are shown in Fig 30. On day 0, medium activity was detected in enzyme acid phosphatase (11) and high activity in enzyme N-acetyl-ß glucosaminidase (18); low activity was detected in wells (2), (3), (4), (6), (7), (8), (12), (14), (17) and (19).

On day 2, unlike *A. niger*, high activity was detected in acid phosphatase (11) and  $\beta$  galactosidase (14), but similarly high activity in N-acetyl- $\beta$ glucosaminidase (18) was noted in both fungi. On day 4, the enzyme profile of *P. digitatum* shows slight. differences to that of *A. niger* notably in enzymes N-acetyl- $\beta$  glucosaminidase (18) where high activity was detected and  $\beta$  glucosidase (17) with medium activity which was not detected in *A. niger* profile; high activity was also found in acid phosphatase (11), naphthol-AS-BI-phosphohydrolase (12), and  $\beta$  galactosidase (14), results which are comparable to *A. niger*.

# Fig.30 Penicillium digitatum Enzymic Activity in Tomato Fruit



Results are mean values of three experiments performed in triplicate

In *R. stolonifer*, (Fig. 31) alkaline phosphatase (2), acid phosphatase (11),  $\beta$  galactosidase (14) and N-acetyl- $\beta$  glucosaminidase (18) were detected with medium activity on day 0, whereas enzymes (3), (4), (6), (7), (8), (12) and (13) showed low activity. On day 2, the highest activity was detected in enzymes (11), followed by medium activity in enzymes (12), (14) and (18). On day 4, unlike the enzyme profiles of *A. nigher* and *P. digitatum* alkaline phosphatase (2) was detected with high activity ;also esterase C4) (3) and esterase lipase (C8) (4) showed medium activity. Acid phosphatase (11) and naphthol-AS-BI-phosphohydrolase (12) and  $\beta$  galactosidase (13) and N-acetyl- $\beta$  glucosaminidase (18) with medium activity comparable to the previous two fungi, and trace amounts of activity in wells (5), (7), and (16).

Colour intensity values and enzymes detected in extracts from control samples for day 0, 1, 2, 4, respectively, are shown in Fig. 32. No enzyme activity was detected in enzymes in wells (5), (9), (10), (15),(16), (19), (20) during the four days. On day 0, low activity was detected in alkaline phosphatase (2), esterase (C4) (3), esterase lipase (C8) (4), leucine arylamidase (6), valine arylamidase (7), cystine arylamidase (8), naphthol-AS-BI-phosphohydrilase (12),  $\prec$  galactosidase (13) and N-acetyl- ß glucosaminidase (18). Medium activity was detected in (11) and (14).

On day 1 no difference was exhibited except for enzymes in wells (8), (12), (18) which slightly increased, and (14) where a slight fall in colour intensity was noted.

On day 2, the enzyme activity increased in enzymes (2), (11), (11), (12), (14) and (18) whereas low activity was detected in esterase (C4) (3), leucine arylamidase (6), galactosidase (13) and ß glucosidase (17).

On day 4 only enzymes in wells (13) and (14) increased their colour intensities.

# Fig.31 Rhizopus stolonifer Enzymic Activity in Tomato Fruit



Results are mean values of three experiments performed in triplicate

# Fig.32 Control Sample Enzymic Activity in Tomato Fruit



Results are mean values of three experiments performed in triplicate

These data suggest this experiment is irreproducible using whole tomato, because of the various interferences from mould contaminants detected in the control samples during storage period. This seems to be quite common as referred in section 2.5 Ayres *et al.*, (1964) reported that the susceptibility of tomato to fungal decay increases, wherever the skin is broken or when injuries caused by packing and grading equipment, or damage caused by rough handling during sorting in transit and in the market occur. Also, a few rotting tomatoes in close proximity to sound fruit constitute an added hazard since injuries that are not evident in green tomatoes are prominent in ripe fruit. It was decided to use tomato juice and crushed tomato as an experimental model because of the greater consistency of the raw material. Any changes in enzymic profile could then be more closely related to fungal activity.

# 4.2.2 Enzyme profile of fungal rot extracts obtained from tomato juice and crushed tomato

The enzyme profile of *A. niger, P. digitatum, R. stolonifer, C. herbarum* and *F. culmorum* were assessed following inoculation into tomato juice and crushed tomato (Fig.33 and 34) using standardized inocula obtained from spore suspensions as described in sections 3.2.1.1 and 3.2.2 and incubated at 25 <sup>0</sup>C for 48 hours.

Comparing both enzyme profiles after the incubation period, some differences were noted as follows: in the control sample of crushed tomato, low activity of enzymes (4), (11) and (12) were detected, wheras in the control sample of tomato juice, low activity was found in wells (3), (11) and (12). In the incubated sample of crushed tomato without any artificial contamination, low activity was detected in wells (2), (3), (4), (11), (12), (14), (17) and (18). The incubated sample of tomato juice (without contamination) shows some low activity in enzymes in wells (2), (3), (4), (11) and (12).

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# Fig.33 Enzymic Activity fungal growth in commercial tomato juice



Incubation conditions: 48h at 25 C Results are mean values of three experiments performed in triplicate

# Fig.34 Enzymic Activity fungal growth in com. crushed tomato



Incubation conditions: 48h at 25 C Results are mean values of three experiments performed in triplicate This may suggest that the hot-break procedure, the preliminary heating given the tomatoes to destroy the enzymes and to protect the constituents of the tomato (especially pectin) from enzymic change (Gould, 1983), probably was not efficiently adequate in these control samples, or that heat processing has inadequate and suggests the APIZYM could be used as a possible monitoring test for assessing heat-treatment adequacy, or to monitor post-process contamination.

Since there is more activity associated with the incubated sample, this suggests that low enzyme activity associated with slow microbial growth was present.

Rot extracts from *C. herbarum* in crushed tomato show low activity in enzymes (4), (11), (12), (14) and (18), whereas in tomato juice low activity of enzymes (4) and (12) were detected. The enzyme activity of *F. culmorum* was similar in both products revealing low activity in enzymes (4) and (12), possibly related to the fact that this organism is slower growing.

*R. stolonifer* enzyme activity in crushed tomato shows high activity in alkaline phosphatase (2), medium activity in naphthol-AS-BI-phosphohydrolase (12), low activity in esterase lipase (C8) (4) and d galactosidase (13). Unlike in crushed tomato , *R. stolonifer* in tomato juice, shows no activity at all in enzyme in well (2), medium activity in enzyme (11) and low activity in enzymes (3),(6) and (12).

