EVALUATION OF BACTERIOLOGICAL TECHNIQUES, SENSORY EVALUATION, GAS CHROMATOGRAPHY AND ELECTRONIC NOSE TECHNOLOGY FOR THE EARLY DETECTION OF *Alicyclobacillus acidoterrestris* IN FRUIT JUICES

Zeenat Harrichandparsad

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REFERENCE DECLARATION IN RESPECT OF A MASTER'S DISSERTATION

I, Zeenat Harrichandparsad (20103348) and Professor B. Odhav and Mr. K. Devchand do hereby declare that, in respect of the following dissertation:

Evaluation of bacteriological techniques, sensory evaluation, gas chromatography and electronic nose technology for the early detection of *Alicyclobacillus acidoterrestris* in fruit juices

1. As far as we can ascertain:

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DEDICATION

I dedicate this thesis to my family without whose unconditional support and encouragement I would not have been able to accomplish my academic achievements.

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LIST OF ABBREVIATIONS

BAM – *Bacillus acidoterrestris* Medium
cfu/ml – Colony forming units per millilitre
DNA – Deoxyribonucleic Acid
FIA – Flow injection analysis
FID – Flame ionisation detector
GC – Gas chromatography
GSC – Gas solid chromatography
HSA – Headspace analysis
HSGC – Headspace gas chromatography
KM – K-Medium
N – Normality
OSA – Orange Serum Agar
PDA – Potato Dextrose Agar
ppm – Parts per million
rRNA – Ribosomal Ribonucleic Acid
SABS – South African Bureau of Standards
SHA – Static headspace analysis
UHT – Ultra-High Temperature
U.S.A. – United States of America

ABSTRACT

Alicyclobacillus acidoterrestris is a spore-forming spoilage micro-organism found in fruit juices whose spores are not destroyed by typical pasteurisation. Once its spores outgrow and multiply in finished juice products, they produce two volatile taint compounds namely guaiacol and 2,6-dibromophenol. In the food industry margins for errors are small and monitoring of products to avert such errors is crucial. Conventional microbiological monitoring is one such technique for spoilage micro-organisms another being automated systems which can detect taints. Both these categories were evaluated in this study with the electronic nose and gas chromatograph being the specific automated systems being assessed. Sensory evaluation was also assessed as a diagnostic tool in the detection of taints. Isolation and identification of what was thought to be *A. acidoterrestris* was a laborious and expensive exercise which eventually proved inconclusive. A pure culture was purchased and juices were then inoculated with two levels of *A. acidoterrestris* spores and incubated. Juices from each level of inoculation were evaluated at different time intervals via the above-mentioned monitoring techniques. Of the three media assessed in the microbiological method, *Bacillus acidoterrestris* medium (BAM) was found to be the most effective for enumerating *A. acidoterrestris* followed by K-medium (KM) then Orange Serum Agar (OSA). While BAM was still indicating the presence of *A. acidoterrestris* KM and OSA were not (counts of <10cfu/g). This illustrated that this micro-organism could be easily overlooked if KM or OSA were being used to enumerate them. Considering that many workers actually do use KM and OSA as their media of choice in enumerating *A. acidoterrestris* (perhaps because BAM is very tedious to prepare) the cause for concern is a real one. Assessment of the resultant taints via sensory evaluation after inoculation and incubation reveals the inability of many panellists to detect taints at levels (as assessed by GC) far above their documented threshold values. While GC is an extremely useful and powerful tool, the level of expertise and skill required to use such an instrument cannot be overlooked nor can the expense involved. With regard to the electronic nose assessment for the presence of the volatile taint compounds, an important finding was that the electronic nose indicated significant differences between test and control samples when panellists performing sensory evaluation did not. This also correlated to an interval when enumeration on OSA illustrated no *A. acidoterrestris* after several days of inoculation and incubation and BAM and KM did. Without implying that the electronic nose has no drawbacks, it has proved, in this instance to be a simple and easy

piece of equipment to use. It can be used to detect taints produced under simulated spoilage conditions at reduced analysis times, levels of expertise, cost and energy.

CHAPTER ONE

1. INTRODUCTION

1.1 IMPORTANCE OF QUALITY IN THE FRUIT JUICE MANUFACTURING INDUSTRY

Over the years, the manufacture of fruit juices has progressed from farm or cottage industry into an efficient technology of modern food processing (Rutledge, 2001). The juice industry has grown in nearly every direction – volume, money, number of ingredients and products, geographical spread, range of markets, and the number and types of packaging (Rutledge, 2001). Fruit juices have become a regular part of the diet of many consumers throughout the world and as a result are a source of huge profits in the beverage market the world over (Rutledge, 2001).

In an increasingly competitive and dynamic commercial environment, it is imperative that all reputable processors in the fruit juice industry satisfy consumer demands for safe, high quality, juices. A whole industry of quality specialists has emerged within the food and beverage manufacturing sector in order to introduce quality assurance programmes which aim to deliver products which deliver consumer satisfaction, develop brand loyalty, stimulate repurchase and resort in a low incidence of consumer complaints. In terms of quality, consumers insist that sensory attributes of juices meet their requirements. These attributes are usually perceived in the following order: first appearance and colour, followed by aroma, then consistency and texture and finally flavour (Lin, 1981). However, most or all of these attributes overlap. Total quality assurance in the food and beverage industry in general is a requirement for consumer satisfaction (Gould and Gould, 1988). With regard to the beverage industry, it is very important that the quality of juices is not compromised in any way and that adverse consumer reactions are minimised if the juice industry is to see continued economic growth in the marketplace (Kilcast, 1993).

The two main aspects of a quality control department in a fruit juice manufacturing plant are to ensure sanitation of the product and the measurement and control of juice sensory attributes as described previously (Kimball, 1999). Ensuring the safety and quality of fruit

juice products is generally not complicated but requires adherence to sanitary and quality principles through all phases of juice processing unit operations (Braddock, 1999).

1.2 PATHOGENECITY AND SPOILAGE OF JUICES AND ASSOCIATED PREVENTATIVE MECHANISMS NAMELY pH AND PASTEURISATION

Failing adherence to sanitary and quality principles, the pathogenecity or spoilage of juices is inevitable. With regards to pathogenic micro-organisms, the acidity of juices (pH values of less than 4.00) gives virtually complete protection against pathogens such as *Escherichia coli* 0157:H7, *Salmonella typhi*, *Shigella*, *Clostridium* and Hepatitis A (Fellers, 1992). Spoilage in juices is governed by the extent to which chemical, physical or microbial/bacterial reactions contribute to changes in their edible quality. Chemical reactions may be enzymatic or non-enzymatic. Physical and/or chemical reactions are those brought about by the contamination of food or beverages by, for example, chemicals, pesticides or herbicides. Microbial spoilage is, by far, the major cause of quality loss in foods and beverages (Springett, 1993). Microbial spoilage of foods and beverages are manifested in products by compromising their sensory attributes, i.e. the production of off-flavours and/or odours (tainting), the formation of slime or visible moulds, colour changes and curdling. Microbial spoilage may also culminate in the alteration of the physical appearance of products e.g. blowing of cans, plastic bottles, cartons or sachets. All of these deteriorative changes are brought about by the metabolic activity of bacteria. Protection of juices against microbial spoilage is provided by pasteurisation. Pasteurisation is usually sufficient to destroy spoilage organisms such as yeasts, moulds and *Lactobacillus* species. This renders the products commercially sterile and unlikely to spoil.

1.3 LIMITATIONS OF PASTEURISATION: SPORES OF *Alicyclobacillus acidoterrestris*

Pasteurisation processes are not sufficient to destroy all bacterial spores. In order to destroy bacterial spores, temperatures higher than those used to kill non-spore-forming spoilage micro-organisms and any remaining pathogens not destroyed by the low pH of the juices would be required (Rutledge, 2001). Higher temperatures, however, are more than

likely to adversely affect the quality of the product with respect to flavour, taste, texture and mouthfeel and, up until recently, such heat treatment was deemed unnecessary as most spores would not proliferate in the low pH range of such products. This notion has been re-visited and has now become invalidated by some relatively recent work on a relatively newly discovered soil-borne, spore-forming spoilage micro-organism, namely *Alicyclobacillus acidoterrestris* (Jenson, 1999). The spores produced by this organism are capable of surviving the typical pasteurisation process of holding at 86°C to 96°C for 2 minutes and are capable of proliferating in acidic products with pH values previously considered below the range for growth of spore-forming bacteria. Thus fruit juices, although always safe to drink, are inherently susceptible to spoilage micro-organisms (Hicks, 1990).

1.4 SPOILAGE OF JUICES VIA METABOLIC ACTIVITY OF *Alicyclobacillus acidoterrestris*: TAINTING OF JUICES

Spoilage of fruit juices contaminated by microbes is brought about via their growth and metabolic activity during which they break down the structure of specific compounds in the product, producing a range of metabolites which subsequently taint (adulterate) the product (Baigrie, 1993). By definition, a taint is a taste or odour foreign to the product in which it occurs (ISO, 1992). This definition focuses on those food or beverage taints which can be perceived by the human senses, particularly by their odour or flavour, even at the extremely low threshold levels of parts per million (ppm = 10^6), parts per billion (ppb = 10^9) or even parts per trillion (ppt = 10^{12}). The metabolic activity of *A. acidoterrestris*, once it begins to proliferate in the juice in which they are present, results in the production of two tainting compounds, namely guaiacol and 2,6-dibromophenol. These compounds confer an off-odour/taste and thus results in the spoilage of the juices in which they are produced. Because of the financial implications of the aesthetic unacceptability of the two tainting compounds in juices, these acidophilic, thermophilic spore-formers have become a major quality control target microorganism Chang and Kang, 2004).

1.5 DETECTION OF *Alicyclobacillus acidoterrestris* USING TRADITIONAL AND MODERN TECHNIQUES

It is often to assumed that if the microbiological test results of juices are satisfactory (obtained using standard culturing techniques), then those products will not be subject to problems. However, extra caution needs to be exercised where *A. acidoterrestris* is concerned as the presence of these micro-organisms is often not detected during routine screening procedures because of their special requirements to proliferate to detectable levels. To avoid the consequences that would result in the event of *A. acidoterrestris* contamination, adequate procedures need to be in place to detect potential problems of the kind attributable to *A. acidoterrestris* at an early stage. The most obvious procedure that could be implemented are bacteriological techniques, since modified methods that are used for the routine screening of other micro-organisms are inadequate for this bacterium. However, it has been established on a laboratory scale that it is neither the quickest nor the cheapest exercise to isolate and identify *A. acidoterrestris* (Jenson, 1999). Therefore alternate methods of detecting contamination of fruit juices via *A. acidoterrestris* would be sought after. A pragmatic alternative would be to establish production of taints within juices as an indication of such contamination.

Traditionally, taint production was established in two ways, namely gas chromatography and/or sensory evaluation using human panellists both of which would, in this context, be classified as modern, chemical techniques for the detection of bacteria (Fox *et al*., 1990). Taint compounds such as guaiacol and 2,6-dibromophenol are detected by panellists at 2ppb and at ppt levels respectively. These levels have been established via subjecting juices with these compounds to gas chromatography. This demonstrates how these two techniques can be used in conjunction with each other. The information derived from gas chromatography in the analysis and identification of taints is beneficial. However, the levels of tainting compounds are often very low and this makes the analysis and identification of taints in this way (i.e. gas chromatography) a challenging and often time-consuming exercise usually requiring a unique combination of scientific skills and analytical expertise (Baigrie, 1993).

The application of sensory techniques as a routine screening procedure in, for example, detecting taints of the kind produced by *A. acidoterrestris* under simulated conditions is also very challenging (Lawless and Heymann, 1999). The time required to assemble a panel, to prepare samples for testing and to analyse and report the results of sensory data is appreciable. However, a family of instruments collectively referred to as 'electronic noses', which can be operated easily and quickly, have recently made an appearance in the marketplace. Electronic noses, like gas chromatography, falls under the category of analytical microbiology within the scope of this research project.

Electronic noses are already being used in many industries including the food and beverage, automotive, cosmetics and tabacco industries as well as by environmental safety institutions. As the name suggests, the design of this instrument is based on the design of the human olfactory system making them capable of assessing the volatile aromas of products such as fruit juices in a way that correlates with the way in which humans would respond to the olfactory properties of the product. They are devices used to analyse the content of a particular headspace through the classification of odours. These instruments are therefore capable of pointing to deviations such as taints like those produced by micro-organisms in products and can also be used as a qualitative quality control tool. The development of such instruments is of great value in minimising total reliance on sensory evaluation and gas chromatography for routine evaluation of products. Routine evaluation refers to repetitive testing that is performed in a production facility, such as a juice manufacturing plant, for the purposes of assessing both raw materials and finished products before they leave the plant. The use of such an instrument would be of advantage especially where a limited number of people are available to conduct sensory evaluations on products or when repetitive sensory evaluations cannot be sustained. It should not be concluded that electronic noses are a complete replacement for sensory evaluations of products. In initial stages of testing, these two technologies should be used in conjunction with each other to establish any correlations which might exist between the two.

1.6 AIM

The aim of this project is to evaluate different techniques to detect *Alicyclobacillus acidoterrestris* and its tainting compounds.

1.7 OBJECTIVES

This research has the following objectives:

(a) to isolate and identify *A. acidoterrestris* from juices;

(b) to optimize parameters for the isolation and growth of *Alicyclobacillus acidoterrestris*. These include:

- effect of juice type
- effect of juice flavour
- quantification of inoculum size

(c) to use the optimised parameters to evaluate the detection of *Alicyclobacillus acidoterrestris* spoilage by:

- standard bacteriological methods
- sensory evaluation using olfactory techniques
- electronic nose technology, and
- gas chromatography

(d) to compare the detection methods listed in (c).

CHAPTER TWO

2. LITERATURE REVIEW

The literature review covers several theoretical and practical issues regarding the spoilage micro-organism, *A. acidoterrestris* and the detection of spoilage via *A. acidoterrestris* using sensory evaluation; gas chromatography and electronic nose technology.

2.1 *Alicyclobacillus acidoterrestris* THE SPOILAGE MICRO-ORGANISM

2.1.1 Historical overview of the isolation of a thermophilic, acidophilic micro-organism

The genus *Bacillus* is a large collection of aerobic, rod-shaped, gram positive to gram variable, endospore-forming bacteria/micro-organisms (Wisotzkey *et al*., 1992). The bacteria of this genus exhibit the ability to grow under extreme conditions including growth in hot, cold, acidic, alkaline and salty environments and are thus sometimes referred to as thermophilic, psychrophilic, acidophilic, alcalophilic and halophilic micro-organisms respectively. The existence of thermophilic bacteria within the genus *Bacillus* has been well documented (Smith *et al*., 1952 loc. cit. Darland and Brock, 1971).

With the exception of *Bacillus coagulans* which is capable of growth at the acidic pH of 4.2 (Walls and Chuyate, 1998), *Bacillus* species are primarily adapted to alkaline or neutral habitats (Darland and Brock, 1971). A thermophilic *Bacillus* species isolated by Uchino and Doi (1967) from hotsprings in Japan was then tentatively identified as a strain closely related to *B. coagulans* because of its acidophilic nature. Four years later, Darland and Brock (1971), working in the United States of America (U.S.A.), isolated a *Bacillus* species which had physiological and morphological characteristics similar to those isolated from the Japanese hotsprings by Uchino and Doi (1967). The U.S.A isolates were also from hotsprings. However, Deoxyribonucleic acid (DNA) base composition work carried out by Darland and Brock (1971) illustrated that the isolates had higher Guanine and Cytosine content than *B. coagulans*. In addition to this finding, they also established that their strain was more acidophilic than *B. coagulans*. Because of the distinct differences

between *B. coagulans* and the new isolates, the new isolates were given the name *B. acidocaldarius*. Unusual fatty acids, omega (ω)-alicyclic fatty acids, were discovered in the bacterial cell membranes of *B. acidocaldarius*. Such fatty acids are not found in the cell membrane of *B. coagulans* which lends further support to the distinction of the new isolate from *B. coagulans*. Ten years after this event, bacteria which was thought to be *B. acidocaldarius* was isolated from garden soil, as opposed to having been previously isolated from the thermal environments of hotsprings (Hippchen *et al*., 1981 loc.cit. Jenson, 1999). Taxonomic studies on these micro-organisms revealed that they had a lower optimum growth temperature of 22°C which was significantly different from the 1971 *B. acidocaldarius* isolate found in the hotsprings which had a lower optimum growth temperature of 45°C (Walls and Chuyate, 1998). The isolates from the soil were thus given a new name, *B. acidoterrestris* (Deinhard *et al*., 1987 a), to distinguish them from *B. acidocaldarius*.

2.1.2 The creation of a new genus (*Alicyclobacillus*) for thermophilic, acidophilic micro-organisms

The spoilage of juices by *B. acidoterrestris* prompted more extensive studies on these micro-organisms. These studies revealed that those organisms that are obligately thermophilic and acidophilic were distinctly different from the other species found in the genus *Bacillus* in that their spores were more resistant to acid and heat (Orr and Beuchat, 2000). More importantly, as revealed when isolates were classified as *B. acidocaldarius*, was the presence of ω-alicyclic fatty acids as the major fatty acid component in the cell membranes of these micro-organisms (Deinhard *et al*. 1987b). This lipid type was found to be unique to these thermophilic, acidophilic micro-organisms and has not been found in any other established *Bacillus* species examined to date. It is for this reason that Wisotzkey *et al.* (1992) proposed that the thermophilic, acidophilic micro-organisms be placed in a new genus, namely *Alicylcobacillus*. Further reclassification of these micro-organisms under a new genus was further warranted because of 16S subunit ribosomal Ribonucleic acid (rRNA) sequence data which distinguish them from the 16S subunit rRNA sequences of *Bacillus* species, suggesting that these organisms are very far removed from other species currently considered members of the genus *Bacillus* (Wisotzkey *et al.*, 1992). The word *Alicyclobacillus* has German origins with '*aliphos*' being the German

word for fat and '*kyklo*' being the German word for circle, hence the definition of the alicyclo component of the new genus name refers to the circular fatty acids found in the cell membrane of the micro-organisms found in this genus. The *bacillus* component of the new genus simply refers to the rod shape of the respective micro-organisms in question (Wisotzkey *et al*., 1992). All the thermophilic, acidophilic, rod-shaped spore-formers are grouped under the genus *Alicylclobacillus*.

2.1.3 Organisms found in the genus *Alicyclobacillus*

Up until recently there were three species found in the genus *Alicyclobacillus*. These are *Alicyclobacillus acidoterrestris*, *A. acidocaldarius* and *A. cycloheptanicus*. *A. acidoterrestris* and *A. acidocaldarius* seem to be very closely related, and both contain ω-cyclohexyl fatty acids, that is, they contain 6-carbon ω-alicyclic fatty acids in their cell membranes. *A. cycloheptanicus*, as the name suggests, contains ω-cycloheptyl fatty acids, that is 7-carbon ω-alicyclic fatty acids in its cell membrane. The use of model membrane systems have made it possible to illustrate that membrane lipids composed of ω-alicyclic fatty acids of the type found in *A. acidoterrestris* and *A. acidocaldarius* pack densely, resulting in low rates of diffusion of the respective membranes at high temperatures. It is suggested that this property of the densely packed lipids in the cell membrane of these micro-organisms provides an advantage when cultures are grown at high temperatures. Six novel species have recently been formally assigned to the genus, namely *A. vulcanalis* (Simbahan *et al.,* 2004), *A. pomorum* (Goto *et al.,* 2003), *A. sendaiensis* (Tsuruoka *et al.,* 2003), *A. acidophilus* (Matsubara *et al*., 2002), *A. herbarius* (Goto *et al*., 2002) and *A. hesperidium* (Albuquerque *et al*., 2000). Of these nine species currently found in the genus *Alicylcobacillus* the only one responsible for taint is *A. acidoterrestris*.

2.1.4 Characteristics of *Alicyclobacillus acidoterrestris*

(a) Physical characteristics: The vegetative cells of *A. acidoterrestris* are 2.9-4.5μm in length, with a width ranging from 0.6 to 0.8μm. They produce terminally or sub-terminally located spores very rapidly, generally within 24 hours in liquid and solid media and in juices.

(b) Temperature growth range: Jenson (1999) has reported growth of *A. acidoterrestris* from around 20°C to 60°C with the optimum temperature being cited as 42°C to 53°C. Extremes of 12°C to 80°C have also been reported as permitting growth of the micro-organisms albeit at reduced rates. The thermal resistance of these micro-organisms may be attributed to the presence of the ω-alicyclic acid, namely ω-cyclohexane comprising the cell membrane. These lipids are closely packed in the membrane core and contribute to the thermal resistance of the micro-organisms by stabilising the cell membrane, preventing the inactivation that would otherwise result.

(c) pH Growth range: While Jenson (1999) found that the *A. acidoterrestris* were capable of growing at pH values ranging from 2.5 to 6.0, the optimum pH values for growth of the organisms are considered to be around 3.5 to 5.0. Just as the cell membrane structure confers the characteristic of thermal resistance to *A. acidoterrestris*, it also confers the characteristic of low pH tolerance to these micro-organisms. However, it is the protein component of the cell membrane rather than the lipid component which confers to the organisms the ability to grow in such low pH values. The protein is very stable and maintains its folded structure under conditions of low pH.

2.1.5 Sources of contamination of fruit juices by *Alicyclobacillus acidoterrestris*

Because of its role as a spoilage organism of shelf-stable products, studies on *Alicyclobacillus acidoterrestris* have become more focused on its spoilage mechanisms (Jenson, 1999). In other words, instead of continuing investigations on the morphological, physiological and biochemical aspects, studies now focus on *Alicyclobacillus acidoterrestris* as a spoilage micro-organism. Issues such as sources of contamination by *A. acidoterrestris* and/or its spores; preventing contamination; or employing corrective measures once contamination has occurred; and detection of *A. acidoterrestris* and the tainting compounds have become important.