The enzyme activity of *A. niger* in crushed tomato shows high activity in enzyme (12) and (13), medium activity in enzyme (18) and low activity in enzymes (2), (3) and (4). In tomato juice *A. niger* shows high activity in enzymes (11) and (12), medium activity in enzyme (4) and low activity in enzymes (2),(3),(4),(13),(15) and (18).

*P. digitatum* in crushed tomato show medium enzyme activity in well (12), low activity in enzymes (2),(3), (4),(13),(15),(18). In tomato juice *P. digitatum* the highest activity in  $\beta$  galactosidase (14), low activity

in enzymes (2), (3), (11), (12), (17) and (18).

Different patterns of extracellular enzymes were detected in these products.

4.2.3. <u>Significance of differences in enzyme profiles associated with moulds</u> growing in tomatoes and their products

Table 9 shows some differences in the profiles obtained from tomato fruit inoculated with *A. niger*, *P. digitatum* and *R. stolonifer* over 4 days of storage at room temperature. These discussion results are tentative because of latent infection in tomato fruits affecting results in control sample (Table 10). Enzymes (17) and (18) could be used to distinguish *P. digitatum* from *A. niger* and *R. stolonifer* in tomato fruit, because enzyme (17) was detected neither in *A.niger* nor in *R. stolonifer* over the 4 days of storage, but it gave medium activity in *P. digitatum* on day 4, and the enzyme (18) gave high activity in *P. digitatum* on day 4, whereas in *A.niger* and *R. stolonifer* it gave only medium activity.

Regarding the profile of *R. stolonifer*, enzymes (2) with high activity, (3) and (4) with medium activity would help to differentiate this species from *A. niger* and *P. digitatum* because these enzymes were not detected at this level on any of the sampling days, except enzyme (2) which was only detected on day 2 in both fungi (*A. niger* and *P. digitatum*) with medium activity, unlike in *R. stolonifer*.

Comparing Tables 11 and 12 the following conclusions can be drawn, regarding the profiles of the various fungi in tomato juice and crushed tomato respectively.

*C. herbarum* and *F. culmorum* gave the same low activity in tomato juice which could be related to the fact that these microorganisms are slow growing organisms. In crushed tomato *F. culmorum* maintained the same profile as in tomato juice, but *C. herbarum* gave very slight differences which

#### ENZYMIC ACTIVITY DETECTED IN TOMATO FRUIT INOCULATED WITH A.niger, P.digitatum AND R.stolonifer DURING STORAGE USING THE APIZYM SYSTEM

#### **TOMATO FRUIT**

A niger					P.digitatum			R.stolonifer				
W	D0	D1	D2	D4	D0	D1	D2	Đ4	DO	D1	D2	 D4
2 3 4		2.0	3.5			2.2	3.5		3.0	2.0		4.5 3.5 3.7
5 6 7						2.0			2.0	2.0		
/ 8 9				2.5	2.0	3.0	3.0	3.5	2.0	3.0	2.2	
11 12 13	3.0	2.5 3.0 2.0	3.0 4.0	5.0 5.0 3.0	3.5 2.0	3.0 3.0 2.0	4.5 3.2	5.0 4.5 3.2	3.0	3.0 2.2	4.0 3.0	5.0 5.0 3.2
14 15 16	3.5	3.0		5.0	2.0	3.0	4.0	4.5	3.5	3.0	2.5	5.0
17 18 19 20	2.5		4.5	3.0	4.0	2.0 3.5	4.5	3.5 4.0	2.5	3.0	2.5	3.5

Results represent mean values of 3 experiments performed in triplicate. Only medium and high activity are presented.

#### Legend:

W = well D = day

<1 Trace 1-2 Low activity (5-10 nM substrate hydrolysed) 2-4 Medium activity (10-30 nM substrate hydrolysed) 4-5 High activity (30-40 nM substrate hydrolysed)

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#### ENZYMIC ACTIVITY DETECTED IN CONTROL SAMPLE OF TOMATO FRUIT DURING STORAGE USING THE APIZYM

Weil	DO	D1	D2	D4
2 3 4		2.2	3.5	
5 6 7		2.0	2.0	•
8 9 10	2.0	3.0	3.0	2.2
11 12 13 14	3.0 2.0 3.5	3.0 3.0 2.0 3.0	4.2 3.2 2.0 4.0	2.5 4.5
15 16 17 18		2.0 3.5	4.5	3.0
19 20				

### **CONTROL SAMPLE OF TOMATO FRUIT**

Results represent mean values of 3 experiments performed in duplicate. Only medium and high activity are presented

### Legend:

<1 Trace 1-2 Low activity (5-10 nM substrate hydrolysed) 2-4 Medium activity (10-30 nM substrate hydrolysed) 4-5 High activity (30-40 nM substrate hydrolysed)

D = Day

Well	Δ	 B	С	D	 F		 G
					<b>-</b>	•	
2 3 4 5 6 7 8 9						2.0 3.0	
10 11 12	2.0	2.0			3.0 2.0	5.0 4.5	2.0
13 14 15 16 17 18 19 20							4.5

#### ENZYMIC ACTIVITY ASSOCIATED WITH FUNGAL GROWTH IN COMMERCIAL TOMATO JUICE USING THE APIZYM SYSTEM

TOMATO JUICE

Results are mean values for 3 experiments performed in triplicate. Only medium and high activity are presented.

#### Legend:

<1 Trace

1-2 Low activity (5-10 nM substrate hydrolysed) 2-4 Med. activity (10-30 nM substrate hydrolysed) 4-5 High activity (30-40 nM substrate hydrolysed)

A - Control sample

- B Sample incubated at 25 °C
- C Sample inoculated with C.herbarum
- D Sample inoculated with F.culmorum

E - Sample inoculated with *R.stolonifer* F - Sample inoculated with *A.niger* 

- G Sample inoculated with *P.digitatum*

#### **ENZYMIC ACTIVITY ASSOCIATED WITH FUNGAL GROWTH IN** COMMERCIAL CRUSHED TOMATO USING THE APIZYM SYSTEM

Well	Α	В	С	D	E	F	G
2 3 4 5 6 7		2.0	2.0		4.0		
8 9 10 11 12		2.0			3.0	5.0	3.0
13 14 15 16		2.0			2.0	4.0	2.0
17 18 19 20						2.5	

**CRUSHED TOMATO** 

Results are mean values of 3 experiments performed in trilicate. Only medium and high activity are presented.