Generally, soil is one of the main sources of contamination of foods or beverages derived from plants (Zottola, 1993). Fertile soil contains millions of bacteria per gram and it is not difficult to see how agricultural products which are derived from plants grown in or around soil can become contaminated with large numbers and types of bacteria (ICMSF, 1988). It

is thus suggested that the most likely source of juice contamination by *A. acidoterrestris*, which is a soil-borne micro-organism, is via unwashed or poorly washed fruit which has been contaminated by soil during harvest and is thus introduced into the manufacturing plant (Pontius *et al*., 1998). Once associated with the fruits, micro-organisms (vegetative cells and spores) are known to adhere to fruit produce in grooves, crevices and cuts on the surface (Seo and Frank, 1999) and it then becomes difficult to remove them via washing. This is especially the case when the fruits are covered with natural cutin as is the case with many of the fruits used to manufacture fruit juices. The cutin makes the respective fruit surface hydrophobic and provides protection for vegetative cells and spores present in the grooves and crevices. Not only has *A. acidoterrestris* been found to be present in soils and subsequently, fruit surfaces, but it has also been isolated from water used in fruit processing facilities in both ingredient water used in producing juice as well as in condensate water generated in processing juice. Therefore the source of contamination may not always be soil or fruit as ingredient water, and water used to wash the fruit are also implicated (McIntyre *et al*., 1995; Wisse and Parish, 1998). Isolation of the bacteria from water is not entirely surprising as *A. acidoterrestris* is a soil-borne organism, so an association with water – via run-off or the water table – is to be expected. Once the bacteria enter the manufacturing environment, they are difficult to eradicate and can become a chronic source of contamination.

2.1.6 *Alicyclobacillus acidoterrestris* spores

2.1.6.1 The effect of acidity and pasteurisation on *Alicyclobacillus acidoterrestris* spores

Shelf-stability of certain acidic fruit juices stored at ambient temperature is maintained by the combination of low pH and pasteurisation. The naturally low pH of the juices prevents many types of micro-organisms such as yeasts, moulds and a few groups of aciduric bacteria which might cause spoilage from growing therein (McIntyre *et al*., 1995). Additionally, the spores of most spore-forming spoilage bacteria will not germinate at pH < 4.10 (Pontius *et al.* 1998). Therefore, the likelihood of products with pH values equivalent to or lower than this being spoiled by spore-formers is small and has been limited to a few micro-organisms, for example, *Bacillus coagulans* and *Clostridium*

pasteurianum, both of which have been reported as being capable of growing at pH 3.80 (Blocher and Busta, 1983).

The heat labile spoilage organisms such as yeasts, some types of moulds and lactic acid bacteria that are not inhibited by the low pH of the products are generally destroyed by pasteurisation by a hot fill and hold process. This process has been deemed suitable to pasteurise these products and render them commercially sterile, hence shelf-stable and unlikely to spoil. The hot-fill and hold process used to pasteurise products with a low pH entails processing the juice into its respective packaging at an appropriate temperature, usually 86°C to 96°C. The pack is then closed or sealed and inverted so that all inner parts of the packaging are pasteurised by contact with the hot drink for a specified time, typically for 2 minutes (otherwise referred to as holding). The pack and its contents are then cooled as rapidly as possible which can be achieved either by blast-chilling with air or by immersion in, or spraying, with a cold liquid such as water or brine (Lewis and Heppell, 2000). The time and temperature combination mentioned is sufficient to destroy the organisms previously thought to be the cause of spoilage of products with a pH < 4.10 as well as to destroy pathogens such as *E. coli* 0157:H7, *S. typhi*, *Shigella* and *Clostridium* (Walls and Chuyate, 1998). For this reason, it was quite perplexing when the German spoilage incident (Cerny *et al.*, 1984 loc. cit. Eiroa *et al.*, 1999) occurred with juices packaged and pasteurised in this manner (i.e. hot-fill and hold); the spoilage could not have been caused by not yeasts, mould or lactic acid bacteria. It is only now known that spores of *A. acidoterrestris* were able to survive the typical pasteurisation process given to fruit juices (Walls and Chuyate, 1998). These spores were capable of germinating and multiplying thus causing spoilage of products with a pH previously considered below the range for growth of spore-forming bacteria (Walls and Chuyate, 1998).

2.1.6.2 The decimal threshold (D) value for *Alicyclobacillus acidoterrestris* spores

Having done heat resistance studies with the spores of *A. acidoterrestris*, Splittstoesser *et al*. (1994) concluded that these spores are able to survive the typical hot-fill and hold juice pasteurisation process. This conclusion was based on the observation that the Decimal Threshold Value (i.e. the D-value which is the time necessary to reduce the viable spore population by 90% at a given temperature) for *A. acidoterrestris* spores at 95°C was 2.4 to 2.8 minutes. Considering that the maximum temperature reached during the hot-fill and hold process is 96°C and that the holding time is only two minutes, it is easy to see how *A. acidoterrestris* spores are able to survive the hot-fill and hold process given to juices (Walls and Chuyate, 1998). It is, infact, suggested that the hot-fill and hold process serves to stimulate spore germination and outgrowth (Jenson, 1999). If this occurs and incubation conditions are suitable for multiplication of the micro-organisms, it is likely that spoilage will result. In other words pasteurisation using hot-fill and hold process to sterilise and prevent occurrences such as spoilage, actually increases the potential for spoilage (Jenson, 1999). The higher temperatures and times required to eliminate *A. acidoterrestris* spores from acid products are very likely to adversely affect the quality of the product.

2.1.7 Juices susceptible to spoilage by *Alicyclobacillus acidoterrestris*

Pettipher *et al*. (1997) have stated that fresh (not heat treated) or pasteurised (hot-fill and hold) juice products which are stored unpreserved at ambient temperature, are susceptible to spoilage if contaminated with *A. acidoterrestris*, especially since most such products are in the pH range in which the micro-organism is capable of growing (pH 2.5-6.0). It can, however, be said that juices pasteurised by means of Ultrahigh temperatures (UHT), irrespective of their preservative or pH status, are safe from spoilage (Pettipher *et al*., 1997). *A. acidoterrestris* were first implicated as spoilage organisms in Germany in 1982 and documented in 1984 (Cerny *et al*., 1984 loc. cit. Eiroa *et al*., 1999). This incident involved the large-scale spoilage of aseptically packed, shelf-stable apple juices during distribution and storage following an exceptionally long, hot summer. It has since been established that spoilage tends to occur most often in the spring or summer (Orr *et al*., 2000). Not only does *A. acidoterrestris* occur in citrus juices such as orange and grapefruit

juices but in other acidified juices as well, for example, apple, pear, peach, white-grape and berry juices. Moreover, *A. acidoterrestris* spores are more likely to be found in juice concentrates as opposed to single strength juices. The reason for this is that the higher sugar content of concentrates increases the heat resistance of spores (Splittsoesser *et al*., 1998). However, high sugar levels will inhibit the germination and outgrowth of spores. Once concentrates are diluted the potential for germination and outgrowth is re-established.

2.1.8 The tainting compounds produced by *Alicyclobacillus acidoterrestris*

Spoilage of juices brought about by the presence and proliferation of *A. acidoterrestris* is in the form of a strong flavour taint in the juices. In the 1990s *A. acidoterrestris* was implicated in the spoilage of fruit juices in the U.S.A., Japan, Europe and Brazil. In the U.S.A, hot-filled apple juices and apple-cranberry juice blends spoiled as a consequence of *A. acidoterrestris*. In Japan, when fruit juices spoiled, Japanese workers attributed the spoilage to the metabolic production of the 'offensive smelling' chemical compound guaiacol by the *A. acidoterrestris*. Guaiacol, the chemical structure of which is represented in Fig. 1(a), has also been described as having a medicinal, antiseptic or phenolic odour and, according to Pettipher *et al.* (1997), can be detected in fruit juices at levels of 2ppb by sensory means. Guaiacol was detected in orange juice and apple juice when approximately 1×10^5 colony forming units (cfu) of *A. acidoterrestris* were present per ml of juice (Pettipher *et al*., 1997). European workers, like their Japanese counterparts also established the presence of guaiacol in spoiled juices and went on to say that there was also a flavour taint chemical produced by *A. acidoterrestris*. The chemical was later identified as 2,6-dibromophenol (Fig. 1(b)) which is produced in juices at parts per trillion levels. Bromophenol production by these bacteria is of particular significance to the food industry as these chemicals are halophenols generally known for their ability to cause flavour taint in foods (Jenson, 1999). Bromophenols have only been recently identified as the compound likely to be causing disinfectant taints as chlorophenols were considered to be the major source of disinfectant taints at levels below 1ppb (Saxby, 1993).

Essentially then, the guaiacol and 2,6-dibromophenol are volatile chemical compounds produced by *A. acidoterrestris* which causes tainting and spoilage by conferring a bad odour and taste to the juices.

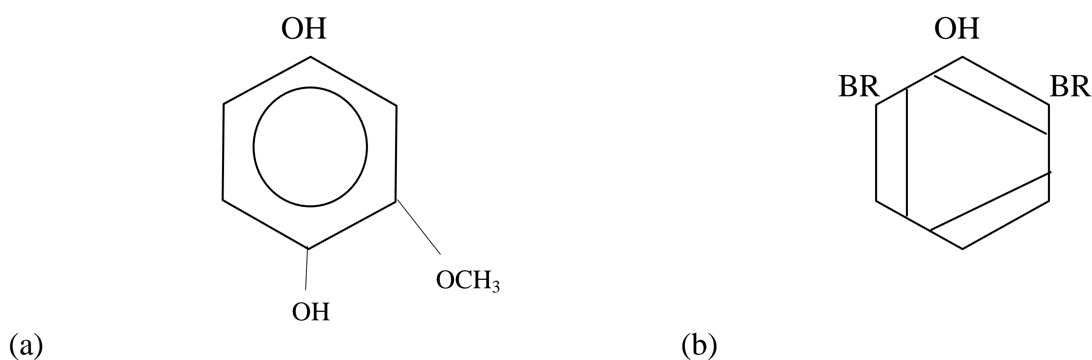


Figure 1: Chemical structures of (a) guaiacol (Crawford and Ohlson, 1978) and (b) 2,6-dibromophenol (Buckingham, 1982)

2.1.9 Bacteriological tests for *Alicyclobacillus acidoterrestris*

The reason that *A. acidoterrestris* is not, as mentioned earlier, detected during routine screening procedures is that the numbers of *A. acidoterrestris* spores are often very low in processed samples. Hence, sensitive detection procedures which will induce germination and enrichment of any spores which may be present in the juice are required. Most authors recommend application of a heat shock treatment, with heating to 80°C for 10 minutes and cooling thereafter in a waterbath at around 40°C (Jenson, 1999). Direct plating after the heat shock treatment is ineffective in detecting such low numbers of spores, and enrichment procedures must therefore be adopted. The enrichment procedure entails incubation of the samples for 2 days at 40-50°C. Agar media for the plating of the heat shocked, enriched juices has been the subject of much debate. Orange Serum Agar (OSA) is one of the media types which some authors (Pettipher *et al*., 1997) have found to be most effective for isolation/detection and enumeration of *A. acidoterrestris* when adjusted to pH 4.0. In contrast, Walls and Chuyate (1998) found K-Medium (KM) with a pH of 3.7 to be superior to OSA. On OSA, *A. acidoterrestris* has a distinctive appearance, being a colony with a very flat interior with raised annulus and a button centre. Colonies have a translucent cream colour initially. If a heat shock treatment has been used colonies require longer than normal (2 days) incubation periods. Plates are often incubated for a minimum of 5 days under such conditions (Jenson, 1999). Other media types which have been used for the detection and enumeration of *A. acidoterrestris* include *Bacillus acidoterrestris*

Medium (BAM), Malt Extract Agar and acidified Potato Dextrose Agar (PDA). Presently, none of these media can be recognised as unconditionally superior.

2.1.10 Implications of spoilage of fruit juices by *A. acidoterrestris* for the fruit juice industry

The potential for *A. acidoterrestris* to cause extensive spoilage to fresh or pasteurised shelf-stable acidic products, particularly those to which preservatives have not been added, should not be underestimated. Such products (except for those that are UHT pasteurised) are most susceptible to spoilage by this bacterium. Although spoilage does not occur very often, it can prove very costly for manufacturers when it does. Surveys conducted in the food industry indicate that manufacturers are usually unaware of *A. acidoterrestris* spoilage problems until they receive consumer complaints (Gouws, 2004). Even when they receive complaints, manufacturers attribute spoilage of the products to direct chemical contamination from an external source rather than to microbial contamination and proliferation of micro-organisms. Such assumptions are made because no gas is produced which is a feature often used as an indicating spoilage. This makes sense as *A. acidoterrestris* is not a gas producing spoilage organism. In addition to this, manufacturers may easily fail to detect the presence of *A. acidoterrestris* during routine quality screening procedures because these organisms have specific requirements in terms of the acidity of the media, elevated incubation temperatures and the enrichment techniques which they require to proliferate to detectable levels. *A. acidoterrestris*, for all these reasons, represents a new challenge to the juice industry. It is imperative, that if present, these organisms are detected early so that large-scale marketplace losses may be averted by the implementation of corrective action procedures. The next 3 sections deal with techniques which, in addition to the bacteriological tests described in section 2.1.9 may be able to serve this purpose. These include sensory evaluation, gas chromatography and electronic nose technology.

2.2 SENSORY EVALUATION

2.2.1 General

Our senses enable us to perceive our environment. There are two predominant ideas of the number of senses humans have, with the most common number being five, namely the senses of sight, smell, taste, touch and hearing (Matheis, 1994). It goes without saying that tests using the five senses have been conducted for as long as there have been human beings and there is no definite beginning to the field. Infact everything that could be used and consumed by humans has been subjected to some type of sensory test by humans since time immemorial. Present day sensory testing is, of course, more formal and developed than that practised in the past. Sensory testing extends from evaluating the quality of water, shelters and even weapons to assessing environmental odours, personal hygiene products and testing of pure chemicals. Apart from having these applications, sensory techniques are routinely and extensively used in characterisation and evaluation of foods and beverages (Stone and Sidel, 1993). Within the food and beverage industry, sensory evaluation has been defined as "a scientific discipline used to evoke, measure, analyse and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing" (Institute of Food Technologists, 1975 loc. cit. Kapsalis, 1987).

All consumers make judgements about the products they eat and drink. Much of the richness and subtlety of the consumer's sensory experience with food and beverages comes from the odours/aroma and flavours which they exude (Coren and Ward, 1989). The food and beverage industry was one of the earliest supporters of sensory evaluation. Sensory evaluation is used in the industry to establish difference between products and to characterise and measure sensory attributes of products such as flavour, texture, colour, size, density and aroma (Hubbard, 1996). It has also been used in industry to establish whether product differences are acceptable or unacceptable to the consumers either for product development or in quality control or quality assurance processes. In product development or quality control, understanding, determining and evaluating the sensory characteristics of products are important in many applications. These include shelf-life studies, product matching, product mapping, product specification, product reformulation,

determining product acceptability and testing for taint and taint potential (Lyons *et al*., 1992). Of these applications, the one which is of relevance to this study and which will be discussed further is testing for taint and taint potential in products as a component of quality control.

2.2.2 Sensory evaluation in testing for taint

Food companies who operate under the stringent standards of Good Manufacturing Practices (GMPs) as well as quality control and quality assurance programmes normally detect grossly tainted products before they leave the factory by means of employing sensory panels. However, some taints develop sporadically, for example, once they have been distributed to retailers or by direct consumer complaints, as is often the case when *A. acidoterrestris* is the contaminant that caused the taint. Whether taints develop in products on factory premises or sporadically on shop shelves, employment of a sensory panel to attempt to identify the taint on site would be required (Baigrie, 1993).

Characteristic food taints are often detected at sub-ppm levels, even ppt levels by the olfactory sense (sense of smell), the gustatory sense (sense of taste) or, in many situations a combination of both senses (Coren and Ward, 1989). However, the concentrations at which taint compounds can be detected varies considerably between individuals. Selection of a panel to detect taints normally involves a measurement of their threshold to that taint. While the term threshold is often used 'loosely', according to International Standards Organisation (ISO) 5492 (ISO, 1992), there are in fact, four different types of thresholds. These are detection threshold which is the minimum value of a sensory stimulus needed to give rise to a sensation; recognition threshold which refers to the minimum value of a sensory stimulus permitting identification of the sensation perceived; difference threshold which refers to the value of the smallest perceptible difference in the physical intensity of a stimulus and finally, terminal threshold which is the minimum value of an intense sensory stimulus above which no difference in intensity can be perceived. The one of relevance to this study and which is commonly used in dealing with taints, is the detection threshold.

The wide range of human thresholds or sensitivities to chemical stimuli is a major reason for the difficulties in preventing taints and in positively identifying the causes of such

taints. Studies carried out in the past have illustrated that the difference between the 1% of people most sensitive and 1% least sensitive to tainting compounds is in the order of 10^6. If one in every 100 consumers (effectively equivalent to the 1% most sensitive) complains to a supermarket that a food has, for example, an antiseptic taint, this would be regarded as an unacceptably high percentage. It can therefore be seen that the food industry must ensure that any potential tainting compound is not only below the mean detection threshold level but also below the levels that this small but highly important percentage of sensitive consumers can detect. In using sensory methods for taint identification and prevention, it is therefore important to try and utilise human subjects who are known to represent, as far as possible, those highly sensitive consumers.

Even if threshold measurements are conducted under consistent conditions there are some drawbacks in the use of human panellists. For one, variations in measured thresholds must be expected as a result of human subjects used as measuring instruments producing variable results over time. Additionally, the range of human sensitivities is enormous. Furthermore human subjects are very prone to bias. It is well known that members of a panel vary innately in their responses when assessing sensory characteristics of different commodities. These drawbacks are accounted for by the implementation of repeated measures, employment of fifteen to fifty panellists so that outcomes of the evaluation are statistically representative and, the interpreter of the results respects and is aware of the biases which govern panel attitudes. Subjects need to be trained fully to understand what they need to do if there is any hope of obtaining an objective set of results (Baigrie, 1993).

Sensory evaluation could serve to confirm that a product is tainted and may provide some clues as to the nature of the taint. Such sensory evaluation may be either gustatory or olfactory or a combination of these provided that no harm will be caused to panellists due to the ingestion of tainted products. Once the presence of a taint has been established, the next step is to ascertain its chemical structure (in other words, what the chemical taint actually is and is commonly established by GC) and to then determine the source (e.g. bacterial etc.) of the tainting chemical.

2.2.3 Sensory Methods

2.2.3.1 General

Sensory methods can be grouped into two categories, namely affective (or hedonic) testing, which is subjective, and analytical testing which is objective. Both of these categories are as important as that of the chemical, physical and nutritional aspects of foods and beverages (Gridgeman, 1984). Affective testing measures liking or preference for a product based on the spontaneous, personal reaction its sensory properties evoke in panellists (Kapsalis, 1987). Although panellists in affective testing are sometimes captured by a specific aspect of a product (especially if it is a bad, unexpected or unpleasant one), they are in fact expected to perceive a product as a whole pattern, without investing much thought or dissection of the products profile (Lawless and Heymann, 1999). This measure is logical and necessary information before substantial capital can be invested in equipment, production, distribution and advertising of a product (Stone and Sidel, 1993). Affective testing provides a good indication of a product's potential prior to the development of packaging, price, advertising and market segmentation. Of those sensory methods which fall in the analytical category, there are the descriptive and discriminative/difference types. Descriptive tests are used to obtain a total description of the sensory characteristics of a product, and to use these characteristics to quantify differences between products (Lewis and Heppell, 2000). Rating the descriptors for the sensory characteristics of appearance, odour, flavour, texture and after-taste will generate what is often referred to as the 'sensory profile' of the product. The discriminative tests, as applicable to this study, are used as a means of determining whether there are perceptible differences between two or more products (Lyons *et al*., 1992). Discriminative tests can therefore be used to establish thresholds and, as a class of tests, represents one of the most useful analytical tools available to the sensory professional (Stone and Sidel, 1993), especially since such tests involve comparative judgements which can be very sensitive in determining small differences between products.

2.2.3.2 The triangle test

The triangle test falls under the category of discriminative testing. If a high-sensitivity panel is not attainable, as many panellists as possible must be used. The minimum number of panellists for a triangular test is fifteen. Triangular presentations involve presenting panellists with three coded samples, with two of the samples being of the same type and the third different. Panellists have to indicate either which is the odd sample or, less frequently, which two samples are most similar. It is not advisable for the person conducting the experiments (sensory analyst) to make a note of individual responses on a single response sheet but rather to present each panellist with their own scorecards. An example of a scorecard that could be used in a triangle test is represented in Fig. 2. Codes assigned to the samples should not give any hint as to the identity of treatments applied to the samples. Samples are presented in a random manner, the position of the similar sample being changed throughout the test (Lees, 1975). Since the panellist is looking for the odd sample, the samples should differ only in the variable being studied with all other differences being masked. Because of this, the triangle test is limited to products which are homogenous and would thus be suitable for use in this research. The triangular test is statistically powerful with only a $^{1}/_{3}$ chance of identifying the odd sample by chance which probably accounts for the claim for the greater sensitivity of the triangle test as compared to other tests such as paired comparison tests and duo trio tests. Forcing panellists to make decisions (an approach which was adopted in this research) is recommended for three reasons. Firstly, the necessity for a firm answer is known to bring about a slight inclination towards one of the items in the trial. Secondly, nothing is ‘lost’ because genuine guesses will be random. Thirdly, by making available a category of ‘do not know’ or ‘not sure’, bored or lazy panellists might be encouraged to choose this category too often (Gridgeman, 1984).

Triangular presentations have been suggested for use in situations of quality control work where, for example, products are being tested for consistency. It can be used in instances of taint evaluation or preventative taint evaluation. Using this approach to determine whether there is a statistically significant perceivable difference between the samples, the sensory analyst simply counts up the number of correct responses and consults the appropriate tables. Significant differences between the ‘different from control’ samples are

TRIANGLE TEST

Name: **Date:**

In front of you are 3 coded samples, two are the same and one is different. Starting from the left, smell the samples and circle the corresponding code of the sample that is different from the other 2. You may re-evaluate the samples, i.e. you may go back to and re-smell any of the samples to make a more confident decision. You must make a choice, whether or not you can establish which is the different sample.

Thank you.

Codes 624 801 199

Figure 2: Example of a scorecard for the triangle test (Adapted from Stone and Sidel, 1993)

established when the number of correct answers identifying the different sample, exceeds a certain level with respect to the particular number of panellists used. In terms of sensory testing for taint, preventive testing is a powerful means of limiting problems from arising from, for example, changes in environmental conditions (Lyons *et al*., 1992). Preventative taint testing involves exposing the product to a severe but not unrealistic situation that could produce the taint. Such testing is thus suitable for testing for micro-organisms such as *A. acidoterrestris* which often produces taint when, for instance, environmental conditions change and when the juices in which it is present are already on the shelves of retailers. Conducting preventive taint testing on batches of juices before they leave the factory premises has the benefit eliminating or reducing the negative effects of spoilage on consumers. However, it goes without saying that this is not the most feasible of options.

2.2.4 Considerations for sensory testing

In conducting a sensory study there are several requirements which need to be met. One of these requirements regards the room in which the evaluations are to be conducted. Firstly, the room should be free of distractions and air-conditioned or at least ventilated well enough to prevent foreign odours from lingering. Secondly, there should be no noisy equipment in the vicinity of the test room. Thirdly the lighting in the room should be uniform and well-diffused. Provisions for differences in the colour of products being

evaluated should be made in the lighting system especially since colour contributes substantially to decisions or judgements made by the panellists and which could interfere with the variable under study even when this is not the variable under study. However, if this is not possible and provided that there are only small differences in the colour amongst the products being compared, then dimmed light, dark containers or dark/black paper or cloth under clear glass containers can be employed (Gridgeman, 1984). It must be noted, however, that dimmed, or even coloured lighting might also have an effect on judgements. As tempting as it is to save time and avoid frustration by presenting samples to panellists at their point of work instead of waiting for them to present themselves at the test area, this is an unsatisfactory procedure as external influences affect judgement and it is difficult to prevent discussion between panel members. In sensory evaluations, every effort should be made to make samples from different treatments identical in every characteristic except that being judged (Kapsalis, 1987). It is advisable that samples are the same size or volume and they should always be presented at room temperature, unless they are products which need to be consumed hot. Serving containers which do not impart any odour to the product should be used and marking pens should not be used to apply codes. If the product is to be tasted, then the taste vessels should not impart any taste to the product either. Containers should be identical so that no bias is introduced from this source. Finally, it is important to note that, regardless of the method used, the risk of panellist fatigue and carry-over effects should be minimised by limiting the number of stimuli presented in any given session and by allowing sufficient time between samplings. No more than 4 to 8 stimuli should be presented in a single session to any one panellist (Kilcast, 1993).

Sensory evaluation has taken time to become accepted as a distinct, recognised scientific discipline. It is, however, an inexact science (Meilgaard *et al.*, 1990) and experimental design needs to be based on a thorough knowledge of the chemical and physical factors behind the sensory attribute of interest. There are many possible explanations for the results of sensory tests. Misinterpretations, however, can be minimised by expanding the knowledge base about our senses and the nature of product attributes.

2.2.5 Advantages of sensory methods in a quality control programme

Sensory methods have the following characteristics which make them unique in a quality control programme. Sensory methods:

(a) are the only methods that give direct measurements of perceived attributes;
(b) provide information that assist in better understanding consumer responses;
(c) can measure interactive perceived effects; and
(d) can provide "integrated" measurements by people versus "discrete" measurements provided by instruments (Muñoz *et al*., 1992).

2.2.6 Disadvantages of sensory methods in a quality control programme

Some of the disadvantages of sensory methods in a quality control programme are:

(a) time involved;
(b) expenses incurred;
(c) effect of environmental and emotional factors on panellists responses;
(d) effects of biases and physiological and psychological factors on the product's results;
(e) possible lack of accuracy and precision
(f) attrition; and
(g) need to handle and resolve personal factors that affect the operation of the programme (Muñoz *et al*., 1992).

2.3 GAS CHROMATOGRAPHY

2.3.1 General

The term chromatography covers those separation techniques in which the separation of the compounds of interest is based upon the partition or distribution of the compounds or analytes between two phases in a chromatographic system (Fowlis, 1998). Chromatography as a method of instrumental analysis is capable of producing data which, once interpreted, may describe the qualitative and quantitative composition of mixtures. Qualitative and quantitative data are obtained from chromatographic analysis only after

separation of the various compounds in a mixture (Schomburg, 1990). Gas liquid chromatography (GLC) is a form of chromatography which makes use of a gaseous mobile phase and a liquid stationary phase. Gas solid chromatography (GSC) makes use of a gaseous mobile phase and a solid stationary phase. Together these two are frequently lumped together under the single heading of 'Gas Chromatography' (GC) (Peters *et al.*, 1976). Said in another way, GC is a form of chromatography in which the mobile phase is gaseous and the stationary phase is either solid or liquid.

Gas chromatography is an analytical technique for the investigation of volatile compounds. No instrument has made more impact on organic analytical chemistry than the gas chromatograph (Goddard, 1997). GC has been in existence now for just over 50 years. Its use was first documented in an analysis of liquids in a paper by James and Martin (1952). Within a few years of this publication it became the most popular instrumental method for analysis. At first its most popular application was in the petro-chemical industry but it was gradually adopted in every area where analysis of mixtures of organic (and sometimes inorganic) materials was required (Cowper and DeRose, 1983). It is not surprising, then, that gas chromatographic methods have found wide acceptance for food and beverage analysis.

As mentioned in the introduction to this report, GC can be used in the diagnosis and detection of micro-organisms by detecting and identifying natural metabolites produced by the respective microbes. These metabolites can cause taints in the products in which they are produced. For many decades now, GC has been an almost indispensable tool in the food and beverage industry as it can be used as a quality control tool to identify metabolic products either on a qualitative basis or, more significantly, quantitative basis.

2.3.2 Assessment of data obtained from a chromatogram

Qualitative measurement refers just to the identification of compounds based on their retention times. Quantitative measurement refers to establishing amounts of compounds and is an essential part of taint studies. In terms of taint studies, quantification is achieved by comparing responses acquired for each solute eluent monitored from the sample to those obtained with standard solutions of the tainting chemical either alone or mixed with

the test substance (Lees, 1975). Identical retention times given for the standard solute and the taint compound in the product which would allow for identification while the size (height or area) of the respective peaks is proportional to the concentration. In these cases it is assumed that complete extraction of the taint compound from the tainted food or beverage has been achieved. Quantitative measurement in GC is dependent upon both detector and chromatographic system parameters including the detector gas used, the flow rate of the gas, the detector temperature, the method of sample introduction, the method of sample preparation or collection, the column type, the ambient temperature and pressure in the surroundings in which the tests are being conducted, the inertness of the system and signal path and data system considerations (Larson, 2001).

2.3.3 Advantages of gas chromatography

There are several advantages in the use of GC in industry. Firstly, if a few compounds are present in the sample to be analysed, GC analysis can be carried out quickly, to achieve high sample through put even with multicomponent samples. Secondly, peak area determinations are performed with high precision and accuracy by means of automated data handling systems. The precision of these measurements depends on the reproducibility of the analysis, because a series of samples must be analysed and the results compared. Thirdly, automation of the GC process is simple, and instruments offering this facility are already available. Finally, a wide range of thermodynamic systems, from dilute solutions to concentrated mixtures is covered by the wide dynamic range of detectors.

2.3.4 Disadvantages of gas chromatography

As powerful and popular as GC is as an analytical tool, it does suffer from a few weaknesses. For one, it requires that the compounds to be analysed be volatile. Secondly, and as a direct consequence of the first limitation of the technique, it is not an easy task to extract, enrich and to separate compounds of interest, for example taints, without losses which makes accurate quantification difficult. Another major problem is the lack of definitive proof of the nature of the detected compounds as they have been separated. For most GC detectors, identification is based solely on retention times in the columns. Since many compounds may possess the same retention times, we are left in doubt as to the

nature and purity of the compound in the separated peak, unless the method is used in conjunction with sensory evaluation (McMaster and McMaster, 1998). Fourthly, failures in gas chromatographic analysis can be attributed to the slow build-up to working temperature at the column head, a column length which is too short or insensitivity in setting of the detector.

Further complications arise when attempting to find methods of speeding up the process of GC. Minimal-time operation in GC has been a research topic for many years and many methods have been proposed. Such methods have included use of shorter columns, use of open columns with reduced inner diameter, use of faster temperature programming, use of pressure/flow programming, use of packed columns with small particles, application of more selective stationary phases, use of hydrogen as the carrier gas and operation of the column at above-optimum linear velocities. However, the wide variety of options for faster GC has been found to seriously complicate the situation for the analyst aiming to reduce the time of the analysis as it is not an easy task to select the best approach. There is no single approach that will result in a significant time reduction for all applications (Cramers *et al.,* 2001).

2.3.5 Headspace gas chromatography

2.3.5.1 Theory of headspace gas chromatography

The separation of volatile organic compounds is most easily carried out by GC and no serious problems are encountered if the whole sample can be vapourised (Kolb, 1984a). However, samples which are involatile but contain volatile constituents at low concentration levels need a ‘clean-up’ or modification prior to the actual GC analysis in order to prevent solid residue remaining in the inlet system. The danger associated with this is that, being a heated zone during the run, the solid residue may decompose and be represented on the chromatogram making it seem as if such compounds were actually present in the sample. Subsequent injections may also be affected by solid residue if decomposition does not occur immediately. Most of the time this modification involves some type of extraction procedure such as liquid solvent extraction, solid-phase extraction or supercritical-fluid extraction. When compounds of interest are extracted with a liquid

solvent, the compounds of interest will be distributed between the sample and the solvent, and the conditions are adjusted to favour distribution towards the solvent phase. However, if the compounds to be analysed are highly volatile, inert gas can be used equally well as a solvent for extraction. Just as in extraction with a liquid solvent, conditions can be adjusted to favour distribution of the volatiles towards the gas phase. Gas would be an ideal 'solvent' for volatile compounds, especially since it is easy to handle and available in much higher purity than most liquid organic solvents used for extraction of compounds to be analysed by conventional GC. The purity of the gas as a solvent avoids trace impurity interferences during the GC run. In addition to this a gas as a solvent does not cause a solvent peak with tailing in a chromatogram as a liquid, organic solvent would (Kolb, 1984b). A gas extract of this nature is ideally suited for analysis by GC and this combination is called "headspace gas chromatography" or HSGC (Kolb and Ettre, 1997). Contrary to classical GC where a peak area depends only on the concentration of substance, the vapour pressure of the substance as well as the activity co-efficient within the prepared mixture play an important role in headspace analysis (HSA) (Wittkowski, 1989).

Smith *et al*. (1982) provide a comprehensive definition for HSGC. They state that "HSGC is an indirect method for the determination of volatile constituents in liquids or solids by gas chromatographic analysis of the vapour phase which is in thermodynamic equilibrium with the sample in a closed system". In practice HSA consists of two steps. First, the sample to be analysed is placed in a glass vessel – usually a vial – so that there is a gas volume above the sample. The vessel is closed by sealing with a rubber septum. The vial is heated in a constant temperature bath until equilibrium for the sample vapour phase is established for each volatile component. The laws of physics dictate that part of the volatile compounds of the sample will be present in the gas phase in contact with the sample proper. Secondly, a known aliquot of the vapour phase is extracted with the inert carrier gas as the solvent and transferred to the chromatographic column for separation using a gas-tight syringe in the case of manual transfers. Automatic transfers are also possible by means of pressurisation of the sample vial and time or volume control (Kolb and Ettre, 1997). By using an aliquot of the gas phase, the volatile compounds can be analysed without interference from the non-volatile matrix. Figure 3 illustrates the two steps. The aliquot of the vapour phase transferred to the gas chromatogram is essentially

the sample. In HSGC it is the concentration of the volatile solute which is determined, initially obtained as a peak area taken from a chromatogram.

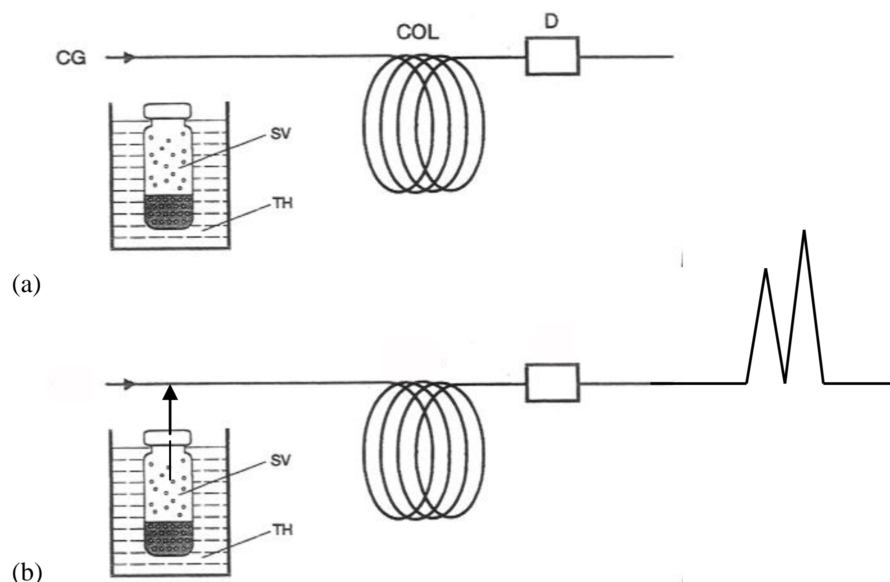


Figure 3: Principles of static headspace gas chromatograph illustrating (a) equilibration and (b) sample transfer. Key: CG – carrier gas; SV – sample vial; TH – thermostat; COL – gas chromatographic column and D – detector (Adapted from Kolb and Ettre, 1997)

2.3.5.2 Gas extraction techniques

Gas extraction techniques can be carried out in three ways. Firstly, as a single step extraction (static headspace extraction), secondly by stepwise repeating of the extraction (multiple headspace extraction) or thirdly, by stripping the volatiles by a continuous flow of an inert purge gas (dynamic headspace extraction). Static headspace extraction involves putting the sample (solid, liquid or gas) into the headspace vial, which is closed immediately and remains closed until a single aliquot of headspace gas has been withdrawn from the vial and transferred directly to the gas chromatographic system, thus guaranteeing sample integrity. The headspace gas is then analysed via GC and the information obtained from this single aliquot is used to make conclusions about the nature and concentration of the volatile analytes that were present in the original sample. Multiple headspace extraction is essentially the same as static headspace analysis except that it is done on a repeated basis. The most frequently used form of dynamic headspace

extraction involves passing an inert gas through a liquid sample and the extraction of the analytes is brought about by the gas bubbles generated by the inert gas. The gas effluent is usually passed through a trap containing an adsorbant, which retards or ‘traps’ the analytes carried there by the purge gas. Analysis of taints via headspace analysis makes use of adsorbants such as Tenax and Chromosorb. Analysis of the ‘trapped’ compounds is then brought about by heat desorption of the volatiles directly on a gas chromatograph or by extraction with a solvent (Maarse, 1993). Dynamic headspace analysis is also commonly referred to as the purge-and-trap method.

2.3.5.3 Comparison of extraction techniques

For any one sample to be analysed, a decision needs to be made as to which of these gas extraction techniques would be the most appropriate for the analysis. In making such decisions, the simplicity of the operation, the degree of automation, the flexibility for changing requirements, the sensitivity and the quantification needs to be considered.

The static HSGC is the most simple extraction. Automation has been available since 1967 and therefore this presents few limitations in terms of which extraction variant should be adopted for the analysis of any one sample. Static headspace extractions are also favoured over the other two extraction techniques when it comes to the flexibility of the system in adapting to varying sample properties. This is especially the case because, in principal at least, it is only necessary to determine the purely physical parameters (i.e. time and temperature) to achieve the necessary state of equilibrium in the vial in static HSGC. As far as sensitivity is concerned, dynamic HSGC appears to be favoured as the exhaustive extraction procedures provides a resultant gas extract which is often considered to be the same as the sample. The sensitivity range of most chromatographic systems extends down to the level of parts per trillion (10^{-12}) and therefore static systems need not necessarily be ruled out as compounds at such a low level can be detected. Static HSGC has been questioned in terms of the effect matrices have on quantitative aspects. However, Kolb and Ettre (1997) argue that this should not be the case as static HSGC has worked effectively in an experiment to quantitate ethanol in blood, a complex matrix in itself.

2.3.5.4 Applications of headspace gas chromatography

Static HSGC is a universally applied analytical method in a wide variety of substrates ranging from food contaminants, to environmental applications and biological samples, having been originally developed for the determination of alcohol in blood (Wittkowski, 1989). The application of HSGC in the food and beverage industry includes quality and production control of the alcoholic and fermentation by-products in breweries (Geiger, 1984), assessment of the odour of spices and spice mixtures (Koller, 1984), determination of aroma compounds in dairy products to ensure their acceptability (Hild, 1984) and in the food and beverage packaging industry. To be more specific with the application of HSGC in the packaging industry, it has been used in the detection of contaminants from the plastics (Jeffs, 1984) and foils (Lüthi, 1984) used in packaging.

2.3.5.5 Advantages of headspace gas chromatography

Headspace analysis has the advantage of lower risk in terms of compounds being decomposed thermally (Maarse, 1993) The advantages of this method compared with conventional preparation of the sample for analysis includes the simplicity of the preparation, comparatively minimal analysis time and high sensitivity (Smith *et al*., 1982). The general advantage of HSGC over general GC is that, with the former, there is no need to be concerned about nonvolatile or high boiling sample components, as they will not enter and contaminate the gas chromatograph.

2.3.5.6 Disadvantages of headspace gas chromatography

Although HSGC has permitted major advances in the field of volatile analysis, many investigators have been confronted with serious problems as direct injection can only be used for the detection of major components with high vapour pressure as well as occurrences such as deterioration of column performances when concentrated liquid samples are injected into the system (Nitz and Julich, 1984). Headspace sampling is an attractive method which has permitted major advances for analysing the volatiles in foods and beverages. It is simple, rapid and measures the odours in the proportions typically presented to the human nose. The primary disadvantage, however, is that the method lacks

sensitivity as typically, only the most volatile and abundant compounds may be determined using this technique. Also headspace sampling is not typically used with capillary columns as the gas flow through these columns is quite low (Leahy and Reineccius, 1984).

2.4 ELECTRONIC NOSE TECHNOLOGY

2.4.1 The development of artificial chemical sensing systems – electronic noses – based on the biological sensing system

Smell is arguably the most mysterious yet most evocative of our senses. Nature has endowed humans with an exquisitely sensitive chemosensory system capable of recognising around ten thousand scents ranging from the pleasurable fragrance of freshly cut jasmine to the putrid smell of rotting eggs, each of which evokes different reactions from different individuals (Axel, 1995). We are capable of recognising and discriminating various odours as information is transmitted from the nose to the brain where it is decoded. This process is brought about by the interaction of volatile odourant molecules with the membrane of odour receptors in the nose causing changes in their ionic conductance (Thomlinson *et al.*, 1995). This culminates in the generation of electrical signals which are then fed to the olfactory bulb, the part of the brain responsible for the processing of olfactory information and output in the form of odour recognition (Persaud, 1990).

There are one thousand types of odour receptors each of which are individually expressed in thousands of neurons (Axel, 1995). However, at least thirty of these thousand types present in the human nose overlap in terms of specificity towards different classes of volatile odourant molecules (Persaud, 1990). In other words, the odour receptors have very poor selectivity and show broad patterns of response as interpreted by the brain (Persaud and Travers, 1991). Odours are thus actually achieved by processing the signals obtained from many receptors that have these broadly different ranges of selectivity for different odourant molecules. By comparing incoming odour patterns generated in this manner with previously learnt odour patterns, it is then possible for humans to discriminate different odours (Persaud, 1990).

Over the last few years, much has been invested in investigating a system capable of being used to mimic the human nose (Amrani *et al*., 1993). Essentially, this mimicking entails applying the design concepts of human/biological olfactory systems to an artificial chemosensory system and not the actual transduction mechanism of the biological sensing systems (Amrani *et al*., 1993). Such a mimicking system would thus have a multi-element array of non-specific chemical sensors, a feature extractor and a pattern classification system for distinguishing between odours equivalent to the odour receptors, olfactory bulb and brain of the biological olfactory system respectively (Persaud, 1990). Extensive research on, and development of, new sensors, as well as advances in the fields of microelectronics and computer technology has made the development of such artificial sensing systems, otherwise known as electronic noses, possible (Persaud and Travers, 1991).

Being constructed in much the same way as the odour receptors of the biological olfactory system, the sensors of electronic noses are also able to discriminate, not only odour classes but also odours of different products with complex chemical make-up. Just like the human nose, the electronic nose can create an 'odour image' of a product based on whatever combination of aromas a product exudes without actually being able to identify any one aroma in particular (Zannoni, 1995).

The advent of electronic noses capable of emulating or mimicking the discriminative capacity of the human olfactory system has greatly enhanced technology devised for sensing volatile chemicals. Consequently, volatile chemicals emitted from products can be assessed in such artificial sensing systems.

2.4.2 Specific sensors versus sensor elements in non-specific sensor arrays

Research into the development of electrically conducting organic polymers for use as sensors capable of sensing volatile odourant molecules has been going on for some time with very positive and encouraging outcomes. Automated sensing systems such as electronic noses have been developed using such electrically conducting organic polymers as odour sensors. Although the mechanism of odourants interacting with the sensors is 'ill-defined' (Thomlinson *et al*., 1995), years of gathering data in trying to understand the

mechanism have made it possible to design polymer sensors with the desired characteristics for sensing odourants. Such sensors are designed on the basis of substituting appropriate chemical groups on the polymer backbone. They are usually aromatic or heteroaromatic compounds such as thiophene, polyaniline and polypyrrole, with polypyrrole being the most intensively studied and commonly used of these compounds (Persaud and Travers, 1991). AromaScan is one of three major manufacturers of commercial electronic noses, others being Alpha M.O.S. and Neotronics.