#### Legend:

- <1 Trace
- 1-2 Low activity (5-10 nM substrate hydrolysed) 2-4 Med. activity (10-30 nM substrate hydrolysed) 4-5 High activity (30-40 nM substrate hydrolysed)
- A Control sample
- B Sample incubated at 25 °C
- C Sample inoculated with C.herbarum
- D Sample inoculated with F.culmorum
- E Sample inoculated with R.stolonifer
- F Sample inoculated with A.niger
- G Sample inoculated with P.digitatum

were always in the range of low activity, and therefore of minimum significance. However, if the general profile of all moulds detected in these two tomato products is considered, then these so called minimmum differences could be very important, and help to distinguish these two species from the other ones.

Enzyme (2) was determinant not only to distinguish *R. stolonifer* in crushed tomato from tomato juice, because it was only detected in crushed tomato, but also to differentiate this mould from *A. niger* and *P. digitatum* in crushed tomato.

Enzymes (4) and (11) were important to distinguish *A. niger* in tomato juice from *A. niger* in crushed tomato, as it only developed in tomato juice, whereas enzyme (13) was detected with high activity only in *A. niger* in crushed tomato. Also these enzymes could be used to differentiate *A. niger* from *P. digitatum* and *R. stolonifer*.

In regard to *P. digitatum*, enzymes (14) and (15) were significant in the identification of this mould in tomato juice where only enzyme (14) was detected with high activity, and in crushed tomato where just enzyme (15) was detected but with low activity.

Curiously, enzymes (13), (14), and (18) which were consistently detected in the three moulds in tomato fruit, with medium to high activity, are not present in tomato juice and crushed tomato, as, for instance, these three enzymes were never detected in *R. stolonifer*.

These results show that it is possible to tentatively identify moulds and therefore detect their presence in tomato products, using their enzymic profile, rather than the Howard Mould Count. Thus, the Apizym system may be quite useful not only as a method of assessing raw material quality in processed tomato products, but also as a possible monitoring test for assessing heattreatment adequacy. Moreover, it can be used to monitor post-process contamination. Furthermore, the results obtained with the APIZYM system suggest that perhaps a more rapid and cheaper enzyme test strip could be developed that would contain only the most significant enzymes and could be used as a rapid screening technique for tomato products.

#### 4.2.4. Diagnostic tablets

As has been stated before the Apizym system is designed for the detection of 19 individual enzymes. However, after analysing the previous results obtained with this method, it was concluded that not all the 19 enzymes were detected in the different mould species used throughout this study. Thus, it was decided to use some of the most relevant enzymes detected in the different moulds in the form of diagnostic tablets, trying at the same time to get an easier test with reduced cost.

The enzymes selected were esterase lipase (C8)(4), which was relevant to detect *R. stolonifer* in tomato fruit and *A.niger* in tomato juice, ß galactosidase (14), which proved to be important to detect *P. digitatum* in tomato juice, and N-acetyl- ß glucosaminidase (18), which was important for *A. niger* in crushed tomato. Not all of the most important enzyme substrates were available from the supplier in tablet form. As this was a preliminary assay a wide range of moulds was used to test its efficiency, as described in section 3.2.5., confirming or not, the result obtained with Apizym system.

Extracts of *M. hiemalis, A. niger, Alternaria sp., P. digitatum, F. culmorum, and R. stolonifer* obtained from tomato juice as described in section 3.2.3, together with a control sample were used for diagnostic tablets after the Apizym result has been obtained. Both results were then compared (Table 13).

## COMPARISON OF DIAGNOSTIC TABLETS/APIZYM SYSTEM IN ASSESSING TOMATO JUICE QUALITY

SAMPLE			ENZYME	REACTION		
			A State	DIAC	NOSTI	C TABLET
	4	14	18	4	14	18
Control	<1	-	-		-	-
Alternaria sp.	-	4.5	3.0	-	+	÷
A. niger	<1	4.5	5.0	· –	· +	+
F. culmorum	<1	4.0	3.0	-	-	+
M. hiemalis	-	-	<b>.</b>	-	-	-
P. digitatum	<1	5.0	4.0	-	+	+
<i>R. stolon</i> ifer	-	4.0	<b>-</b> ·	-	-	-

Note: These results are mean values of 3 experiments performed in triplicate.

### Legend:

- 4 = esterase lipase (C8) 14 = β galactosidase 18 = N-acetyl-β glucosaminidase a:diagn

No enzyme activity was obtained for control sample in enzymes (4), (14) and (18) using Apizym, and a negative reaction was obtained with the correspondent diagnostic tablets.

In Alternaria sp. and A. niger a negative reaction corresponded to enzyme (4) in both diagnostic tests which was in agreement with the lack of activity obtained using Apizym, whereas positive reactions were obtained for enzymes (14) and (18) in both fungi, using both tests.

In *F. culmorum* results agreed for enzymes (4) and (18) using diagnostic tablets and Apizym system, but no agreement was obtained regarding enzyme (14) where Apizym gave high activity but, the correspondent diagnostic tablet gave a negative reaction.

In *M. hiemalis* and *P. digitatum* the results obtained using both tests totally agreed, as negative reactions were obtained with diagnostic tablets and Apizym system in *M. hiemalis*, and a positive reaction in enzymes (14) and (18) corresponded to high activity in *P. digitatum* in Apizym system. A negative reaction obtained with diagnostic tablets corresponded to trace of activity detected by Apizym in *P. digitatum*.

In *R. stolonifer* there was agreement between the results obtained by diagnostic tablets and Apizym, for enzymes (4) and (18), but again disagreement was revealed with enzyme (14) where high activity was detected by Apizym, but diagnostic tablets gave a negative reaction.

#### 4.2.5. Diagnostic tablets - general discussion

Although in c. 90% of the tests, there was agreement with the Apizym results, the actual value of this test for application in laboratory as a rapid screening technique requires further investigation. Some other techniques should be applied such as spectrophotometric analysis in order to quantify and confirm the results obtained in this study.