The electrochemical characteristic of the polymers which make them suitable for use as sensors is that they behave as semi-conductors each of which has an inherent charge (Amran *et al*., 1995b). When volatile odourant molecules are adsorbed and desorbed from the surfaces of sensors manufactured from the polymers, they display reversible changes in their inherent charge (Persaud and Travers, 1991). Alternatively, it can be said that the sensors change in resistance when there is interaction between odourants and sensors. Automated sensing systems measure changes in resistance of the sensors relative to a baseline, usually obtained through a response to purified air (Atkinson and Hammond, 1994). The change in resistance is proportional to the way odourant molecules fit into the polymer structure.

Electronic noses have not been constructed with sets of sensors each of which is capable of responding to a specific chemical. The impracticality of constructing instruments in this manner is not difficult to understand. For example, if analysis of a sample with two volatile odourants was required, then two sets of sensors, specifically designed to respond to those two chemicals, would be required (Marsili, 1995). Many products, however, have much more than two odourants, often tens to hundreds of them, and it is easy to see the problem that would arise if analysis of such samples were to be carried out via instrumentation constructed with specific sets of sensors. For this reason, electronic noses are not constructed in this way. Instead they consist of a non-specific sensor array of polymer sensor elements. In this instance, if there were an array of, for example, twenty sensor elements in the sensing system, and the backbone of each of the twenty polymer sensor elements were substituted with different chemical groups, then each sensor would respond differently to any odour class or product odour (Persaud and Travers, 1991). In other words, because sensor elements constructed in this way would be different to each

other, the resistance change of each sensor element in the array would be different and unique in its response to odourants. It is this ability of all the sensor elements to respond to any one odourant but at different magnitudes which allows a set of descriptors, odour images or 'fingerprints' to be produced once the data has been recorded and processed.

The concept behind employing this instrument in some industries is to detect abnormalities from the control specification of products (Atkinson and Hammond, 1994). The adsorption and desorption kinetics of the sensor elements in the array are adequate when all that is required is checking for odourants that signal the degradation of a product or signal product contamination, and the identification and quantification of each component in a complex mixture is not required. The capability of the multitude of sensor elements in electronic noses cannot exceed that of the tens of millions of sensing cells in our noses. However, in spite of the small number of sensor elements in electronic noses, they can still be used successfully to detect out of specification products. In this case, all that is required is comparison of sensor array output of a control to that of an unknown (Atkinson and Hammond, 1994).

2.4.3 Analysis of samples using an electronic nose

2.4.3.1 Capturing of data

It has been demonstrated that instruments, most of which on the market have exploited the electrochemical properties of polymers in the construction of sensor arrays, are capable of detecting organic odourants with a molecular weight range of between thirty and three hundred. This is essentially the same molecular weight range detectable by the human olfactory system (Marsili, 1995). The broad overlapping response of the different polymer sensor elements in an array also makes the systems which incorporate them very similar to the olfactory sensor arrays of the human olfactory system. The sensor elements of these arrays are also completely unresponsive to non-volatile components.

It has been suggested that systems with arrays of sensor elements as described in the previous section, have the optimum configuration for a practical sensing system (Pisanelli *et al*., 1994). The sensing system of an electronic nose consists of an array of between 4

and 32 polymer sensor elements (the latter having made commercialisation of the product possible) together with associated electronic circuitry and data acquisition software (Taylor, 1994; Amrani *et al*., 1995a). Visual displays of changes in resistance for each of the sensor elements in response to their interaction with volatile odourants are made possible by means of these advances in computer technology. These visual displays are either an X-Y plot with time on the X-axis and averaged response of individual sensor elements on the Y-axis or a histogram with sensor element number shown on the X-axis and percent change in resistance of the respective sensor element on the Y-axis (Taylor, 1994). A histogram of this nature, once processed using the relevant software, is effectively a fingerprint of the odour being analysed. Irrespective of the type of visual displays, the extent of the response is dependent on the affinity for and concentration of volatile odourants at each sensor element in the array. The concentration of volatiles presented to a sensor array is very important. For example, if a volatile chemical which fits poorly into the structure of a polymer sensor element is present at a higher concentration than a volatile chemical that fits well, or even perfectly into the polymer structure, then the former volatile chemical can create a response signal equal to that of the latter. Electronic noses are capable of detecting volatile odourants at the parts per billion level (ppb) (Taylor, 1994).

A two minute time period is often employed during sampling using electronic noses, one minute to reach steady state and one for actual sampling. As with GC, there are two basic types of sampling techniques. One is static headspace analysis (SHA) and the second is flow injection analysis (FIA). FIA involves a known gas constantly being pumped across a sensor. Then a known concentration of the air or headspace gas to be tested is injected into the gas stream before the sensor. SHA is the more common practice since it is easier to perform. It involves allowing the headspace above a certain sample to become saturated with the volatile odours of the sample which are then pumped across the sensor for analysis. In each method, the temperature, humidity and pressure can be controlled. Data acquired by whichever method used, is recorded in the memory of the computer on which the relevant software has been loaded and on which measurements are recorded. Once recorded, the data can be processed using the software to produce a fingerprint for a particular substance.

2.4.3.2 Data reduction

Once a sample has been analysed several times, the data produced are, in effect, multidimensional and it is therefore essential to reduce this data reliably and correctly into a more easily understood form (Persaud *et al*., 1996a). The first step in data reduction is the selection of the stable portion of the odour profiles generated during each analysis to yield average responses from each sensor for each analysis (Thomlinson *et al.,* 1995). At this point, the patterns of response are normalised, meaning that the responses of the individual sensor elements are relative to the summed responses of the entire array (Persaud and Travers, 1991). Normalisation of data removes instrumental odour intensity effects from the sensor element responses. Instrumental odour intensity is proportional to the concentration of volatiles presented to the sensor elements (Persaud *et al*., 1996).

The second step of data reduction is using output obtained from the first step and then subjecting it to cluster analysis, one of the several types of pattern recognition techniques which separates clusters of single points in a two- or three-dimensional space, most often in the form of Sammon Maps, which can be easily evaluated by human observation (Sammon, 1969). Better discrimination of family clusters, which at this point would be specific groups relating sample types, is achieved by re-implementation of mean instrumental odour intensity lost during normalisation of data. Mean instrumental odour intensity refers to the sum of all the sensor elements divided by the number of elements (Thomlinson *et al*., 1995). During re-implementation, a scaling factor of one hundred is used.

The ability of artificial chemical sensing systems to reduce multidimensional data into two- or three-dimensional clusters makes these instruments appropriate for use in the assessment of raw materials as well as process monitoring because cluster analysis allows easy comparison of overall aromas for the different samples analysed (Taylor, 1994). Furthermore, the extent of variance in the aroma profiles of products from a control can be measured by the instruments software. This is expressed in terms of a quality value which is very similar to standard deviations in statistics.

2.4.4 Application of electronic noses in the food and beverage industry

With the exception of saltiness, sweetness, bitterness and sourness, the flavour of most foods and beverages are attributable to the interaction of human sensory organs with the volatile and semi-volatile organic constituents therein. If it is agreed that volatile flavour components constitute aromas, then it can be said that many foods and beverages contain hundreds of these aroma chemicals. Aroma is an important sensory component of foods and beverages and it is very important that it is measured or assessed in a reliable and objective manner (Taylor, 1994). It would be an extremely challenging and sometimes frustrating exercise to analyse products with many aroma chemicals by traditional methods such as GC should there be, for example, a need to study and resolve an aroma related problem. Under such circumstances (i.e. an aroma related problem), an ideal tool would be an electronic nose. The electronic nose would not separate or resolve all individual volatile components in such a problem sample, but would rather use an array of sensor elements that would respond to each volatile odourant/aroma molecule in slightly different ways, in a similar manner to the function of the human nose (Marsili, 1995).

Electronic noses are proceeding at an increasingly rapid pace (Apps, 2001). Up until recently, novel chemical sensors were being developed largely for use in clinical and defence sectors (Rogers, 1993). Novel chemical sensors have now found their application in the food and beverage industry. The development and applications of chemical sensors in sensing instruments or electronic noses for this industry is a topic of increasing practical importance (Rogers, 1993). Currently, the biggest market for electronic noses is the food and beverage industry where applications are varied and where they can augment methods such as GC and sensory panels (Giese, 2000; Ouellette, 1999). One of these applications is inspection for food quality of both raw and finished products; monitoring of the cooking process; monitoring of fermentation processes; monitoring of processes such as mixing, flavouring and blending; studying food storage, freshness and ageing; evaluation of the maturation and ripening of cheese, wine and meat products and monitoring of product-packaging interactions (Mielle, 1996). In each of these cases, results are obtained by assessing changes in the composition of the odour emanating from the products and comparing this with controls. Detailed characterisation of what specific changes have occurred is not necessary.

Some of the specific studies include that conducted by Pisanelli *et al*. (1994) who used an electronic nose with a conducting polymer sensor array consisting of twenty conducting polymer sensing elements to assess the quality of food raw materials such as wheat grain, odours from different stages of cheese ripening and to differentiate between different types of cheese. Taylor (1994) used an electronic nose to discriminate between hop varieties as well as to differentiate between normal beer and beer which deviated from specifications in terms of volatile compounds. Assessments of the odour quality of coffee (Gardner *et al*., 1992), beer (Pearce *et al*., 1993) and liquors (Alshima, 1991 loc.cit. Thomlinson *et al*., 1995) using electronic noses have also been reported. Recently, Keshrie *et al.* (1998) evaluated the use of the electronic nose in detecting and differentiating grain spoilage fungi on the basis of the odour volatiles which they produce. They found that the electronic nose which they used produced results which allowed differentiation of the species based on an analysis of volatiles produced by each of the species. Keshrie *et al*. (1998) also established the improved ability of the electronic nose to differentiate amongst the species over the three-day test period. Madsen and Grypa (2000) used an electronic nose to assess spice origins. In an analogous study to that of Keshrie *et al*. (1998), Korel and Balaban (2002) used an electronic nose to assess the aroma of milk inoculated with *Pseudomonas fluorescens* or *Bacillus coagulans* and odours were correlated with microbial loads and sensory evaluation. The electronic nose was found to be capable of discriminating differences in odour due to microbial load and these results correlated well with those obtained from sensory evaluation.

Although the electronic noses in use today are far from replacing the human olfactory system on which their design is based, the applications for this technology are endless. These instruments are capable of fulfilling the need in the food and beverage industry for objective automated quality monitoring system. It is suggested that, although they will not replace traditional methods such as the use of sensory evaluation and GC, electronic noses will make it possible to resort to traditional methods only when required, for example if a defect in a product needs to be analysed further (Mielle, 1996). As can be concluded from the applications listed above, one of the quality control applications is the detection of microbiological spoilage organisms on the basis of the characteristic odours which are a result of their metabolism in specific products. It should be apparent that this is the basis

on which *Alicyclobacillus acidoterrestris* could be detected in juice products since they emit characteristic odours as a result of their metabolism.

2.4.5 Advantages of electronic nose technology

The advantages of this technology include its objectivity which would allow for improvement of quality control from raw material to finished product; and its rapid identification of defective products enabling early detection of process problems. The capability of the instrument is not affected by subjective factors such as working conditions and emotional state (Ouellette, 1999). The technology is user friendly allowing decision-making with clear cost-saving potential. It could eliminate the health risk associated with smelling certain toxic chemicals. The potential of real time analysis by electronic noses in certain applications would allow 24hr measurement of odour quality. Final advantages include good adsorption and desorption kinetics at temperatures around 35°C, their low power consumption and resilience to poisoning by volatiles (Persaud *et al.*, 1996b).

2.4.6 Disadvantages of electronic nose technology

The main limitation of electronic noses in the food and beverage industry is the high sensitivity of their sensors to compounds such as ethanol and carbon dioxide which have the effect of masking desired responses to compounds. In this regard they cannot be used in the field of alcoholic or sparkling beverages without the considerable time required and costs incurred for removing the ethanol and carbon dioxide from such products before analysis. This would defeat two of the purposes of the instruments, that is, the reduction in analysis times and cost. Another drawback with the electronic nose is the difficulty experienced when it comes to varying environmental conditions which may cause the sensor responses to drift.

CHAPTER THREE

3. METHODS

3.1 ISOLATION OF *Alicyclobacillus acidoterrestris* FROM FRUIT JUICES

3.1.1 Samples and sample preparation

Hundred ml Schott® bottles with caps screwed on were sterilised by autoclaving at 121kPa for 15 minutes. Once the bottles had been sterilised, juices (Table 1) were prepared for decanting into the bottles while working in a laminar flow hood. This was done by puncturing each of the samples of juice with a flamed, hot inoculating needle on the edge away from the straw insertion point on each of the boxes in order to facilitate the transfer of the juices from the boxes into the Schott® bottles while maintaining atmospheric pressure. A sterile disposable blade was then used to cut away the pieces of foil at the straw insertion points. Approximately 40ml of the juices were aseptically added to the sterile Schott® bottles and the bottles were recapped.

3.1.2 Heat shock treatment of the juices

Heat shock treatment as described by Jenson (1999) was used on the samples prepared to induce germination of any spores present. This was done by placing the Schott® bottles into a water bath at 80°C. Once the juice itself reached 80°C it was kept at this temperature for 10 minutes. In order to establish whether the juices had reached 80°C, another Schott® bottle with approximately 40ml of juice was covered at the mouth with heavy-duty aluminium foil and placed in the water bath along with the other (test) juices. A thermometer was then inserted through the foil covering into the juice. The temperature of this juice (referred to as 'temperature control' hereafter) allowed temperature of the test juices to be monitored as the treatment of the former was a simulation of the latter. This procedure ensures that microbial contamination of the test juices via an external source is minimised. During the entire period of heating of the juices, the bottles (including the temperature control) were agitated every two to three minutes in order to distribute the heat

evenly in the sample. Once heating was completed, the bottles were immediately placed in a 40°C water bath to cool to this temperature.

Table 1: List of 250 ml box juices tested for the presence of *Alicyclobacillus acidoterrestris*

Type	Flavour	Best before	Batch number
Woodland Select	Orange and Other Fruit[1]	27/10/2000	200041
Woodland Select	Orange and Other Fruit[2]	27/10/2000	200041
Minute Maid	Orange, Pear and Grape	12/05/2001	W522:31B19
Minute Maid	Orange, Pear and Grape	12/05/2001	W522:31B19
Minute Maid	Orange and Peach	27/05/2001	W502:59B21
Minute Maid	Orange and Peach	27/05/2001	W502:59B21
Minute Maid	Orange and Peach	27/05/2001	W502:59B21
Minute Maid	Orange and Peach	27/05/2001	W502:59B21
Minute Maid	Orange and Peach	27/05/2001	W502:52B21
Minute Maid	Orange and Peach (Calcium Enriched)	27/05/2001	W502:53B21
Minute Maid	Apple	11/07/2001	W502:59B21
Minute Maid	Orange	12/05/2001	W522:31B19
Minute Maid	Naartjie Orange	25/08/2001	W500:13B34
Minute Maid	Orange (Calcium Enriched)	27/07/2001	W512:06B30
Minute Maid	Naartjie Orange (Calcium Enriched)	25/08/2001	W500:13B34
Minute Maid	Apple	22/06/2001	W500:32B25
Minute Maid	Orange and Peach (Calcium Enriched)	27/05/2001	W502:52B21
Minute Maid	Orange and Peach (Calcium Enriched)	27/05/2001	W502:53B21
Minute Maid	Orange and Peach (Calcium Enriched)	27/05/2001	W502:54B21
Minute Maid	Orange and Peach (Calcium Enriched)	27/05/2001	W502:58B21
Minute Maid	Orange and Peach (Calcium Enriched)	27/05/2001	W502:59B21
Minute Maid	Naartjie Orange (Calcium Enriched)	25/08/2001	W500:13B34
Minute Maid	Orange, Pear and Grape	12/05/2001	W522:31B19
Minute Maid	Apple	05/12/2001	W520:50A48
Minute Maid	Orange and Other Fruit (Calcium Enriched)	27/05/2001	W502:58B21

[1] Blown sample
[2] Unblown sample

3.1.3 Enrichment and cultivation conditions

As mentioned in the literature review, direct plating techniques are ineffective in detecting the low number of *A. acidoterrestris* spores that are usually present in juices even after heat shock treatment. An enrichment procedure (Jenson, 1999) was therefore used prior to plating. Enrichment was achieved by placing the juices subjected to the heat shock treatment into an incubator at 45°C ± 2°C for 48 hours. After the incubation period, ten fold dilutions of the juices were prepared. One ml of juice samples themselves and 1ml of the ten fold dilutions were pipetted into appropriately labelled petri dishes. Approximately 20 ml of sterile, tempered Orange Serum Agar (OSA) prepared as per manufacturer (Oxoid) instructions and adjusted to pH ≈ 4.00 using 0.938 (N) sodium hydroxide (Appendix A) was then poured into each of the plates. The plates were gently swirled several times to mix the media and the dilutions, were allowed to set after which they were inverted and placed in a 45°C incubator for a total of seven days.

After incubation, OSA plates on which colonies (isolates) had grown were selected for sub-culturing onto *Bacillus acidoterrestris* medium (BAM) plates (Appendix B) in order to obtain pure cultures using a four-way streak. Each of the colonies growing on the OSA plates was sub-cultured in this manner onto BAM plates. Plates were placed in a 45°C incubator until good growth was observed. Further sub-culturing of the micro-organisms in this manner was carried out until pure cultures were obtained.

3.2 IDENTIFICATION OF *Alicyclobacillus acidoterrestris*

3.2.1 Phenotypic characterisation

Each of the pure cultures were first subjected to the gram stain in order to determine their gram response. The method used is the one described by Tortora *et al.* (1997). Preparation of stains are outlined in Appendix C. The second phenotypic characterisation test conducted was the spore stain as described by Acuff (1992). Preparation of reagents for spore staining is outlined in Appendix D. The prepared slides in both cases were examined under the oil immersion lens of a light microscope. The third test conducted was the catalase test. Immediate observations as to whether or not oxygen bubbles had formed

in the drop of hydrogen peroxide added to colonies were made. Further observations at intervals of 1 minute were made thereafter to assess whether oxygen bubble formation was evident.

3.2.2 Identification of pure cultures

After conducting the phenotypic characterisation tests (gram and spore stains and catalase test), BAM plates with sub-cultures of the isolates were sent to the Nelson R. Mandela School of Medicine, Microbiology Department for identification. The isolates were again subjected to the gram stain and then plated onto Egg Plates, followed by sub-culturing onto Nagler plates. Examination of plates was conducted to assess for zones of clearing.

Due to the results obtained for experiments conducted in section 3.2.1 and 3.2.2, a pure culture of *A. acidoterrestris* was purchased from the South African Bureau of Standards (SABS) Test House at this point and the phenotypic characterisation tests described in section 3.2 were also carried out on this culture. This pure culture was also sub-cultured onto nutrient agar to assess its ability to grow on media with neutral pH. It was sub-cultured onto twenty BAM plates using the spread plate technique and plates were incubated at 45°C for 7 days. Thereafter spores were harvested from the sub-cultures.

3.3 HARVESTING AND PRELIMINARY ENUMERATION OF *Alicyclobacillus acidoterrestris* SPORES

The method used was as described by Orr and Beuchat (2000). All work was conducted using sterile apparatus and under aseptic conditions. Approximately 2 ml of sterile distilled water was deposited onto the surfaces of the twenty BAM plates on which *A. acidoterrestris* had been sub-cultured. The colonies were gently rubbed off the surfaces of the plates using a sterile glass spreader and the resulting suspension of vegetative cells and spores was filtered through sterile glass wool into a sterile glass tube using a vacuum pump system. In order to maximise spore recovery, a further 2 ml of sterile distilled water was added to the same set of twenty BAM plates, rubbed again and the suspension was also filtered through the sterile glass wool into the same glass tube as before. The filtrate was centrifuged at 6000 rotations per minute (rpm) for 20 minutes and the supernatant

discarded. Washing of the pellet was conducted by resuspending it in 5 ml of sterile distilled water and mixing vigourously. This was topped up with another 25 ml of sterile distilled water and the suspension recentrifuged at 6000 rpm, this time for 10 minutes. This washing procedure was repeated 6 times. The final pellet was suspended in 50 ml of sterile distilled water and roughly 10 ml deposited into each of five sterile screw-capped McCartney bottles.

At this point a preliminary enumeration was conducted to obtain an idea of the number of spores per ml of suspension. Enumeration was done using peptone water as the diluent and pre-poured BAM, OSA and K-Medium (KM) prepared as per Walls and Chuyate (1998) (refer to Appendix E).

Serial dilutions of the spore suspension from one of the McCartney bottles were prepared to the order of 10^{-4}. From each dilution, 0.5 ml was pipetted onto pre-poured BAM, OSA and KM plates and spread over the surface of the agar using a sterile glass spreader. Once this was done, the 10^{-1} dilution was heat treated by placement in a water bath at 80°C along with a peptone blank which was used to monitor temperatures reached as described for the temperature control for the juices (section 3.1.2). Once the spore suspension dilution reached 80°C, it was kept at this temperature for 10 minutes. Thereafter serial dilutions were prepared again to the order of 10^{-4}. The heat-treated dilutions were plated in the same manner as pre-heated dilutions, i.e. 0.5 ml on pre-poured BAM, OSA and KM plates. All plates were incubated at 42°C for 10 days. The number of colonies were then counted and the relevant conversions done to establish a rough idea of the number of spores per ml of suspension. All counts in this research were conducted using rules for counting outlined in Appendix F (Swanson *et al.*, 1992). The spore suspensions in the McCartney bottles (including the one used) were stored at –20°C until further use.

3.4 USE OF SENSORY EVALUATION (OLFACTORY TECHNIQUE) IN THE OPTIMISATION OF PARAMETERS (JUICE TYPE/FLAVOUR AND INOCULUM LEVEL)

Prior to inoculation of the actual test set it was necessary to establish whether the 200 ml Wilde juices available for the study would be suitable for inoculation and what level of

inoculum would be suitable. Wilde juices are fruit juices available in several flavours to which no preservatives have been added. Suitability testing was done, firstly, by selecting two flavours with pH values within the range known to permit growth of *Alicyclobacillus acidoterrestris*. These were Tropical Juice Blend (pH 3.01) and Passion Fruit Juice Blend (pH 2.49). Secondly, it was necessary to prepare the two flavours for inoculation. This was done by applying clear silicone to the straw insertion point of the juices to be used for the test followed by placement of a semi-transparent rubber disk on top of the silicone on each of the juices. The disks were then pressed down securely and excess silicon smeared around the edge of the disks to create a leak-proof seal between the inside of the juice box and its surroundings even after inoculation. The juice boxes were left for 24 hours to allow the silicon to dry completely. One of the spore suspensions (prepared, enumerated and stored as described in sections 3.3) was removed from the freezer and allowed to thaw at room temperature. The suspension was then heated to 80°C and kept at this temperature for 10 minutes. One ml of the spore suspension (with a spore level as determined in section 3.3) was drawn into a sterile 10 ml syringe to which a sterile syringe needle had been fitted. The needle was then inserted through the leak-proof seal of the juices at the foil straw insertion point (which could be seen through the semi-transparent disk and clear silicon). The contents of the syringe were emptied into the juice. This was done for both the Tropical Juice Bend and Passion Fruit Juice Blend flavours, using separate syringe needles on each occasion. The two flavours, together with their respective controls (i.e. with no inoculum added) were placed in a 45°C incubator for 15 days. Treating the boxes aseptically, the leak-proof seals were thereafter removed and portions of the test sample and control sample for each flavour were subjected to sensory evaluation to establish whether tainting was evident at the level of inoculum used and, if so, in which flavour of juice.

The sensory evaluation conducted at this point was only aimed at establishing whether there was a difference between the inoculated juice sample (test) and control, and a double-blinded paired comparison test was therefore employed. Each of 9 panellists were asked to sniff the control, then the test, and to state whether or not they were able to detect any difference between the test and control samples. Based on the findings of the sensory evaluation test, bacteriological tests were also conducted using serial dilutions to the order of 10^{-5} in peptone water. One ml of each of the dilutions was plated on BAM, OSA and

KM plates and incubated at 45°C. In addition to this, 10 ml portions of each of the test juices and controls were decanted into sterile McCartney bottles, capped and placed back into the 45°C incubator, and again subjected to a paired comparison after 15 days.

3.5 PREPARATION AND INOCULATION OF JUICES TO INDUCE SPOILAGE

Based on the outcome of the experiments conducted in the previous section, the juices selected for the study were 200ml Wilde Tropical Fruit Juice Blend. Leak proof seals for juice samples to be used for the study were prepared as described in the previous section. For the actual inoculation it was decided that two levels of inoculum would be used. Having roughly established the level of spores in the spore suspensions and that this level would result in the formation of an off-odour (section 3.4), it was decided that one level of inoculum would be that of the stock spore suspension directly ($\pm 10^7$ colony forming units [cfu].ml^{-1}) and the second would be a tenfold dilution thereof (a level of $\pm 10^6$ cfu.ml^{-1}). At this point it was, however, necessary to establish the exact level of spores that were inoculated into the juices in order to calculate the number of spores per millilitre of juice. This was done by first heating the stock spore suspension to 80°C in a water bath and holding it at this temperature for 10 minutes. Thereafter, dilutions of this heated sample were prepared to the order of 10^{-6} with sterile distilled water as the diluent. The 10^{-4}, 10^{-5} and 10^{-6} dilutions were selected for plating. 0.1 ml of these dilutions were plated onto pre-poured BAM, OSA and KM plates and placed in a 45°C incubator for 10 days as done previously (section 3.3) and the number of spores.ml^{-1} of suspension determined. This count was then divided by 200 (the volume of the juice samples inoculated) to determine the theoretical number of spores.ml^{-1} of juice. This information is vital for comparison with counts done at a later stage which are reported as cfu.ml^{-1} of juice.

After having plated the dilutions as above, 2 pre-poured nutrient agar and 2 PDA plates without their lids were laid out in the vicinity of the flame in the laminar flow hood to assess the sterility of the air in this area over the duration of the inoculation. One ml of the heated stock spore suspension was drawn into a 10ml sterile plastic syringe along with 9 ml of air from the vicinity of the flame in the laminar flow hood. Each of nine Wilde Tropical Juice Blend juices was inoculated with the 1 ml inoculum and 9 ml of air. The tenfold dilution of the heated spore suspension (i.e. the 10^{-1} dilution in distilled water) was

also inoculated in this manner into each of another nine Tropical Juice Blend juices. All the juices were placed in a 45°C incubator along with 6 controls (no spore inoculum but with 9ml of air added), one for each sampling day. The nutrient agar and PDA air plates were incubated at 37°C for 2 days and 25°C for 5 days respectively. It was decided that a representative count on a Tropical Juice Blend juice from the same batch would be tested to ensure that the juices were free of other contaminants. In addition to adopting the technique used in the isolation of *A. acidoterrestris* (section 3.1), a direct plating technique of the 10^{-1} and 10^{-2} dilutions of the representative juice was employed using BAM plates to check for any other micro-organisms which might have been present prior to inoculation which could have affected the outcome of the study.

The juices with the lower level of inoculum (i.e. the tenfold dilution of the heated stock spore suspension) as well as a control were evaluated at 7-day intervals up until 21 days using bacteriological techniques, sensory evaluation and electronic nose technology (AromaScan™). The inoculated samples were analysed in triplicate on each sampling day. The juices inoculated with the higher level of inoculum (i.e. the heated stock spore suspension) were evaluated in the same manner except at 10-day intervals up until 30 days. These samples were also analysed in triplicate.

3.6 BACTERIOLOGICAL TESTS

The bacteriological tests on the juices were conducted in a laminar flow hood using pre-poured BAM, OSA and KM. 0.1 ml of the serial dilutions prepared in peptone water to the order of 10^{-6} were spread-plated on these three media types. Plates were incubated at 45°C for a minimum of 7 days prior to counting whereafter counts were calculated as per Appendix F.

3.7 SENSORY EVALUATION OF THE JUICES (BY OLFACTORY MEANS)

The sensory panel consisted of 16 females and 8 males ages ranging between 20 and 51 years of age. Evaluations were conducted between 11:00 and 13:00 on the test days. Red 30ml plastic cups were used in an attempt to mask potential differences in the colour of the samples which may have influenced panellists. Two of the three coded cups each

contained 10 ml control juice and 1 cup contained 10 ml test juice. The cups were covered with aluminium foil for presentation to the 23-24 panellists. The triangle test was employed whereby panellists were asked to sniff the three samples and decide which sample was different from the other two. A choice was required even if the panellist was not able to discern a difference. This was done for the reasons described in the literature review so as to obtain a full complement of data. Triplicates of each set (i.e. the set with the lower level of inoculum and that with the higher level of inoculum) of samples were analysed on each sampling day. Samples were at room temperature (23±1°C) at the time of evaluations. Minimum numbers of correct judgements (i.e. identification of the cup with the test juice) in order to establish significance at the various probability levels for the triangle test were obtained using Table G1 (Appendix G) sourced from Stone and Sidel (1993). Panellists were informed that at no point was the product to be ingested as juices has been inoculated with bacteria

3.8 ANALYSIS OF JUICES USING ELECTRONIC NOSE TECHNOLOGY (AROMASCAN™)

The method involved drawing 40ml of the test sample from inoculated stocks and placing it into a sterile 100ml Schott® bottles. The bottle was closed with an omnifit cap which has special openings. The tubing attached to the inert carrier gas, in this case nitrogen gas, was inserted through one of the openings to just above the surface of the juice sample in the bottle. The tube which was to carry the volatiles generated in the headspace of the bottle into the AromaScan (Appendix H [1]) analyser was attached to the second opening. Once the sample was attached to the AromaScan, it was then ready to be analysed. The data acquisition software was opened and the following acquisition parameters, and as can also be seen in Appendix H (2), were entered in the relevant boxes to analyse the samples:

Sampling interval: 5 seconds. This means that although the instrument was analysing the volatiles continuously, the profile generated was only updated every 5 seconds during the analysis time. This was a matter of preference.

Humidities (purge and reference): 70% RH. This value is as per Gammatec (supplier) instructions.

Cycles: 1. This means that the process of referencing (which is when the instrument is equilibrating), sampling, washing and then referencing again occurred only once. Although each sample analysed was subjected to 6 such cycles to get a more significant representation of the volatiles being emitted, leaving this value at 1 allows labelling and identification of individual cycles. If the value had been set at 6, then the referencing, sampling, washing and referencing again would have been continuous for 6 cycles and individual cycles could not have been labelled.

The other parameters used were referencing, which was initially 40 seconds, followed by the all-important sampling interval of 120 seconds during which time the volatiles generated in the headspace of the sample bottle were actually allowed to interact with the sensors. Washing of the sensors using water vapour, which serves to eliminate residues from the surfaces of the sensors leaving the sensors clean for subsequent analyses, was set at 10 seconds. This was followed by an additional referencing step of 120 seconds. During the switch from the first referencing to the sampling interval, the valve attached to the gas supply tubing was closed and the valve attached to tubing leading to the AromaScan analyser was opened.

Once the acquisition parameters had been set, the gas supply was turned on and the analysis started. Once turned on, the inert carrier gas serves two simultaneous functions during the first referencing step. One of these is the establishment of a baseline resistance by its interaction with the sensor elements in the sensor array i.e. it is regarded as a reference interaction. The other function is the creation of conditions of equilibrium in the atmosphere above the sample in the Schott® bottle. Conditions of equilibrium are created by the pressure of the gas within the bottle whereby volatile organic compounds (analytes) diffuse from the sample in the atmosphere above the sample and at the same time diffuse from the atmosphere back into the sample. When the two diffusion rates equal, the concentration of the analytes remains equal between the sample and the atmosphere above it, i.e. a headspace at equilibrium is created. Once these two functions have been served (after 40 seconds as defined by the value entered for the first reference in the acquisition parameters), the gas supply valve was closed and the valve attached to the tubing leading from the sample to the AromaScan analyser was opened. The volatiles in the headspace

were thus allowed to interact with and cause changes in the baseline resistance of the sensors during what is defined as the sampling interval.

Analysis generated profiles as exhibited in Appendix H (3), with each of the areas representing referencing, sampling, washing and referencing again as defined by the acquisition parameters. Each sample was analysed 6 times to generate 6 such profiles which were automatically recorded. Once the profiles for 6 cycles had been captured for each of the test and control samples on the test days using the above-mentioned acquisition parameters the data, with the exception of the first of the 6 cycles, was grouped (i.e. only 5 of the 6 cycles were used). This was done using the data reduction software of the AromaScan. This software allows data from each run to be opened at will and permits selection of the portion of the profiles corresponding to the 120-second sampling interval by entering values which correspond to the start and end of sampling (in seconds) in the respective boxes (Appendix H [4]). It then allowed addition of the sampling data from the 5 cycles for each sample. Grouping of data results in a window appearing automatically on screen. Here comparisons can be made between each of the 5 cycles in terms of how the volatiles from the sample interacted with the sensors. This was done by using the superimpose function. Comparisons can be made using line graphs (Appendix H [5a]) or bar graphs (Appendix H [5b]). Grouped data was then manually saved as a group on the computer using appropriate labels. This effectively constitutes the database which permits comparison of data captured from 5 of the 6 analyses of one sample to that of another. Such comparisons were done using 2-dimensional Sammon Maps to which instrumental odour intensity are implemented and are the final products of analysis.

For any two samples compared on a Sammon Map, scientific comparisons were made on the basis of quality values which are generated by the AromaScan software. Quality values of greater than 2 for two compared samples, indicates a significant difference between the samples. In terms of this research, the control sample was compared to each of the three replicates on the different sampling days on Sammon Maps and in terms of quality values. For valid comparisons to be made in terms of distribution of clusters etc., Sammon Maps were formatted so that there was uniformity in terms of maximum and minimum values for each of the X- and Y-axes.

3.9 ANALYSIS OF CHEMICAL TAINT STANDARDS AND JUICES USING HEADSPACE GAS CHROMATOGRAPHY

3.9.1 Preparation of guaiacol and 2,6-Dibromophenol standards

Guaiacol (Sigma) was purchased in its pure form (liquid). Stock solutions were prepared there from for use in GC. Guaiacol dissolves in water. A stock solution of 100 ppm guaiacol was prepared by microsyringing 10 μl of the pure chemical into a 100 ml volumetric flask and made to the mark using distilled water.

2,6-Dibromophenol (Fluka) was also purchased in its pure form (crytalline). This chemical does not dissolve in water. Therefore, 0.01g of the pure 2,6-dibromophenol was first dissolved in 5ml of 0.980N NaOH (Appendix 1) in a volumetric flask and then made to the mark with distilled water.

3.9.2 Analysis of the standards and juice samples

Both standards (100ppm) together with two samples which a significant number of panellists from the sensory evaluation described as being different from the control, were sent to Illovo Sugar (PTY) Ltd. for analysis. The samples were analysed using a Thermoquest CE Gas Chromatograph Trace 2000 coupled to a Headspace 2000 CE headspace autosampler under the following instrument parameters:

Column type: WCOT fused silica
Stationary phase: CP. SIL 8 CB
Length: 50cm
Internal diameter: 0.32mm
Film thickness: 5μm
The headspace conditions were as follows:
Oven temperature: 75°C
Vial equilibrium: 10 minutes
GC cycle length: 15 minutes
Splitless injection: 0.20 minutes

Column (initially):	50°C (1 minute)
Temperature progress:	15°C.min^{-1}
Final temperature:	120°C (5 minutes)
Detector temperature:	175°C
Carrier gas:	Nitrogen (2ml.min^{-1})
Detector type:	Flame ionisation detector

CHAPTER FOUR

4. RESULTS

4.1 ISOLATION AND IDENTIFICATION OF A*licyclobacillus acidoterrestris* FROM FRUIT JUICES

Of all the juices tested for the presence of *Alicyclobacillus acidoterrestris*, the only one which produced positive results in terms of isolation of micro-organisms was a blown Woodland Selectsample (Table 1). While no colonies were detected on the petri-dish on which the juice had been plated without diluting, 5 colonies were detected on the petri-dish on which the tenfold juice dilution had been plated (i.e. 50cfu.ml^{-1}). Close visual inspection of the colonies was encouraging in that the colonies exhibited morphology typical of *A. acidoterrestris* on the OSA plates as described by Jenson (1999). The colonies were creamy, circular and had raised annuli. After sub-culturing each of the 5 colonies which appeared on the 10^{-1} dilution OSA plate, and allowing them to grow in the incubator, colonies were found to be gram variable spore-formers. The catalase reaction yielded bubbles approximately 15 seconds after addition of the hydrogen peroxide to the colonies and isolates were hence established as being catalase positive. The sub-cultures of the 5 isolates on BAM plates were all subsequently identified as the same *Bacillus* species by the technicians at the Nelson R. Mandela School of Medicine, Microbiology Department. They were unable to further identify the isolates. The Gram reaction and weak catalase test response of the isolates cast a doubt as to their identity as did their identification as a *Bacillus* species. The *A. acidoterrestris* culture subsequently purchased from the SABS Test House was found to be gram positive (Fig. 4), catalase positive sporeformers. Spores can be seen as green spots in Figure 5. This culture also exhibited typical *A. acidoterrestris* morphology on the OSA plates when sub-cultured (Fig. 6) and was used for subsequent testing.

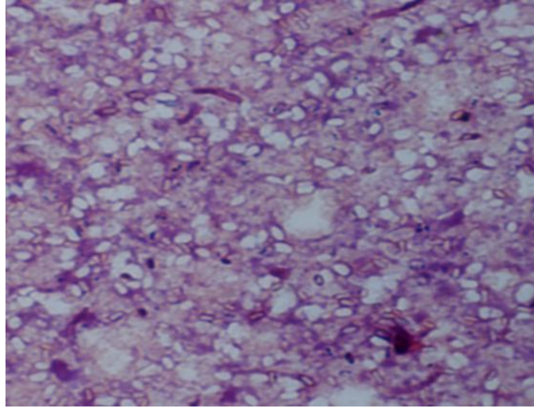


Figure 4: Using the method prescribed by Tortora *at al*. (1997), gram-positive *Alicyclobacillus acidoterrestris* are seen as purple-stained cells on the photomicrograph (x1000 magnification)

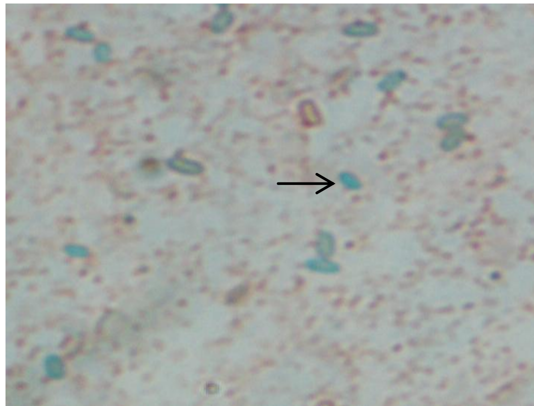


Figure 5: Using the method prescribed by Acuff (1992), spores are seen as green spheres (arrow). Remnants of *Alicyclobacillus acidoterrestris* vegetative cells (stained pink) can be seen on the photomicrograph (x1000 magnification).

Figure 6: Several colonies of *Alicyclobacillus acidoterrestris* on an Orange Serum Agar plate. Raised annuli can be seen (a) as well as remnants of the button centre (b).

4.2 PRELIMINARY ENUMERATION OF *Alicyclobacillus acidoterrestris* SPORES HARVESTED

Table 2: Number of spores (cfu) per ml in harvested suspension before and after heating on three different media types (BAM, OSA and KM)

Agar media	Number of spores before heating ($cfu.ml^{-1}$)	Number of spores after heating ($cfu.ml^{-1}$)
BAM	4.63×10^7	3.34×10^7
OSA	8.50×10^6	7.06×10^6
KM	1.92×10^5	5.50×10^4

4.3 USE OF SENSORY EVALUATION IN THE OPTIMISATION OF PARAMETERS (JUICE TYPE/FLAVOUR AND INOCULUM LEVEL)

Fifteen days after inoculation and incubation, panellists were unable to detect a difference between the test and control samples for both the Tropical Juice Blend and Passion Fruit Juice Blend juices ($p \geq 0.05$) which was perplexing. Considering that the juices were inoculated with $\approx 10^5$ $spores.ml^{-1}$ of juice, the low bacterial counts in table 3 illustrate that the assumption that bacterial growth was being inhibited was likely to be correct.

Table 3: Bacterial counts on juices 15 days after inoculation and incubation

Agar media	Number of viable cells in Wilde Tropical Juice Blend ($cfu.ml^{-1}$)	Number of viable cells in Wilde Passion Fruit Juice ($cfu.ml^{-1}$)
BAM	2.00×10^3	6.00×10^2
OSA	1.08×10^2	7.00×10^1
KM	2.55×10^1	1.10×10^1

Fifteen days after decanting into the McCartney bottles and re-incubation, panellists found that the Tropical Juice Blend had developed an off-odour ($p \leq 0.05$) while the Passion Fruit Juice still had not ($p \geq 0.05$).

4.4 PREPARATION AND INOCULATION OF JUICES TO INDUCE SPOILAGE

Table 4 reflects the level of spores in the spore suspension prior to dilution (i.e. stock) on three different media on the day of inoculation. Also reflected in the table are the theoretical number of spores that would be present per ml of juice upon inoculation. This value was calculated by dividing the number of spores per ml of stock spore suspension after heating by 200 – the volume of the juices inoculated. This step is necessary because all subsequent counts of bacterial levels in the juices will be represented per ml of juice. The theoretical number of spores of a tenfold dilution of the stock spore suspension per ml of juice is also reflected in Table 4.

Table 4: Spore counts in the stock spore suspension, per ml juice and in 10 fold dilution of stock spore suspension

Agar media	Number of spores in stock spore suspension after heating ($cfu.ml^{-1}$)	Theoretical number of spores of stock suspension after heating per ml of juice ($cfu.ml^{-1}$)	Theoretical number of spores of tenfold dilution of stock suspension after heating per ml of juice ($cfu.ml^{-1}$)
BAM	8.60×10^7	4.30×10^5	4.30×10^4
OSA	9.90×10^6	2.45×10^4	2.45×10^3
KM	1.50×10^5est.[3]	7.50×10^2	7.50×10^1

As expected, there was no growth on any of the air plates that had been placed in the vicinity of the flame in the laminar flow hood (0cfu/64cm^2). The representative Tropical Juice Blend juice tested for the presence of micro-organisms also exhibited no growth on any of the plates.

4.5 BACTERIOLOGICAL COUNTS, ELECTRONIC NOSE TECHNOLOGY (AROMASCAN™) AND SENSORY EVALUATION

For purposes of comparisons, results of each of the three different tests types (bacteriological tests, electronic nose technology [AromaScan™] and sensory evaluation [by olfactory means]) are represented together for each test day for each replicate.

[3] Count recorded as estimate as per Appendix F – rules for counting plates without colonies

Sammon Maps corresponding to the quality values listed in tables 6-11 are represented in figures 7-12.