ENZYME ASSAYED FOR N. 1 Control Phosphatase alcaline 2 2-1 3 Esterase (C 4) 2-n Esterase Lipase (C 8) 4 2-n 5 Lipase (C 14) 2-n 6 Leucine arylamidase L-le 7 Valine arylamidase l -v Cystine arylamidase 8 Ł-c 9 Trypsin N-t 10 Chymotrypsin N-Phosphatase acid 11 2-n Naphthol-AS-BI-phosphohydrolase Nai 12 α galactosidase 6-B 13 14 β galactosidase 2-n 15 Nap β glucuronidase a glucosidase 16 2-na 17 β glucosidase 6-B 18 N-acetyl-ß glucosaminidase 1-na 19 a mannosidase 6-Br 20 α fucosidase 2-n

Source: API System SA

		RESULT			
SUBSIKATE	рп	Positive	Negative		
		No colour or colour of the sample if it ha an important colouration			
naphthyl phosphate	8.5	Violet			
naphthyl butyrate	6.5	Violet			
naphthyl caprylate	7.5	Violet			
naphthyl myristate	*	Violet	to an tight.		
eucyl-2-naphthylamide	*	Orange	posed		
valyl-2-naphthylamide		Orange	en ex o an Ì		
cystyl-2-naphthylamide	*	Orange	has be ts. osed t		
benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	allery eagen n exp		
glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	the g the ro		
naphthyl phosphate	5.4	Violet	atrol if tion of has no		
phthol-AS-BI-phosphate	34	Blue	ne cor r addil ailery		
Br-2-naphthyl-2D-galactopyranoside		Violet	ur of th e after the g		
naphthyl-βD-galactopyranoside		Violet	color sourc		
phthol-AS-BI-pD-glucuronide	*	Blue	our or e light ale ye		
naphthyl-2D-glucopyranoside	*	Violet	lo col ntense /ery p		
Br-2-naphthyl-pD-glucopyranoside	*	Violet			
naphthyl-N-acetyl-pD-glucosaminide	در	Brown			
Br-2-naphthyl-2D-mannopyranoside	*	Violet			
naphthyl-北-fucopyranoside	*	Violet			

#### 4.3 ATP photometry

This technique is dependent upon the fact that all cells contain adenosine triphosphate (ATP). It is an important component of all living cells, which can be measured using the luciferin-luciferase enzyme system of the firefly (*Photinus pyralis*). The intensity of light emitted and detected by the photomultiplier, as relative light units (RLU) during the reaction, is directly proportional to the ATP concentration, and therefore to the microbial level present in the sample.

Thus, an attempt was made to use this method as an alternative to the Howard mould count, to detect fungal presence in tomato juice and crushed tomato inoculated with different mould species, and to check tomato juice sterility.

#### 4.3.1 Thermal stability of fungal ATP

A spore suspension of *P.digitatum* was prepared as described in section 3.2.2., and heat treated at 70 <sup>O</sup>C for five minutes. The levels of ATP detected in the spore suspension as RLU, before and after heat treated, is represented in Fig. 35. Although there was a slight decrease in the total amount of ATP present in the suspension, it may be concluded that the ATP concentration was still very high.

A total viable count carried out on OGYEA in triplicate, after 48h incubated at 25 <sup>0</sup>C, gave negative results after the heat treatment of the spore suspension. These results are in contradiction not only with Patterson *et al.*, (1970), who stated that ATP disappears within two hours of a cell's death, but also with Stanley (1982), who reported ATP photometry as a method which only measures viable cells, as non-viable cells do not contain ATP. Furthermore, they suggest that probably the enzymatic system that breaks down the ATP

rig.so mermai stability of lungal ATP

as assessed by ATP Photometry



Rdigitatum

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within the cell, may either take sometime to do it, or depend on the specific enzyme concentration for that reaction. Heat treatment would inactivate those enzymes and so, any ATP present under such circumstances would remain in the system, thus giving misleading final results.

#### 4.3.2 The effect of sample concentration on the level of ATP detected

After several attempts using the ATP assay to detect ATP on tomato juice and crushed tomato using the protocol recommended by Lumac, the results obtained proved that 1/10 dilution of samples was necessary to facilitate ATP assessment of mould inoculated samples of these two products. The data presented in Fig. 36 show that in pure tomato juice and crushed tomato, light output and therefore apparently the ATP concentration after inoculation with a spore suspension of *Penicillium digitatum*, prepared as described in section 3.2.2., and incubation at 25 °C for 48h, is lower than in the 1/10 diluted sample; this phenomenon appears to be quite common in food samples like tomato juice, which contain quenching agents (luciferase inhibitors), or coloured compounds, or are turbid, which absorbs some of the light from the reaction before it reaches the photomultiplier. In both cases the result is the lowering of the amount of light detected by the photomultiplier (Stanley, 1982). It is therefore recommended in assaying ATP levels in such samples that dilutions are prepared in order to assess any possible quenching effects.

### rig.so Ellect of Sample Concentration

as assessed by ATP Photometry



experiments performed in triplicate

4.3.3 ATP concentration in tomato juice and its relationship with the Howard mould count

Results obtained from ATP assessment as described in section 3.5.3 (Method 1), on control and samples of diluted tomato juice inoculated with five different moulds, (incubated at 25 <sup>0</sup>C for 48h and 96h), were compared with Howard mould counts obtained from the same samples.

Fig.37 shows the relationship between these two screening methods after 48h incubation: the lowest amount of RLU (500 RLU) and therefore the lowest ATP concentration corresponded to the control sample without any incubation period or inoculation, where 20% of positive fields fields were obtained by Howard mould count. The highest ATP level (2,721 RLU) was detected in the sample inoculated with *Aspergillus niger* to which 52% of positive fields corresponded.

Samples inoculated with *Rhizopus stolonifer* and *Penicillium digitatum* revealed the second and third highest ATP levels (1,927 RLU and 1,922 RLU, respectively) corresponding to the two highest Howard mould counts (60% and 64% respectively). The incubated control sample with 1,557 RLU and 36% of positive fields had values comparable to the samples inoculated with *Cladosporum herbarum* (1,550 RLU and 40% positive fields) and *Fusarium culmorum* (1,463 RLU and 44% positive fields).

These results show that the (tested) sample of tomato juice is non sterile as according to the instructions for the technique the value for the incubated control sample (1,557 RLU) is more than three times the RLU of the control sample (500 RLU). From the Howard mould count this sample was also rejected because 36% of positive fields were counted, which is higher than the acceptance level (20%).

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FIG.ST ATE CONCEINATION IN TOMATO JUICE

and its relationship with HMC (48h inc)





Fig. 38 shows the same relationship obtained on tomato juice after 96h incubation. There was a general decrease of ATP level in all the samples. However, the opposite situation was detected by Howard mould count as all the samples increased their counts with the exception of *Penicillium digitatum* which maintained the same value, 64%.

These results show the disagreement between these two methods concerning mould assessment in tomato products. Although the percentage of positive fields of the incubated control sample of tomato juice after 96 h incubation is double that obtained after 48h incubation (64% against 36%), the RLU decreased 3 times. This suggests that the concentration of ATP within the cell changes with cellular metabolism or may reflect ATP consumption by enzyme action, as outlined in section 4.3.1.