Table 5: Bacteriological counts on BAM, KM and OSA, significance of triangle test results and quality values for juices inoculated with 4.3 x 10^4 spores.ml^{-1} of juice after 7 days incubation

	Replicate 1	**Replicate 2**	**Replicate 3**
BAM (cfu.ml^{-1})	1.85 x 10^4	7.60 x 10^3	1.77 x 10^4
KM (cfu.ml^{-1})	1.15 x 10^4	8.60 x 10^3	5.20 x 10^3
OSA (cfu.ml^{-1})	1.80 x 10^3	4.00 x 10^2	1.60 x 10^3
Significance ratings	$p \geq 0.05$	$p \leq 0.05$ ($p = 0.01$)	$p \geq 0.05$
Quality value[4]	0.605	0.091	0.669

Table 6: Bacteriological counts on BAM, KM and OSA, significance of triangle test results and quality values for juices inoculated with 4.3 x 10^4 spores.ml^{-1} of juice after 14 days incubation

	Replicate 1	**Replicate 2**	**Replicate 3**
BAM (cfu.ml^{-1})	5.50 x 10^3	3.70 x 10^3	1.02 x 10^4
KM (cfu.ml^{-1})	4.00 x 10^2	1.00 x 10^2	1.80 x 10^3
OSA (cfu.ml^{-1})	3.00 x 10^2	<10est.	<10est.
Significance ratings	$p \geq 0.05$	$p \geq 0.05$	$p \geq 0.05$
Quality value	4.516	14.627	10.570

[4] The quality values generated are as per comparison of each of the replicates with the respective control.

Table 7: Bacteriological counts on BAM, KM and OSA, significance of triangle test results and quality values for juices inoculated with 4.3×10^4 spores.ml^{-1} of juice after 21 days incubation

	Replicate 1	**Replicate 2**	**Replicate 3**
BAM (cfu.ml^{-1})	4.40×10^3	1.15×10^4	6.00×10^3
KM (cfu.ml^{-1})	<10est.	<10est.	<10est.
OSA (cfu.ml^{-1})	<10est.	<10est.	<10est.
Significance ratings	$p \geq 0.05$	$p \geq 0.05$	$p \geq 0.05$
Quality value	0.109	1.288	0.819

Table 8: Bacteriological counts on BAM, KM and OSA, significance of triangle test results and quality values for juices inoculated with 4.3×10^5 spores.ml^{-1} of juice after 10 days incubation

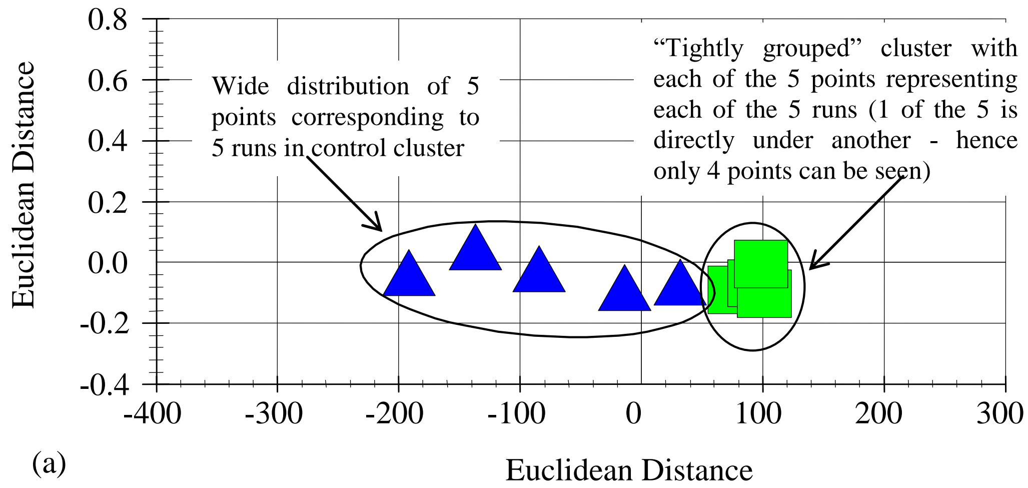
	Replicate 1	**Replicate 2**	**Replicate 3**
BAM (cfu.ml^{-1})	2.26×10^5	1.14×10^5	6.60×10^4
KM (cfuml^{-1})	9.20×10^4	4.20×10^4	7.20×10^4
OSA (cfu.ml^{-1})	1.40×10^3	2.10×10^3	5.40×10^4
Significance ratings	$p \geq 0.05$	$p \leq 0.05$ ($p = 0.01$)	$p \geq 0.05$
Quality value	1.793	1.253	2.999

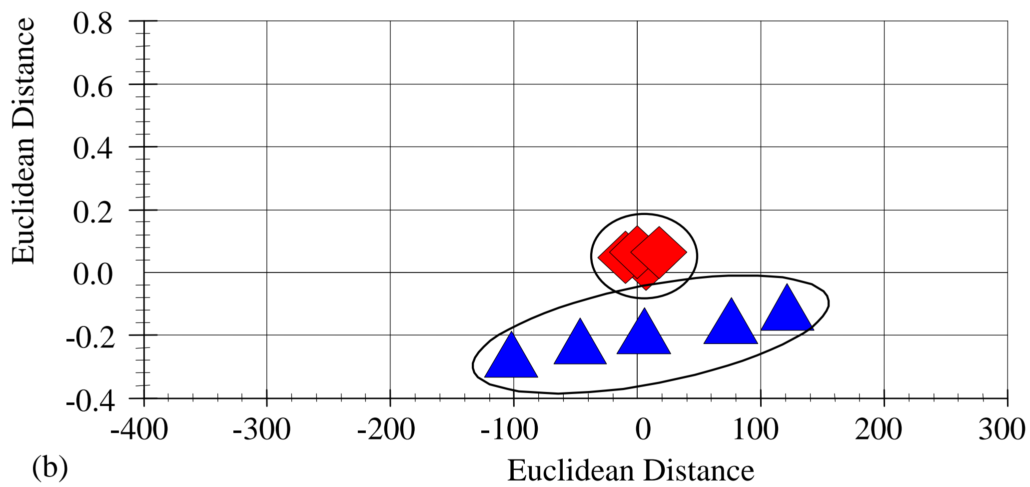
Table 9: Bacteriological counts on BAM, KM and OSA, significance of triangle test results and quality values for juices inoculated with 4.3 x 10^5 spores.ml^{-1} of juice after 20 days incubation

	Replicate 1	**Replicate 2**	**Replicate 3**
BAM (cfu.ml^{-1})	6.10 x 10^4	6.80 x 10^4	3.90 x 10^4
KM (cfu.ml^{-1})	2.50 x 10^3	4.40 x 10^3	7.20 x 10^3
OSA (cfu.ml^{-1})	4.00 x 10^2	5.00 x 10^2	2.00 x 10^2
Significance ratings	$p \geq 0.05$	$p \geq 0.05$	$p \geq 0.05$
Quality value	8.336	16.340	18.639

Table 10: Bacteriological counts on BAM, KM and OSA, significance of triangle test results and quality values for juices inoculated with 4.3 x 10^5 spores.ml^{-1} of juice after 30 days incubation

	Replicate 1	Replicate 2	Replicate 3
BAM (cfu.ml^{-1})	1.87 x 10^4	1.74 x 10^4	3.90 x 10^4
KM (cfu.ml^{-1})	8.00 x 10^2	1.11 x 10^4	3.00 x 10^2
OSA (cfu.ml^{-1})	<10est.	8.00 x 10^2	<10est.
Significance ratings	$p \geq 0.05$	$p \geq 0.05$	$p \geq 0.05$
Quality value	5.045	6.257	1.160





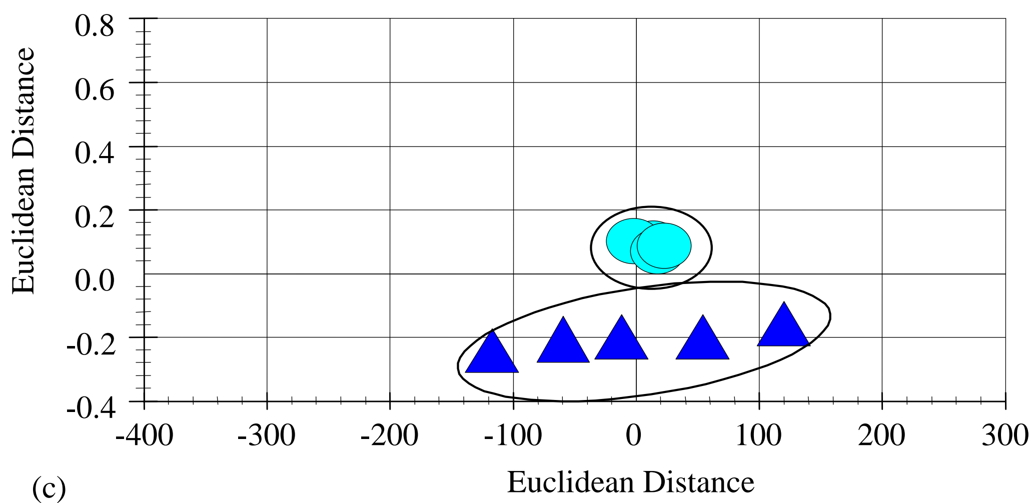
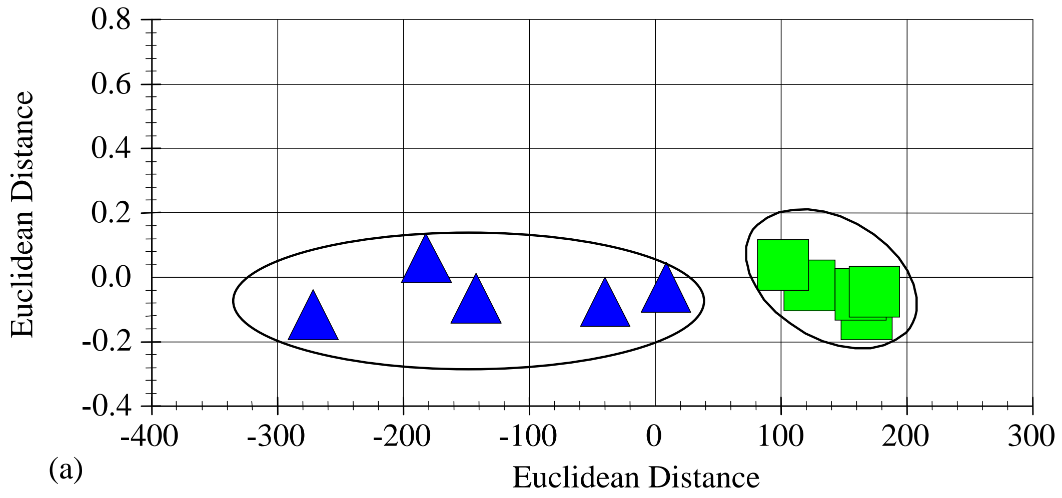
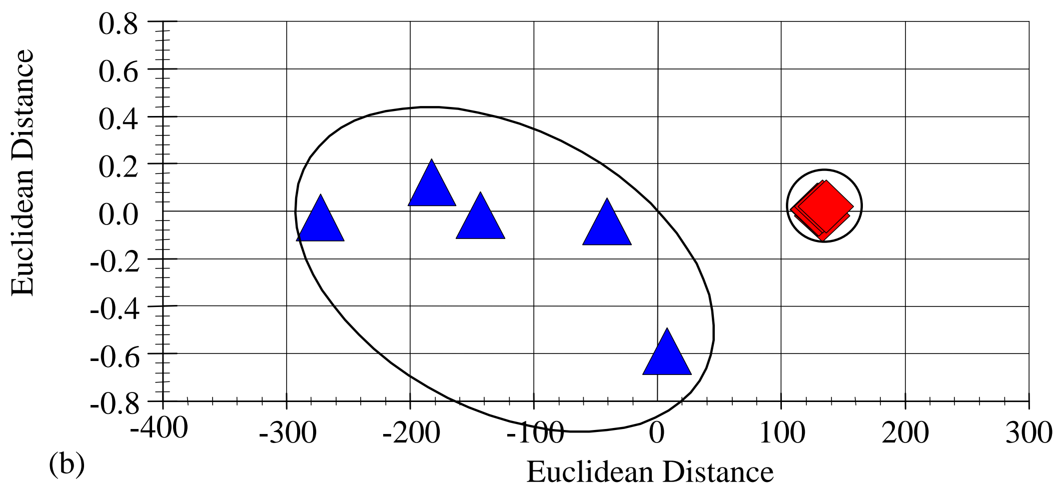


Figure 7: Sammon Maps of control juice (blue triangle) versus juices inoculated with a level of 4.3 x 10^4 *A. acidoterrestris* spores per ml of juice after 7 days incubation (a) control vs. replicate 1 (green square); (b) control vs. replicate 2 (red diamond) and (c) control vs. replicate 3 (cyan oval)





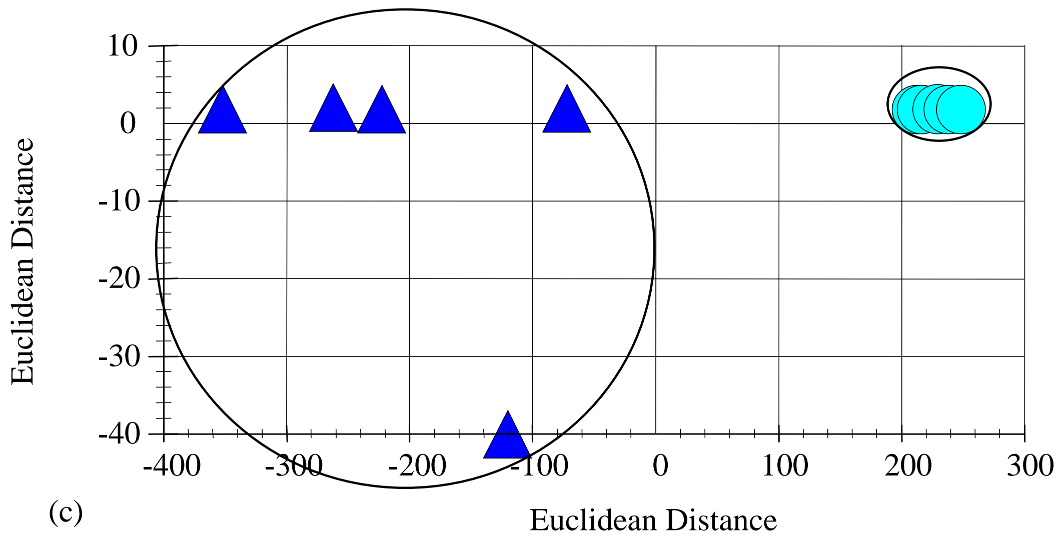
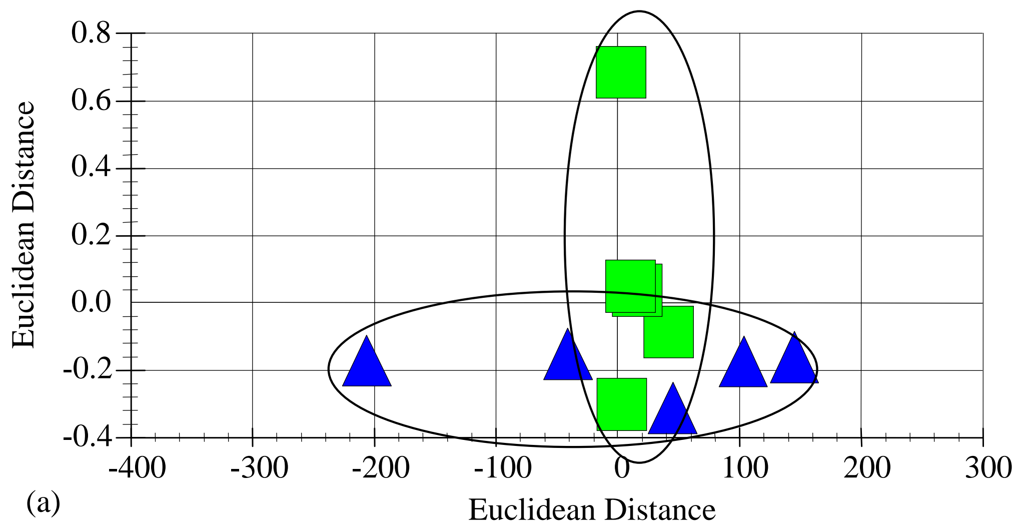
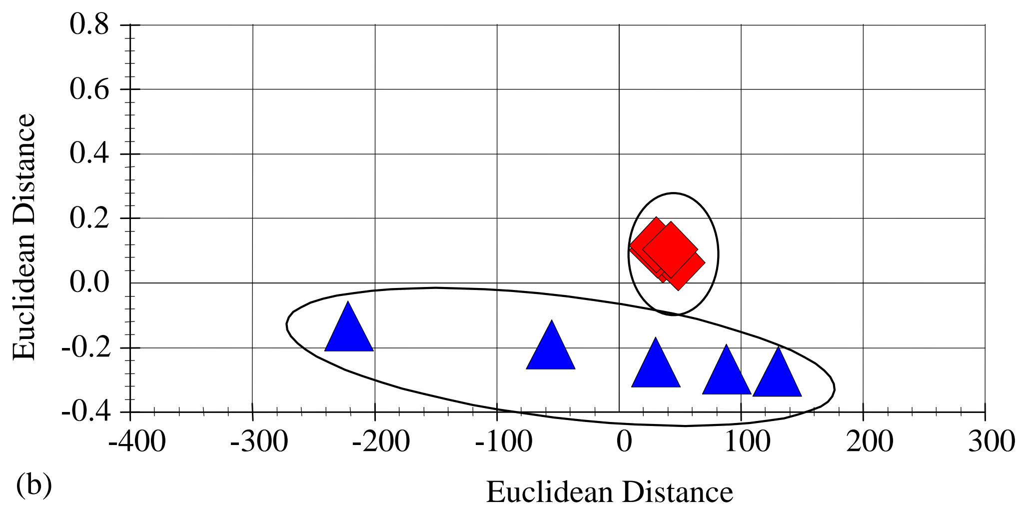


Figure 8: Sammon Maps of control juice (▲) versus juices inoculated with a level of 4.3 x 10^4 *A. acidoterrestris* spores per ml of juice after 14 days incubation (a) control vs. replicate 1 (■); (b) control vs. replicate 2 (◆) and (c) control vs. replicate 3 (●)





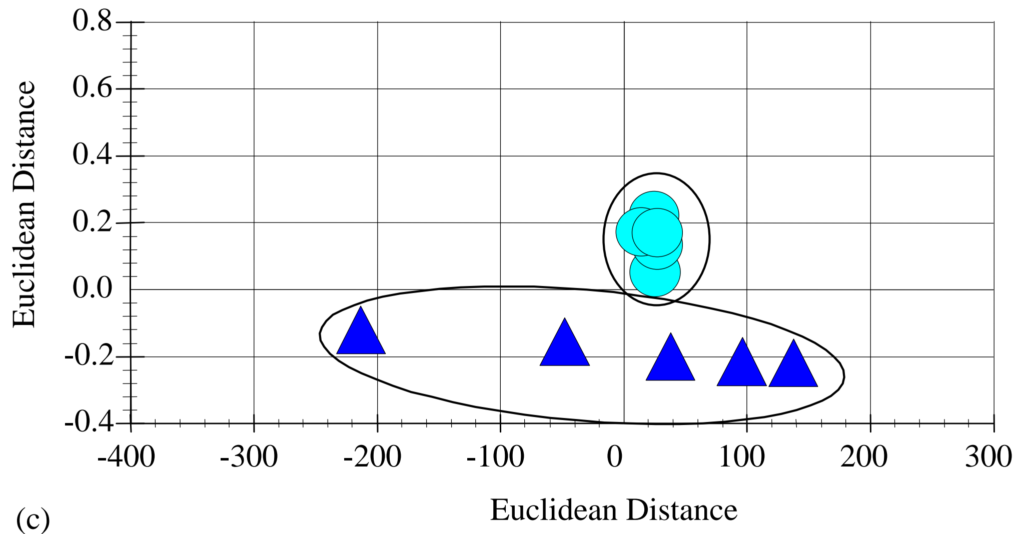
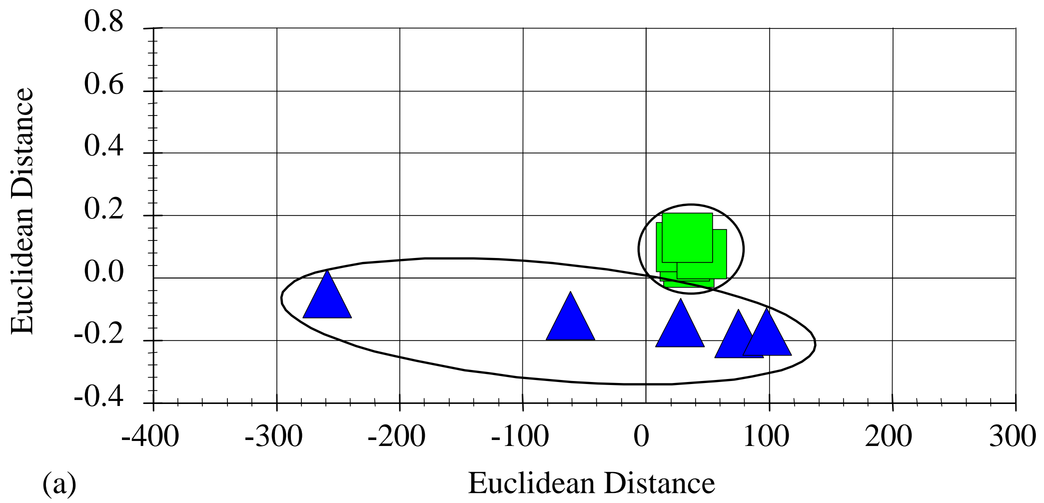
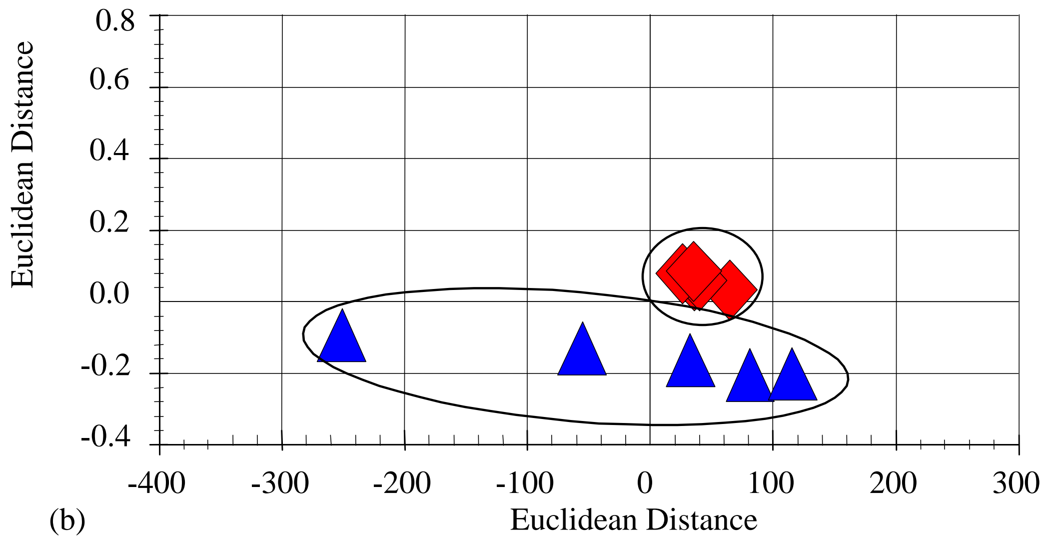


Figure 9: Sammon Maps of control juice (▲) versus juices inoculated with a level of 4.3 x 10^4 *A. acidoterrestris* spores per ml of juice after 21 days incubation (a) control vs. replicate 1 (■); (b) control vs. replicate 2 (◆) and (c) control vs. replicate 3 (⬭)





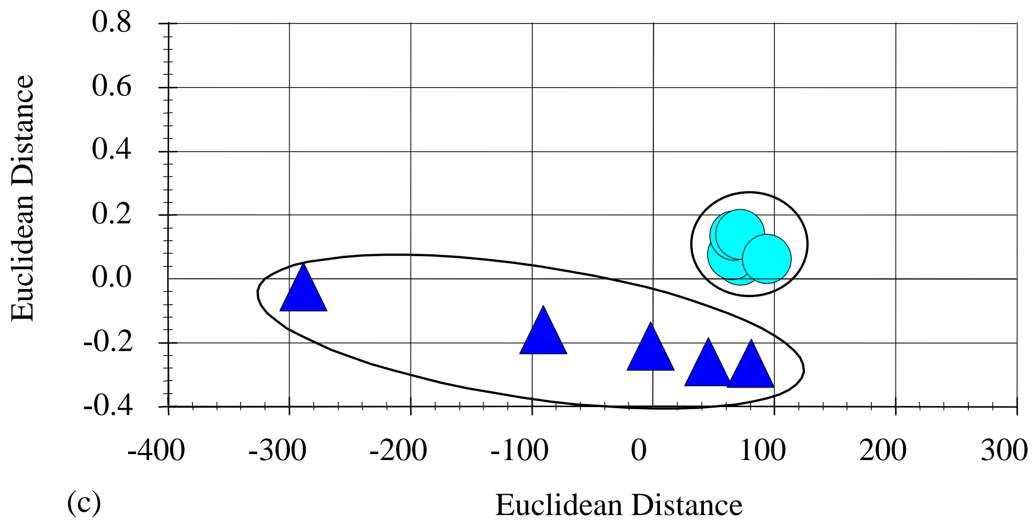
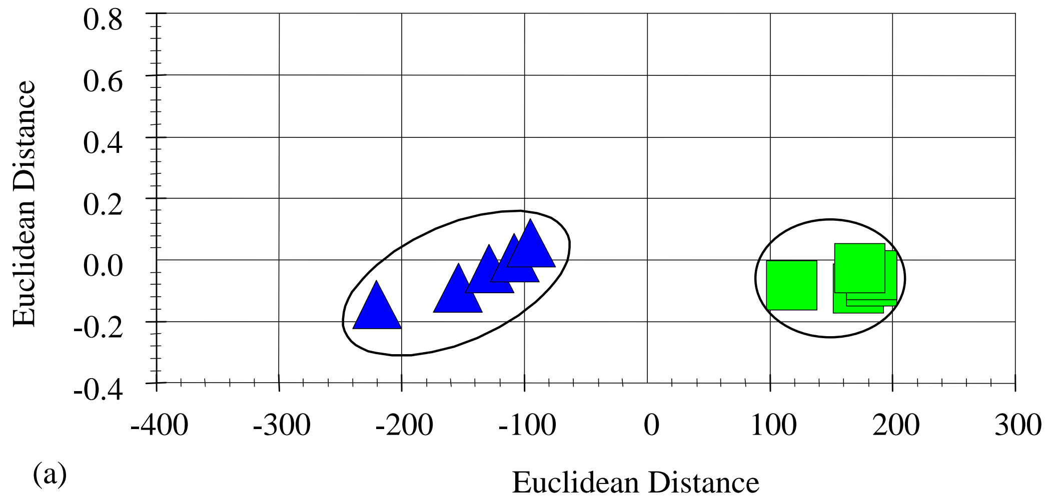
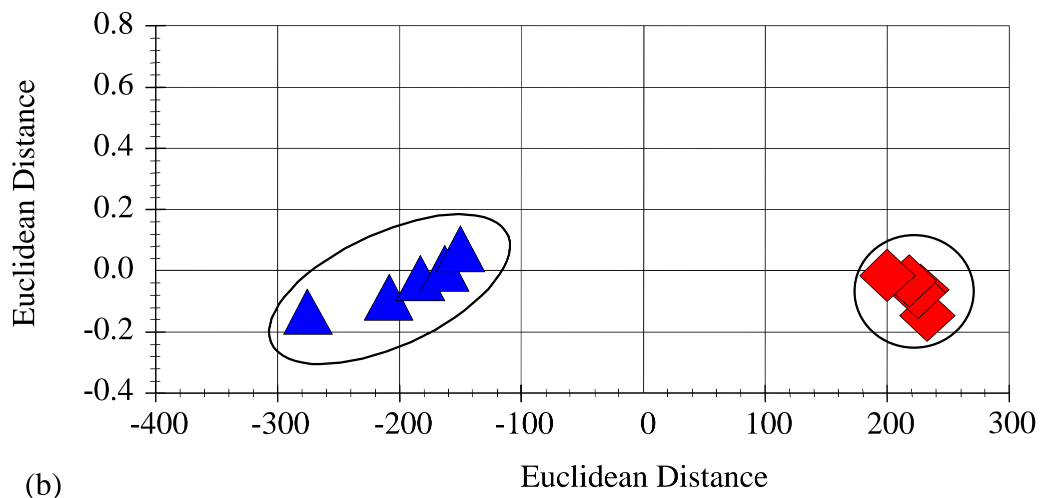


Figure 10: Sammon Maps of control juice (▲) versus juices inoculated with a level of 4.3 x 10^5 *A. acidoterrestris* spores per ml of juice after 10 days incubation (a) control vs. replicate 1 (■); (b) control vs. replicate 2 (◆) and (c) control vs. replicate 3 (⬭)



(a)



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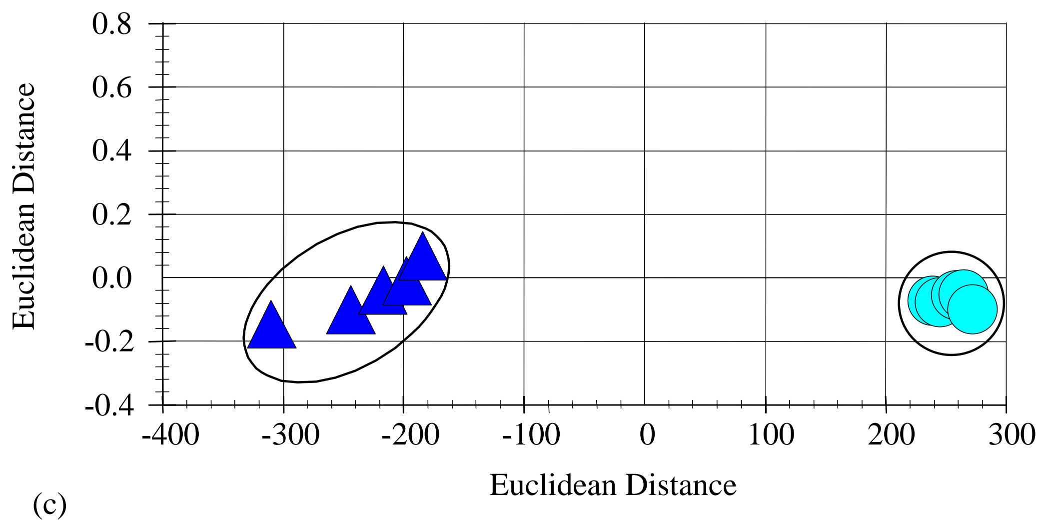
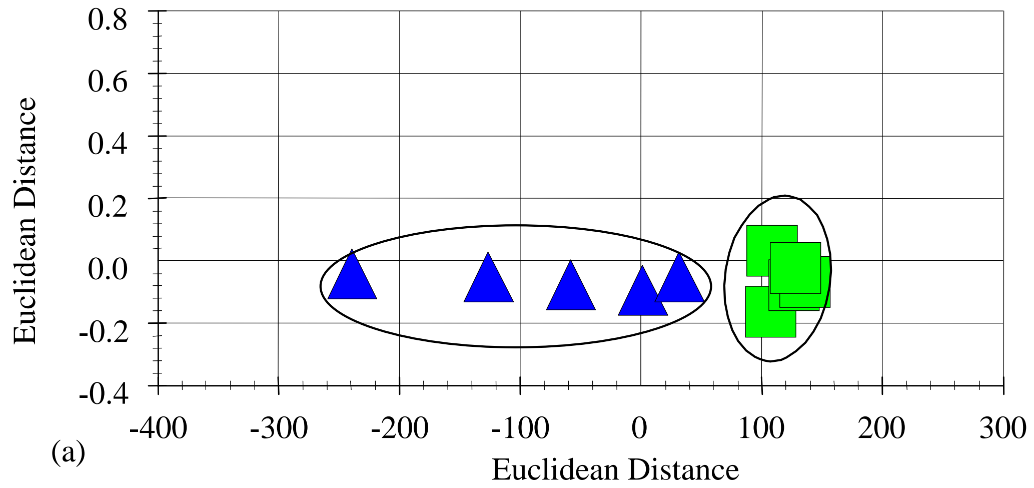
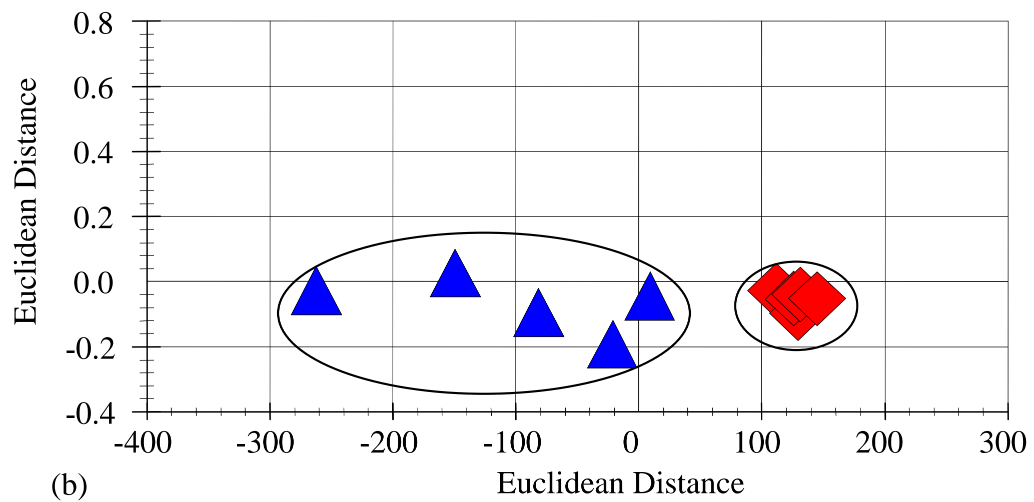


(c)

Figure 11: Sammon Maps of control juice (blue triangle) versus juices inoculated with a level of 4.3 x 10^5 *A. acidoterrestris* spores per ml of juice after 20 days incubation (a) control vs. replicate 1 (green square); (b) control vs. replicate 2 (red diamond) and (c) control vs. replicate 3 (cyan ellipse)





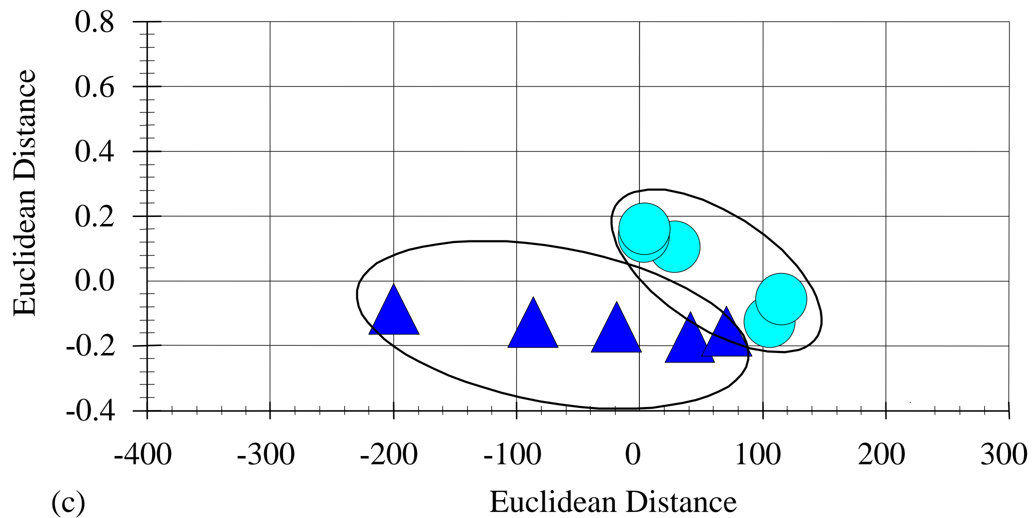
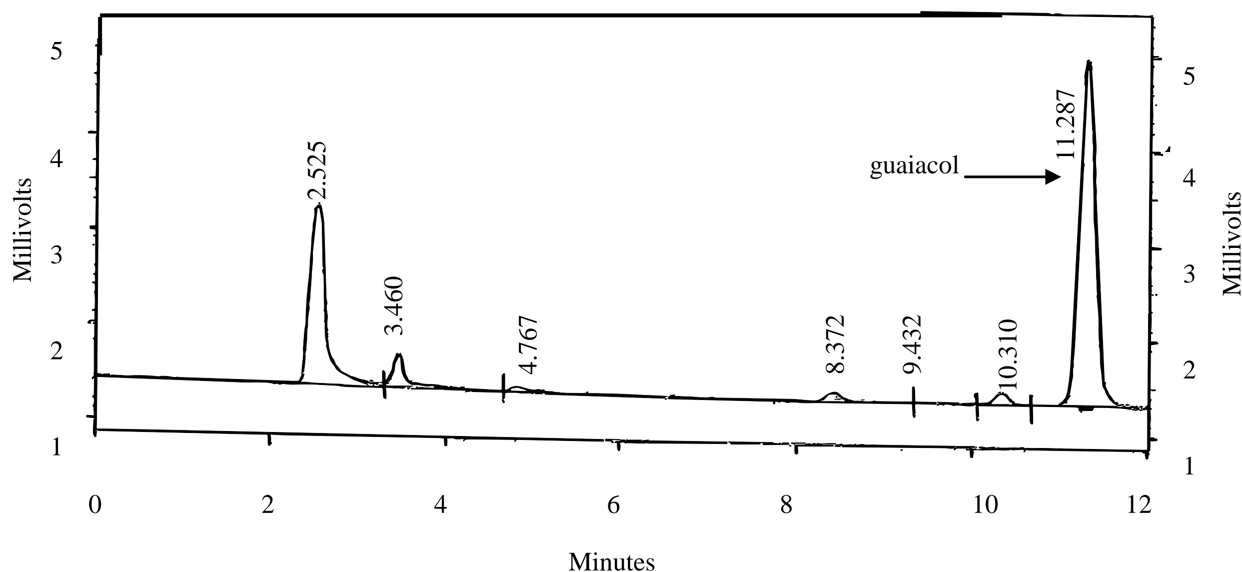


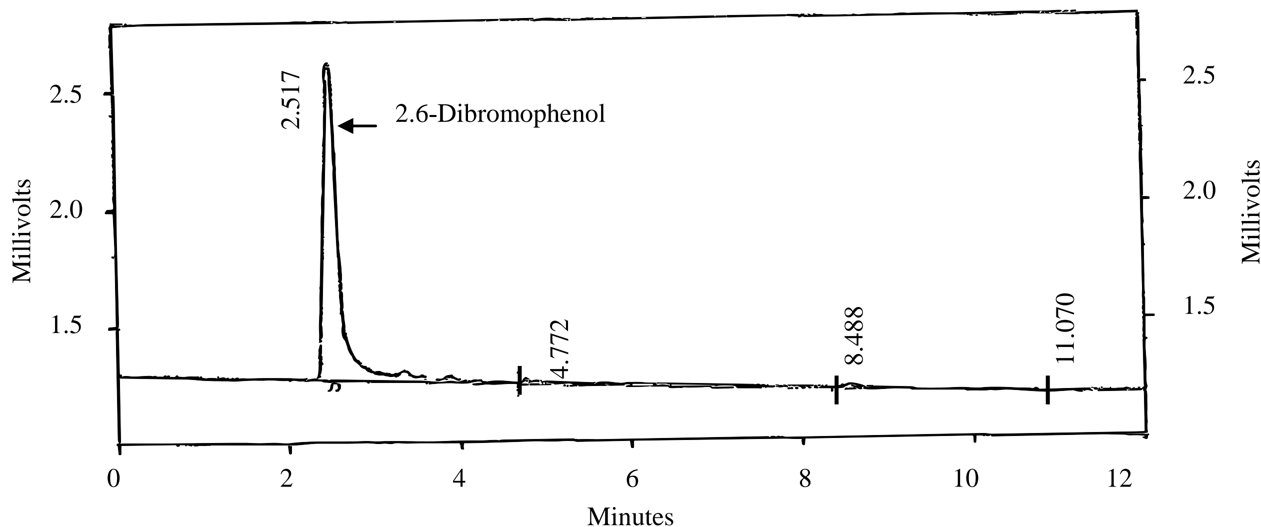
Figure 12: Sammon Maps of control juice (▲) versus juices inoculated with a level of 4.3 x 10^5 *A. acidoterrestris* spores per ml of juice after 30 days incubation (a) control vs. replicate 1 (■); (b) control vs. replicate 2 (◆) and (c) control vs. replicate 3 (⬭)

4.6 ANALYSIS OF STANDARDS AND JUICES USING HEADSPACE GAS CHROMATOGRAPHY

Both 2,6-dibromophenol and guaiacol standards were detected using HSGC. The chromatograms for each of the standards can be seen in Figure 13 and Table 11 lists the retention times and corresponding areas of the standards.



(a)



(b)

Figure 13: (a) Chromatogram of guaiacol standard at 100 ppm and (b) chromatogram of 2,6-dibromophenol standard at 100 ppm

Table 11: Retention times and corresponding peak areas of guaiacol and 2,6-dibromophenol standards each at 100ppm

Standard (100 ppm)	Retention time (minutes)	Peak area (units)
Guaiacol	11.287	4489919
2,6-Dibromophenol	2.517	1760891

The chromatograms of the two juice samples analysed via HSGC are represented in Figure 14.

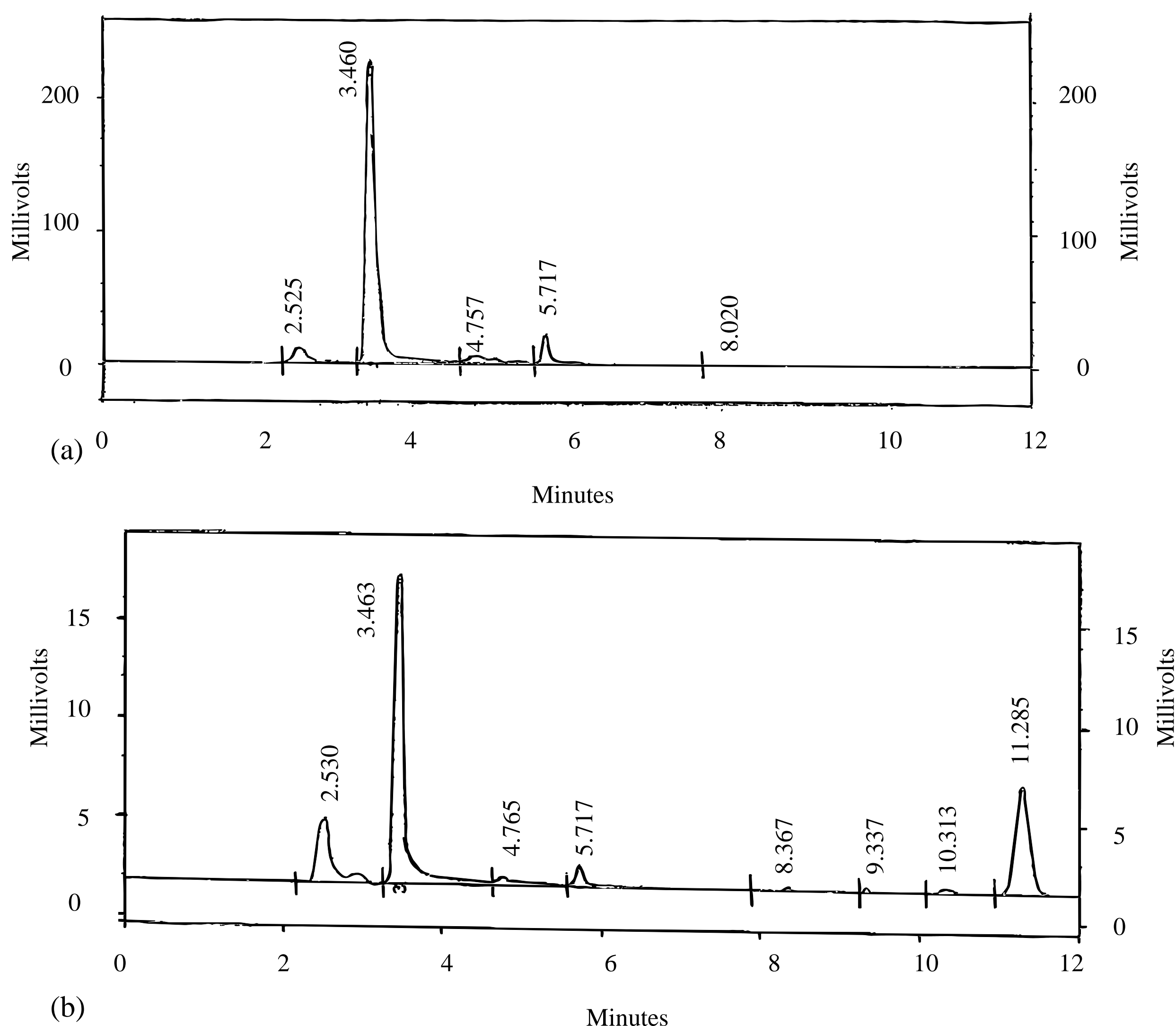


Figure 14: (a) Chromatogram of juice sample (replicate 2) inoculated with 4.3 x 10^4 spores.ml^{-1} 7 days after incubation; (b) Chromatogram of juice sample (replicate 2) inoculated with 4.3 x 10^5 spores.ml^{-1} 10 days after incubation

From the chromatography results for each of the two juice samples, retention times most similar to those of guaiacol and 2,6-dibromophenol standards (as listed in Table 11) and corresponding peak areas are presented in Tables 12 (a) and (b).