### 4.3.4 <u>ATP concentration in crushed tomato and its relationship with Howard</u> mould count

Fig. 39 shows the results obtained under similar conditions described in 4.3.3, using crushed tomato. The incubated control sample had a high ATP level (1,808 RLU) which was only surpassed by *Penicillium digitatum* (2,190 RLU) compared to the lowest Howard mould count (22.4%).

The highest Howard mould count (60%) in the sample inoculated with *Aspergillus niger* corresponded to an RLU value of 1,714. The control sample showed 28% positive fields and 1,555 RLU, which compared with 1,808 RLU obtained in the incubated control sample of crushed tomato does not allow any reasonable conclusion about the sterility of the sample, since although the incubated control value is not three times that of the unincubated control, its initial value appears high. Moreover, the results obtained with Howard mould count seem rather ambiguous as both control and incubated samples show 28% and 22.4% positive fields respectively, which are considerably lower than

rig.38 ATF Concentration in Tomato Juice

and its relationship with HMC (96h inc)





experiments performed in triplicate

R

L

U

п

i

1 \$

% Positive Fields

Fig.39 ATP Concentration in Crushed

Tomato and its relationship with HMC (48h inc.)





R

L

U

п

i

\$

experiments performed in triplicate

% Positive Fields

the acceptance limit for crushed tomato which is 40%. The remaining samples show intermediate values for both Howard mould counts and ATP concentration as assessed by RLU, although again there was lack of agreement between ATP level and mould count.

After 96h incubation (Fig.40) in crushed tomato samples, a general decrease of the ATP concentration was detected in all samples and again the reverse situation was observed with regard to Howard mould count, where *Fusarium culmorum* and *Rhizopus stolonifer* mould count rose to 96% and 92% compared to 869 RLU and 862 RLU, respectively.

### 4.3.5 <u>Comparison between ATP concentrations using two different methods</u> in ATP assay

As mentioned in section 3.5.2 the ATP assessment was determined using two different methods, which was necessary because of a change in the instructions supplied by the manufacturer.

Figures 41 and 42 compare the ATP concentrations detected using the two methods obtained after 48h incubation of tomato juice and crushed tomato treated and incubated under the same conditions as those used in 4.3.3 and 4.3.4 . In tomato juice and crushed tomato all the values of ATP level detected were dramatically lower using method 2. The highest value in tomato juice obtained using method 1 was 2,721 RLU from *Aspergillus niger* (after 48h incubation) whereas the corresponding value using Method 2 was 620 RLU. The same situation applied to the incubated control sample where 1,557 RLU were obtained after 48h incubation using Method 1 and only 105 RLU were detected using Method 2.

Similar trends were noted for all other treatments, which suggest that free ATP, which is normally associated with fruit debris in these products, and somatic ATP were better eliminated in method 2 (as described in section

### FIG.40 ATP Concentration in Crushed

Tomato and its relationship with HMC (96h inc.)





experiments performed in triplicate

% Positive Fields

# Fig 41 Comparison of ATP Levels

Methods 1 and 2 in Tomato Juice(48h inc)





Method

# Fig. 42 Comparison of ATP levels using

Methods 1 and 2 in Crushed Tomato (48h inc.)







3.5.2.) than in method 1, and therefore provided a significant decrease in the final RLU readings.

Regarding crushed tomato, the RLU value for both control and incubated samples is similar using method 1. However, when compared with method 2 the RLU value detected in the control sample of crushed tomato is double the one detected in the incubated sample (Fig. 42), which suggests that probably crushed tomato will need a longer period of incubation (usually, it takes 45 minutes) with F-NRS and F-Somase in order to destroy the non microbial ATP present in this product.

Regarding the Howard mould count in these two samples similar results were obtained to those presented before , *i.e.* there was a general increase in Howard mould counts after 96h incubation as happened before, since at this optimum temperature moulds grow very fast.

# 4.3.6 The statistical correlation between Methods 1 and 2 used in ATP assay

Figures 43 and 44 present the results of regression lines and correlation coefficients (r) between Log 10 RLU and Log 10 CFU/g (carried out as described in section 3.5.3.), obtained using least-squares fit of the data for tomato juice using Method 1 and Method 2 of the ATP technique to assess tomato juice sterility after 48h incubation at  $25 \, {}^{\circ}$ C.

The regression line represented graphically in Fig. 43 (Method 1 after 48h incubation) for tomato juice has a correlation coefficient r = 0.75, whereas the correlation coefficient of the regression line represented on Fig. 44 (Method 2 after 48h incubation) is r = 0.91. These correlations were confirmed by a paired T test at 95% confidence limit, which suggest that Method 2 of ATP technique should be preferred to Method 1 when assessing tomato juice sterility.

# Fig.43 LOG RLU v. LOG CFU/g Method 1 of ATP assay (48h incub.)



Tomato Juice Results are mean values of three experiments performed in triplicate

# Fig.44 LOG RLU v. LOG CFU/g Method 2 of ATP assay (48h incub.)



Tomato Juice Results are mean values of three experiments performed in triplicate 127

Concerning crushed tomato, very poor correlation coefficients were obtained (r = 0.28 for Method 1 and r = 0.10 for Method 2) after 48h incubation which again was confirmed by a paired T test at 95% confidence limit, and therefore suggests that the ATP technique is not a suitable method to assess crushed tomato sterility, as probably the somatic ATP present in the sample is not completely eliminated before the measurement of mould ATP.

4.3.7 ATP assay - general discussion

As mentioned in section 2.7.2.1.3. various authors have obtained encouraging results using ATP photometry when assessing different levels of either bacterial or yeast contamination in foodstuffs such as meat, milk and fruit juices.

However, the technique has not been applied widely to estimation of mould, largely because of the problems of separating both mycelium and conidia from food materials. Only very recently, Gaunt *et al.*, (1985) tried to assess the usefulness of using adenosine triphosphate (ATP) as an indicator of fungal biomass. but several problems have been pointed out regarding this technique by different authors: Stanley (1982) reported this technique, as one of the most sensitive assays, where extreme care must be taken not to introduce ATP into any stage of the analytical procedure. Sharpe *et al.*, (1970) and Bossuyt (1981) have referred to the universal difficulty encountered in the application of this method to foods, because not only of the interference of bioluminescent quenching effects, but also the non microbial source of ATP as affecting light emission, since most food products derived from plant or animal sources contain natural background levels of ATP.

Pitt and Hocking (1985), mentioned the special difficulty to separate fungi from food materials, mainly those derived from plant cells, as they contain high levels of ATP, and the extraction of molecules from fungal cells. Some difficulties were also experienced during this study, not only with regard to the quenching effect and non-microbial ATP extraction and removal, but also related to the fungal ATP extraction which sometimes gave quite low values. Moreover, it was frequently found that dilution counts on plate counts for moulds did not always follow a strict decimal progession, probably because of the fragmentation of mycelium or breaking of spore clumps during dilution, or maybe due to the competitive inhibition when large numbers of colonies are present on the plates.

#### 4.4 Direct epifluorescent filter technique (DEFT)

The direct epifluorescent filter technique is a method which uses membrane filtration and the epifluorescence microscopy. This technique enables the detection of microorganisms, following the application of a fluorescent dye, such as acridine orange, which fluoresces under UV light. It has been applied to assess bacteria in milk, fresh meat and fish and yeasts in beverages, since they may be easily distinguised from the food debris.

According to the general staining pattern of DEFT, all viable cells fluoresce orange under UV light, whereas non-viable cells fluoresce green.

In this study, DEFT was evaluated as an alternative to the Howard mould count to differenciate fungal hyphae in tomato juice by using an image analyser. Two different situations were evaluated: firstly, the technique was used to check tomato juice sterility. Secondly, tomato juice was inoculated with two different mould species, and their presence was assessed by using DEFT.

#### 4.4.1 Assessment of tomato juice sterility using DEFT

DEFT was used to check the sterility in control samples of tomato juice and samples incubated at 25 <sup>O</sup>C for 48 hours, as described in sections 3.6.1. to 3.6.5. Plate counts were also carried out on OGYEA with DEFT assays, as described in section 3.4.1.

The results obtained, showed disagreement between DEFT count and plate count, as regards juice sterility, since there was no growth on the plates, but DEFT values obtained from the microcomputer gave positive results. This is probably due to non-viable cells fluorescing orange and, therefore, being included in the DEFT count.

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Plate 5 Tomato fibrovascular bundles present in tomato juice fluorescing green after DEFT staining (x400).



Plate 6 Mould hypha found in tomato juice fluorescing orange after DEFT staining (x400).



A possible explanation for this is may be because tomato juice processing involves heating the juice as rapidly as possible to a temperature of about 85 °C ("hot break" method described in section 2.3.1.2.4) to destroy enzymes and to obtain a greater extraction of pectin and also pasteurization subsequently.

The effect of heat treatment on acridine orange staining characteristics has been investigated. Rodrigues and Kroll (1986) reported a poor relationship between DEFT count and plate count of yeasts in beverages after pasteurization because all the cells fluoresced bright orange despite being non-viable. These results contradict the accepted staining characteristics of the dye. It is generally held that ribonucleic acid (RNA) fluoresces orange and deoxyribonucleic acid (DNA) fluoresces green (Nicolini *et al.*,1979), and that the former masks the latter if the RNA is present in significant concentrations. It is thought that acridine orange intercalates in the DNA double helix about every third base pair; this does not allow dye-molecular interaction and acridine orange fluoresces orthochromatically (green). With RNA, acridine orange binds to the exposed phosphate groups of nucleotides allowing polymerization which results in metachromatic fluorescence (orange) (Rigler, 1966).

Heating DNA denatures the secondary and tertiary structure and exposes bases to which acridine orange can bind in a similar manner to RNA, and fluoresce orange (Rigler, 1966). This may be the reason why yeasts and certain genera of bacteria fluoresce orange after heat treatment. However, the interpretation predicts that all organisms would fluoresce orange when heated; this is not the case with most species of bacteria (Pettipher and Rodrigues, 1981). A more likely explanation is the partial denaturation of structurally complex (tRNA) or complexed (rRNA), or the inactivity of the relevant nucleases (Schultz-Harder, 1983; Hurst, 1984). Indeed, RNase, but not DNase, treatment of heat treated streptococci cause them to fluoresce green (Kroll unpublished results).

The effects of heat treatment on acridine orange staining are certainly complex and currently investigations on these results are being performed.

Despite the poor agreement obtained in this study, between triplicates of DEFT count and total viable count, the DEFT may still prove useful, because of its rapidity in detecting mould contamination by monitoring the microbiological condition of the product at various stages during its manufacture (Pettipher and Rodrigues, 1982).

Because of the results obtained in the previous assements, possibly due to alterations in acridine staining characteristics caused by heat treatment, it was decided to check the effect of autoclaving on a sample of tomato juice inoculated with a mould, using DEFT and plate count on OGYEA.

# 4.4.2 Effect of heat treatment on an autoclaved sample of tomato juice inoculated with mould

A sample of tomato juice was inoculated with a spore suspension of *Rhizopus stolonifer* as described in section 3.2.2., and autoclaved at 121 <sup>0</sup>C for 15 minutes.

DEFT procedure was followed as described in sections 3.6.1. to 3.6.5., and the plate count on OGYEA performed as described in section 3.4.1.

The results showed disagreement between these two methods, as there was no growth on the plates but DEFT gave a positive count. However, they confirmed the previous statements, since fungal hypha fluoresced bright orange under UV light.


Plate 7 Mould hyphae present in inoculated tomato juice fluorescing orange after DEFT staining (x400).

Plate 8 Mould hyphae found in inoculated tomato juice fluorescing bright orange, after autoclaving at 121 <sup>O</sup>C for 15 minutes and DEFT staining (x400).



### 4.4.3 Mould assessment of inoculated tomato juice using DEFT

Two lots of the same sample of tomato juice were separately inoculated with spore suspensions of *Aspergillus niger* and *Rhizopus stolonifer* as described in section 3.2.2. and assessed by DEFT, as described in section 3.6.1. to 3.6.5. and plate count on OGYEA, as described in section 3.4.1.

In this study good agreement was observed in both cases, between the log DEFT count and the log CFU/g. The regression lines are represented graphically in Figures 45 and 46 with correlation coefficients of 0.97 and 0.91 respectively.

Pettipher and Rodrigues (1982), reported that when good agreement between DEFT count and the total plate count was obtained, the DEFT could be used for quality control during production and in some cases to predict the keeping quality of the product.

Despite the good correlation coefficients obtained between these two counting methods, again DEFT counts slightly overestimated the total viable count. Possibly this is due to the reasons given previously in section 4.4.1, or due to the inability of all the mould clumps to grow on oxytetracycline glucose yeast extract agar (OGYEA) as discussed before in section 4.3.7.

### 4.4.4 DEFT - general discussion

For any new technique to gain acceptance it is advantageous the results are similar to, or correlate with those of standard methods (Pettipher and Rodrigues, 1981). However, Pettipher and Rodrigues (1982) reported the poor agreement obtained in frozen vegetables such as peas, corn and broad beans, due to the effect of heat treament, since before freezing vegetables are blanched at a temperature of about 98 to 99 <sup>O</sup>C to inactivate enzymes.

# Fig.45 LOG DEFT v. LOG CFU Tomato Juice inoculated with A.niger



Results are mean values of three experiments performed in triplicate

# Fig.46 Log DEFT v. Log CFU/g Tomato Juice with Rhizopus stolonifer



Results are mean values of three experiments performed in triplicate

Moreover, they found that heat treatment especially affects products containing a large number of streptococci or micrococci.

Rodrigues and Kroll (1986) considered DEFT to be unsuitable for the enumeration of yeasts in heat treated beverages because non-viable cells fluoresce orange.

In this study despite the slight overestimation of DEFT count over the total viable count, DEFT values based on the number of actively orange-fluorescing clumps of mould meets this requirement to some extent which may be useful in assessing quality control in tomato juice production.

However, the immediate prospects for DEFT seem to lie in new applications, improved sample preparation, possibly improved rapidity and specially improved sensitivity as this technique is not sufficiently sensitive to accurately determine microbial numbers in pasteurized products with counts below 1 x 10<sup>3</sup>/ml.

Also, currently, statistical methods are being evaluated, for analysing the field counts with the aim of removing unrepresentative counts prior to calculation of the final DEFT count, which should have a further reduction in the counting errors (e.g. poor focusing prior to counting) (Pettipher, 1986).

#### 4.5 Impedance monitoring

Impedance microbiology is a rapid automated method, based on the principle that metabolizing organisms alter the chemical and ionic composition of the medium, thereby changing the impedance of the medium; where impedance is the resistance to flow of an alternating electrical current through a conducting medium.

This technique was evaluated to check tomato juice sterility and to assess fungal presence in inoculated samples, instead of using the Howard mould count.

### 4.5.1 Media selection for impedance microbiology

Schaertel and Firstenberg-Eden (1987) found that an agar surface, and therefore a solid medium was preferable for moulds in impedance detection, probably due to the requirements for aerobic conditions and as an anchor for surface mycelial growth.

They also reported that not all of the media recommended by plate count methods for mould growth, such as Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) should be utilized for impedimetric detection, since certain metabolic pathways yield better impedance signals than others (Shaertel *et al.*, 1987).

An attempt was made to select the solid medium with the best (shortest) detection time, by comparing the rate of growth of *Aspergillus niger* in four different media, frequently used to culture moulds on plates.

Figure 47 represents the results obtained comparing the rate of growth of *Aspergillus niger* spore suspension, prepared as described in section 3.2.1.1 in four different solid media, as assessed by impedance: tryptona soya agar (TSA), oxytetracycline glucose yeast extract agar (OGYEA), potato dextrose

### Fig.47 Aspergillus niger Rate of Growth in Different Media



Impedance/Conductance Detection Results are mean values of three experiments performed in triplicate agar (PDA), and malt extract agar (MEA). Good impedance signals were obtained as well as good detection times, where the shortest detection time was achieved using PDA (12.5h) immediately followed by MEA (13.5 h). It was decided to use PDA to assess the relationship between detection time and log of total viable count, carried out as described in section 3.4.1., for *Aspergillus niger, Rhizopus stolonifer, Penicillium digitatum, and Alternaria sp.* 

4.5.2 Detection time obtained with different mould species using PDA

The average of three replicates is represented in Fig. 48, which shows the inverse linear relationship between the log 10 of the total viable count (measured on spore suspension as described in section 3.4.1.) and the impedance detection time for *Aspergillus niger*, *Rhizopus stolonifer*, *Penicillium digitatum* and *Alternaria sp.* in PDA.

Results showed that a general good agreement was achieved

between the detection time and the log total viable count for these four mould species using PDA, with correlation coefficients of 0.99 for all of them except *Penicillium digitatum* where 0.96 was obtained.

### 4.5.3 Calibration curve for inoculated tomato juice using PDA and MEA

Impedance detection time was evaluated for tomato juice inoculated with a mixed mould inoculum as described in section 3.7.3., using PDA and MEA. Fig. 49 and 50 represent the calibration curves obtained by using the least square fit method data. Good correlation between the impedance detection time and log 10 of the total viable count was obtained with correlation coefficients of 0.92 and 0.94 respectively.

Brooks (1986) reported that if a correlation coefficient of this type is obtained for any particular product, the sample may be classified by setting a cut-off point, and determining the correspondent time. Values greater than that one established should be recorded by all acceptable samples. Those showing

# Fig.48 Mould Detection Time Detection Time v. Log 10 TVC



Medium (PDA) Results are mean values of three experiments performed in triplicate

# Fig.49 Tomato Juice Calibration Curve in PDA



Sample contaminated (mixed population) Results are mean values of three experiments performed in triplicate

## Fig.50 Tomato Juice Calibration Curve in MEA



Sample contaminated (mixed population) Results are mean values of three experiments performed in triplicate detection time shorter than the cut-off point, would be considered unacceptable.

# 4.5.4 Assessment of tomato juice sterility by means of impedance microbiology

It was decided to evaluate impedance microbiology in assessing the quality of tomato juice by detection time and its relationship to contamination values. Since there is no limit for tomato juice established by Food Standard regulations, it was decided to use the one for orange juice concentrate (10<sup>4</sup> CFU/g), as being representative.

Two different situations were evaluated by means of the calibration curves obtained in section 4.5.3: firstly, the sterility of several uninoculated samples of tomato juice was checked. No impedance change occurred in any sample, which correlated well with the total viable count, as both showed that the tomato juice was sterile.

However, when Howard mould count was used to assess mould presence in these samples, a coefficient of variation of 12.1% was obtained which shows the inaccuracy of this method. A typical value for Howard mould count in these samples was 22.6%. Jarvis (1977), reported the lack of precision of Howard mould count, where coefficients of variation of 15.0% or more were usually found.

Secondly, two lots from the same sample of tomato juice were separately inoculated with *Aspergillus niger* and *Rhizopus stolonifer* spore suspension, as described in section 3.2.2.

All the samples were rejected as the detection time was around 19h, which is less than the limit detection time correspondent to the pre-established cut-off point of 23h (PDA) and 25h (MEA) for 104 CFU/g. Using least squares analysis, the regression lines for tomato juice inoculated with *Aspergillus niger* and *Rhizopus stolonifer* were obtained, with correlation coefficients of 0.92 and 0.94 respectively, showing the good correlation between detection time and log 10 CFU/g.

Furthermore, an Howard mould count was carried out at the same time on the same samples; a Howard mould count value of 32% was found and 25.8 % was obtained as coefficient of variation, which agreed with Jarvis (1977) results, related to the lack of precision of this method.

### 4.5.5 Impedance detection - general discussion

Detection time is a function of the suitability of a growth medium for a particular microorganism and the initial concentration of the inoculum (Shaertel *et al.*,1987).

Brooks (1986) reported that detection time is generally much shorter than the time required for growth of colonies to visible size in a standard plate count, which entirely agrees with the results of our study, where a minimum of 48h was necessary to get a plate count result, sometimes it takes more time, depending on mould species, whereas using impedance microbiology the result was obtained after 19h, thus assessing the sample contamination level.

Sterility testing can also be quickly detected, providing that the medium chosen will allow detection of any viable microorganisms that might be found in the product, it is necessary only to monitor the impedance for a suitable length of time. Any change of impedance will indicate contamination.

After an initial period of use in parallel with conventional methods the impedance method is now relied upon for a day-to day counting in food industry (Brooks, 1986). Whilst the most obvious use of impedance in microbiology is as a substitute for plate counting, there are a number of applications where the detection time is correlated with some other estimation,

and may be of the greatest importance. Thus the measured impedance change is a function of metabolic activity rather than microbial numbers, which is well-suited to estimating shelf life of perishable products (Bossuyt and Waes, 1983; Shaertel *et al.*, 1987).

Clearly there is a considerable amount of method development work to be done, especially in regarding mould assessment, where very little work has been published to date. However, impedance microbiology proved to be a suitale method to assess mould presence in tomato juice, thus contributing to improved results in microbiological quality control of this product, as a possible reliable substitute for Howard mould count.

### 5. CONCLUSIONS

The Howard mould count, which has been used to determine the level of mould contamination in tomato and tomato products for many years, proved to be a very fastidious and time consuming method but lacking precision when assessing the quality of tomato juice and crushed tomato, where coefficients of variation going from 13.4% to 47.4% were obtained. This is mainly due to the fact that a positive field may contain either a single mould hypha or a large clump of mycelium.

Furthermore, the size of mould filaments present depends not only on the amount of rot in the raw stock, but also the method of processing which may give rise to false positive total counts.

The total viable count has the advantage of its simplicity, its adaptability and its convenience, since it relies on the self-indicating ability of moulds to form colonies and no requirements exist for special equipment or intensive extraction. However, it has serious disadvantages as it is a very time consuming method, as well as labour and materials intensive. Morever, it can give erroneous results on mould counts, as depending on the sampling method, mycelial clumps and sporing heads may or may not be fragmented by homogenization procedures. It will also, of course, only assess viable mould units.

The Apizym system has the advantage of being a rapid diagnostic method (a result may be obtained within 4h) with a low running cost and providing useful information on mould presence in tomato products using their enzymic profile. There is no need for expensive equipment or specialized staff as the results are easy to read. This technique was used to assess fungal presence in tomato juice and crushed tomato and different patterns of extracellular enzymes were obtained for *Aspergillus niger, Penicillium digitatum* and *Rhizopus stolonifer*, suggesting that Apizym could be used to assess raw

material quality in processed tomato products, as well as a possible monitoring test to assess heat treatment adequacy or post process contamination. Three enzymes were selected from the enzymic profiles of the above moulds, and diagnostic tablets which detect these enzymes, were successfully used so rending the technique more rapid and equally differential with even lower costs. However, the amount of data reported in this study is still limited, regarding its possible use in food industry and further work needs to be done to confirm its suitablility for industrial use.

The ATP level can be used to provide an indirect estimate of mould contamination, providing the intrinsic ATP content of the food is very low or can be removed, or that the mycelium and conidia may be separated from the food material (which was very difficult in some cases eg. crushed tomato). Method 2 used in the ATP assay proved to be the most suitable for mould detection in tomato juice, probably because non microbial ATP was better eliminated.

The Direct Epifluorescent Filter Technique (DEFT) showed a good correlation between the logarithm of CFU/g and the logarithm of DEFT count/ml in tomato juice contaminated with *Aspergillus niger* and *Rhizopus stolonifer*, providing results within 30 minutes. Although problems relating to correlation between staining and viability of cells were experienced due to the effect of heat treatment on acridine orange staining characteristics, DEFT proved useful because of its rapidity to detect fungal presence in tomato juice. It could therefore be used to assess quality control in tomato juice production, or to predict the keeping quality of the product.

Impedance monitoring proved to be a suitable method for assessing fungal contamination in tomato juice using either PDA or MEA. It was used to check tomato juice sterility using the relationship of detection time to contamination levels. Using a calibration curve obtained for that product, and setting a cut-off point corresponding to the limit detection time, it was possible to reject

contaminated tomato juice in 19h, which is considerably less than the 48h that the plate count usually takes.

More work needs to be done in this area, which provides more data about mould detection in foods, as very little has been published to date.

#### 6. SUGGESTIONS FOR FURTHER WORK:

6.1. Use of the scanning densitometer and spectrophotometer for the Apizym System and Diagnostic Tablets, might be very useful in quantifying different colour intensities following enzymatic activities. This would enable grading and more rapid evaluation.

6.2. The development of methods to facilitate the physical separation of moulds from food material, or to employ a combination of physical and enzymic methods in order to destroy the non-microbial ATP, might render the ATP assay a more precise technique.

6.3. It would also be interesting to assess mould contamination, by analysing the glucosamine content in the tomato samples by H.P.L.C., as it is a breakdown product from chitin, which is one of the major constituents of fungal wall. The use of gas chromatography or headspace analysis to check mould presence through their metabolites would be useful to provide information about mould contamination, and compare it with the results obtained using other methods.

6.4. To develop new media for detecting moulds and therefore improving the detection time and optimise the signal as to their efficacy to assess mouldy tomato products, would improve the results of impedance microbiology.

6.5. The Vids III system, which consists of a microscope, an image analyser and mouse/digitised pad would permit quantification of hyphal material in terms of total length/area/volume of mycelial material present. This would be comparable to Howard mould count, but would present distinct advantages in terms of the degree of quantification.

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