Table12a: Peak areas and concentration of compounds eluting at retention times similar to guaiacol and 2,6-dibromophenol for juices with 4.30 x 10^4 spores.ml^{-1}, after 7 days incubation.

	Retention times (min.)	**Peak area (units)**	**Concentration (ppm)[3]**
Retention time most similar to that of the guaiacol standard	-	-	-
Retention time most similar to that of the 2,6-dibromophenol standard	2.525	16691814	948

Table12a: Peak areas and concentration of compounds eluting at retention times similar to guaiacol and 2,6-dibromophenol for juices with 4.30 x 10^5 spores.ml^{-1}, after 10 days incubation.

	Retention times (min.)	**Peak area (units)**	**Concentration (ppm)**
Retention time most similar to that of the guaiacol standard	11.285	6717247	150
Retention time most similar to that of the 2,6-dibromophenol standard	2.530	4343260	247

[3] Concentrations calculated on basis of the area of standards and assuming that the compounds being referred to in the tables 12a and b are guaiacol and 2,6-dibromophenol.

CHAPTER 5

5. DISCUSSION

Quality control of juice products is essential as these products are susceptible to pathogenic and spoilage microorganisms that can compromise the health of the consumers. It leads to maintained or increased profitability as the application of quality control on the juice manufacturing process is likely to reduce the amount of off-specification or reject products, ensuring that consumers are content with the products. In juice industry where product volumes are generally large and margins for error are small, improvement in product monitoring can make a dramatic impact on profitability (Lynch, 2001). Consequently, techniques/technologies for monitoring products are becoming increasingly important and measurements via automated systems are now considered essential. Automated systems are thus considered a key area for development. However, in some instances quality control or quality assurance systems other than automated ones are also relied on. Conventional bacteriological monitoring is one such approach.

The discussion begins with this aspect in terms of the results obtained for isolation of *Alicyclobacillus acidoterrestris*. Prior to this, however, it is felt that some clarification is necessary concerning the isolation of *A. acidoterrestris* and enumeration methods included in this study. The isolation method along with methods of characterisation and identification, in effect, demonstrate the presence or absence of *A. acidoterrestris*. Enumeration methods, on the other hand, are used to establish actual counts. Unless micro-organisms have been identified as such, there is no way of knowing for sure whether the counts in the real-life situation are actually *A. acidoterrestris* and such counts would thus be presumptive *A. acidoterrestris* counts (Pettipher *et al*., 1997). In this study, the isolation method was evaluated as one of the methods for detection of the micro-organism (Section 5.1). Bacteriological counts (enumeration) methods were also evaluated in this regard, although to a much lesser extent (section 5.5.1). More specifically, media types have been assessed in terms of bacteriological enumeration. The counts were also used to monitor the levels at which tainting was detectable.

5.1 ISOLATION OF *Alicyclobacillus acidoterrestris* FROM FRUIT JUICES AND CHARACTERISATION AND IDENTIFICATION OF ISOLATES

The sealed juice carton from which the gram variable, catalase positive, sporeforming micro-organisms were isolated was blown as a consequence of bacterial spoilage. The reason that this sample was still chosen for attempted isolation of *Alicyclobacillus acidoterrestris* in spite of the fact that it is not a gas producer, was that the blown state of the sample did not eliminate the possibility that *A. acidoterrestris* may also have been present together with the micro-organism which caused the blowing. This consideration (i.e. that the micro-organisms isolated could be *A. acidoterrestris*) was thought to be substantiated, firstly, when the morphology of the colonies isolated from the blown sample were found to be typical of those formed by *A. acidoterrestris* on OSA. Secondly, as is characteristic of this micro-organism (Jenson, 1999), a low number of colonies (only 50cfu.ml^{-1}) were recovered from the juices even after enrichment. Thirdly, when sub-cultured on BAM (which was thought to be selective for *A. acidoterrestris*), the micro-organisms grew very well. Other reasons for not eliminating the possibility that the micro-organisms isolated were *A. acidoterrestris* were the similarities which the phenotypic characterisation tests illustrated between the isolates and those known to be typical of *A. acidoterrestris*. In spite of all these observations, it would have been unacceptable to assume that the isolates were unquestionably *A. acidoterrestris*. Doing so could have led to conducting the entire research with the incorrect micro-organism perhaps producing results which could not have been explained in terms of the objectives of this study. The pure cultures of the isolates on BAM plates were therefore sent for identification and all were identified as *Bacillus* species. Admittedly, at this point the micro-organisms isolated could still actually have been *A. acidoterrestris*. However this is a relatively new genus and species (1992), and it might therefore have been classified as a *Bacillus* species as earlier workers had done. Other researchers who have experienced difficulties in conclusively identifying *A. acidoterrestris* using an API 50CHB biochemical test kit include authors such as Pettipher *et al*. (1997). Those researchers also stated that ribotyping is capable of providing conclusive identification of *A. acidoterrestris* but the technique is too expensive to be used routinely and that simulating conditions to demonstrate that a taint is produced is a more feasible approach to confirming the presence of this micro-organism.

The inability to conclusively identify the isolated micro-organisms in this study led to the purchase of the pure culture from the South African Bureau of Standards (SABS) Test House. This culture exhibited the same phenotypic characteristics as the isolate from the blown juice sample except that it was distinctly gram positive as opposed to the gram variability of the latter. Its catalase reaction was also more vigourous than that of the latter. Additionally, this culture did not grow on nutrient agar when sub-cultured, illustrating the inability of this organism to grow on an agar medium with a neutral pH as has been pointed out by Jenson (1999) and Splittstoesser *et al*. (1998). All of these findings illustrate that it is not the easiest, quickest or cheapest task to accurately or conclusively detect *A. acidoterrestris* using bacteriological and biochemical techniques, especially considering aspects such as the tediousness of preparing BAM and the other media (OSA and diluent), the combined lengthiness of the incubation period of samples after the heat shock treatment (2 days), the incubation period of the petri-dishes on which samples had been plated (7 days) and the time taken for sub-culturing and identification of the isolates (minimum of 7 days in total). It is therefore more feasible to subject juices to conditions under which *A. acidoterrestris* are known to cause spoilage and establish the presence of spoilage taints which could be linked to *A. acidoterrestris*, even in the real-life situation.

The next 3 focus on the 3 methods used to evaluate taint in this project will be discussed.

5.2 PRELIMINARY ENUMERATION OF SPORES PER ML OF SUSPENSION HARVESTED BEFORE AND AFTER HEATING ON 3 DIFFERENT MEDIA TYPES

It is evident from the results illustrated in Table 2 that there is a difference between the spore count before and after heating with the count after heating being lower. This is to be expected as during harvesting of the spores, vegetative cells are sometimes present in the spore suspension and these form colonies during counting (Splittstoesser *et al*., 1998). Heating the suspension kills off any vegetative cells and the count after heating represents that of spores only and should be the count that is taken into consideration for further use. At this stage, it was important to have established a rough idea of the number of spores harvested so as to ensure that a level of at least the order of 10^7 cfu.ml^{-1} of suspension was

available for use, in other words that the level of spores harvested was not too low (or too high) prior to inoculation to generate a meaningful result. Once a rough idea of the number of spores harvested was obtained, it was easy to do the relevant dilutions to obtain the desired levels for inoculation for the spoilage parameters.

5.3 USE OF SENSORY EVALUATION IN THE OPTIMISATION OF PARAMETERS

The trial experiment was conducted to prevent wastage that would have resulted from inoculating an entire test set only to find that no odour had formed. The reason suggested for the lack of formation of an odour in both of the juice flavours 15 days after initial incubation is the lack of aerobic conditions within the juice boxes, which inhibits the growth of the micro-organisms, metabolism and odour formation. Decanting of the juices into McCartney bottles with a headspace of air above it thus facilitated the development of an odour within the Tropical Juice Blend flavour 15 days after re-incubation. The lack of development of an odour within the Passion Fruit Juice Blend may thus be attributable to some factor other than limited oxygen. It is suggested that an inherent characteristic of the juice caused the death of viable micro-organisms within this flavour after re-incubation and hence there was no odour formation. It can be seen from Table 3 that even when both samples had no available oxygen, the bacterial counts in the Passion Fruit Juice Blend were lower on all three media types when compared to those of the Tropical Juice Blend 15 days after inoculation and initial incubation. As it was only the intention to demonstrate taint/odour formation in this trial experiment on a qualitative level, no further work was conducted.

5.4 PREPARATION AND INOCULATION OF THE JUICES TO INDUCE SPOILAGE

Distilled water was used as the diluent for inoculations because it does not interfere with the make-up of the juices as peptone water does. The spore suspension used for inoculation was not from the same bottle as was used to conduct the preliminary count. Furthermore, the spores were not subjected to freezing on the day on which they were harvested. With these two factors combined, it is clear why it was necessary to perform

another count on the spore inoculum at this point. Preliminary determination of the number of spores (Section 4.2) illustrated that it was necessary to heat samples prior to plating to ensure that only spores were present in the inoculum. At the same time, heating of the spores at this point served to activate them so that there would be maximum growth upon inoculation especially since they had been subjected to freezing.

The reason that 9ml (an arbitrarily chosen quantity) of air was inoculated into the juices was to facilitate aerobic conditions as trial experimentation (Section 4.3) illustrated that aerobic conditions are necessary for odours to develop in the juices chosen for testing. Given that *A. acidoterrestris* are obligate aerobes (Walls and Chuyate, 1998) this is not entirely surprising. This is also perhaps the reason why some workers decant their test juices into bottles with a headspace of air prior to inoculation. Orr *et al*. (2000) have also stated that the role of oxygen in the headspace of packaged products needs to be further investigated as it would affect the rates of microbial growth and odour formation. Additionally, they state that oxygen uptake during testing can be facilitated by agitating the containters in which juices are being tested. This ensures that all microbial cells are exposed to oxygen and not just those in contact with the headspace oxygen. Agitation of the juices during laboratory tests mimics the conditions of motion which juices are exposed to from the point of manufacture and transport to the consumer. The fact that no growth was observed on the nutrient agar and PDA air plates laid out in the vicinity of the flame in the laminar flow hood illustrates that it was unlikely that anything other than the micro-organisms in the inoculum was injected into the juices. Furthermore, none of the juices had been spoiled by production of gas and subsequent blowing of the juices during the incubation period by, for example, the presence of a gas producing spoilage yeast which might have been present in the air. In addition, spoilage could not have been attributed to any micro-organism other than *A. acidoterrestris* as no micro-organisms were detected in the representative juice tested.

5.5 BACTERIOLOGICAL COUNTS, SENSORY EVALUATION AND ELECTRONIC NOSE TECHNOLOGY

5.5.1 Bacteriological Counts

Spread (pre-poured) plates have been found to be more effective than pour plates for optimum enumeration of *A. acidoterrestris* (Pettipher *et al*., 1997) hence the former type was used for this purpose. It can be seen from the counts reflected in Tables 5-10 that BAM is the most effective of the 3 agar media evaluated for the enumeration of *A. acidoterrestris* for the duration of the study. Such was identified and observed during earlier experiments (Tables 2-4). While not many authors prior to Jenson (1999) have referred to BAM in their work with *A. acidoterrestris*, the situation is likely to change if, of course, researchers are willing to overlook the complicated process involved in preparing the media, especially the preparation of the micronutrient solution. After BAM, the next most effective medium for the enumeration of *A. acidoterrestris* is KM and then OSA. Orr and Beuchat (2000) in their study also found that KM was better than OSA as did Walls and Chuyate (1998). This contradicts trends reflected in Tables 2-4 where the OSA was found to be better than KM. It is suggested that the different sources of the KM plates may be the reason for this contradiction. The KM plates which produced the results in Tables 2-4 had been purchased from the SABS Test House while those which produced the results listed in tables 5-10 had been prepared at the laboratory where the study was conducted. Other findings from the bacteriological counts illustrated that OSA plates were the first to exhibit no growth for both levels of inoculum as expressed by counts of <10 $cfu.ml^{-1}$ (Table 6 and Table 10). This was followed by KM at a later stage for the lower level of inoculum (Table 7) while BAM gave positive counts over the entire test period. This finding is indicative of the different levels of sensitivity of the different growth media. Based on these results, the problems that could arise in a large-scale juice manufacturing plant using KM or OSA for enumeration of *A. acidoterrestris* are apparent and can be disastrous to product shelf-life.

Using BAM counts as the basis for comparison, the counts in those replicates which had been inoculated with the stock spore suspension (Tables 8-10) and those with the tenfold dilution thereof (Tables 5-7) dropped in both cases after the first intervals of their

respective incubation periods and continued to decrease over the rest of the test period for both test sets. However, even after having been incubated for 14-20 days, the counts did not drop as much as those of the trial experiment (no oxygen available) which would have had an initial inoculum level very similar to that of the actual test set. Decrease in the counts do not necessarily imply that microbial growth was not occurring. It might simply be that the rate of death exceeded the rate of growth. In an analogous finding, Orr *et al.* (2000) found that over their test period there was little net change in bacterial populations and they attributed this to similar rates of growth and death of *A. acidoterrestris*. This being the case, the taint compounds would still be produced especially since the levels of bacteria present in the test juices (tables 5-10) are within the range said to cause tainting. Evidence that micro-organisms are still growing can be derived from Tables 6 and 7. In Table 7, replicates 2 and 3 interval have counts at the 21 day which are higher (on BAM) than replicates tested at the 14 day interval.

5.5.2 Sensory Evaluation by Olfactory Means and Headspace Gas Chromatography

Significant sensory differences ($p \leq 0.05$) between test and control juices were detected at 7 days (Table 5) and 10 days (Table 8) in only 1 of the 3 replicates in each case. According to the headspace gas chromatography (HSGC) results for replicate 2 at the 7 day interval, there is no guaiacol present at this point while a compound with a retention time similar to 2,6-dibromophenol is present at a level of 948ppm (Table 12a). For replicate 2 at the 10 day interval, a compound with a retention time similar to guaiacol is present at 150ppm while a compound with a retention time similar to 2,6-dibromophenol is present at 247ppm (Table 12b). The levels of the tainting compounds detected by HSGC are far above the thresholds that have been established by previous researchers. At such levels, it would be expected that 100% of the panellists would have selected the respective test samples as the odd one out during the triangle test. This, however, was not the case. Considering that all juices were inoculated under the same conditions, it would have been expected that panellists would have identified the test sample in each of the replicates for the respective test days. It is unlikely that contamination via the standards had occurred during HSGC as the juice samples were analysed prior to the standards. The lack of the expected correlation between olfactory evaluation amongst replicates indicates the difficulties associated with this technique in quality control. Sensory results after the

second and third intervals for both levels of inoculum thereafter reflects no significant differences ($p \geq 0.05$) between control and test samples.

5.5.3 Electronic Nose: AromaScan

There are several conclusions which can be drawn from the quality values listed in Tables 5-10 as well as from the related Sammon Maps (Figures 7-12). The quality values generated for comparisons between controls and each of the respective test replicates on each of the test days are more or less comparable. For example, by looking at the values for the control compared to replicates 1, 2 and 3 tested at the 7 day interval, the values are very similar to each other. This is the pattern throughout the test period. As an extension of this interpretation, it can be clearly seen from the Sammon Maps that for each replicate, the results for each of the 5 runs were almost identical to each other, that is the clusters for each replicate were "tightly grouped" as shown on figure 7a. This illustrates that the AromaScan is capable of producing reproducible results for any one sample. From all of the Sammon Maps it can also be seen that for each of the runs with the controls there is a wider distribution within the clusters (Figure 7a). Zannoni (1995) found patterns in clusters similar to those for the control juices when they analysed cheese samples. While Zannoni (1995) state that further investigations are necessary to understand the reason for these distributions, the author suggests that it is probably attributable to a greater range of volatiles in the control samples as compared to the test samples. It appears that the test samples have a dominating aroma which resulted in the almost identical patterns for each run. This illustrates the ability of the AromaScan to correctly group test samples.

It is easy to see from the quality values that the juices inoculated with the lower level of inoculum, when compared to the control, increase from day 7 to day 14 for all replicates (Tables 5 and 6). This is also the case for comparisons made between the controls and juices inoculated with the higher level of inoculum and tested at 10 days and 20 days (Tables 8 and 9). These increases in quality values are also represented by increased spatial distances between the control cluster and replicate clusters on the Sammon Maps (figures 7 and 8; figures 10 and 11 respectively). The increases are not surprising as the extra incubation time for both the test sets would have allowed further growth and metabolism by *A. acidoterrestris*, producing higher concentrations of the taint compounds

which were subsequently detected by the AromaScan with corresponding increases in quality values. Atkinson and Hammond (1994) have also affirmed that responses of electronic noses depend on the concentration of the volatiles. Lending further credibility to the AromaScan are the higher overall quality values generated when the control juices are compared to the test juices with the higher levels of *A. acidoterrestris* spore inoculum as opposed to comparisons of controls with juices with the lower levels of inoculum. Taylor (1994) found similar discriminative capability of an AromaScan A20S in their evaluation of different hop varieties as well as in their evaluation of out of specification beer.

In addition to the reproducibility indicated in the analysis using the AromaScan, the next issue is what the quality value obtained are actually predicting about the quality of the juices as is relevant to the study. With the exception of replicate 3 at test day 10, at both levels of inoculum there were no significant differences between the control sample and replicate test samples for the first test days for each inoculum level. This is indicated by quality values of less than 2 and is represented by the overlap of the clusters on the Sammon Maps. At the next set of test days, that is, day 14 for those with the lower level of inoculum and day 20 for those with the higher level of inoculum, there are significant differences between the controls and replicate test juices. This is based on quality values being greater than 2. The only foreseeable reason for these differences is that the test juices had been inoculated with *A. acidoterrestris* while the controls had not. Since all other factors to which the test and control juices had been subjected to were exactly the same, this is a plausible reason. The quality values suggest that the products become tainted somewhere between 7 and 20 days after inoculation corresponding to bacterial levels ranging from $\approx 10^3$ to $\approx 10^5$ cfu.ml^{-1}.

Thus far, much has been said by the author about the quality values obtained by analyses at 7 and 14 days at the lower level of inoculum and 10 and 20 days analyses at the higher level of inoculation. Looking at the quality values for day 21 and 30, for both levels of inoculum, it can be seen that there was an unexpected drop in values. The values for day 21 (Table 7) indicate that the AromaScan is not detecting any difference between the control and test replicates. This may be attributed to the possibility that the volatile taint compounds could have been degraded to less volatile or non-volatile components as a result of the prolonged incubation. The day 30 quality values (Table 10), however, says

that there are still differences between the control and test replicates. This may be attributable to the fact that these samples were inoculated with a higher level of inoculum, hence higher levels of the taint compounds would have formed. The taint compounds would thus have required a further incubation period for quality values to reflect no difference between the control and test replicates for the reasons suggested above. It is unlikely that the drop in microbial counts played a role in the drop in quality values as a decrease in microbial counts will not result in a decreased level of the taint compounds already produced at an earlier stage.

CHAPTER 6

6. CONCLUSION

Alicyclobacillus acidoterrestris is capable of surviving the typical pasteurisation process given to fruit juices by means of their spores which are able to outgrow, multiply and cause spoilage by the production of the volatile taint compounds, guaiacol and 2,6-dibromophenol. *A. acidoterrestris* is not the easiest or quickest micro-organism to isolate and identify because of all its special requirements. Establishing the presence of the volatile taint compounds is thus a more practical way of detecting *A. acidoterrestris* and can be done by sensory evaluation, GC or electronic nose technology.

Sensory evaluation and GC have provided the mainstay of quality control in many industries including the fruit juice manufacturing industry. While GC is used to derive information about odours of a product, this method cannot be used to tell if the component has an odour or not and data is often difficult to correlate with sensory information (Giese, 2000) as has been the case in this research. Giese (2000) also states that results from GC often require thorough analysis and that, in many instances, much of the information is irrelevant. Although a very powerful tool, sensory panel usage in quality control for the detection and identification of taints is very costly and time-consuming. In contrast to the limitations of sensory panels and GC, electronic noses provide a more rapid, less costly and safer way to conduct odour measurement. Odour/aroma is an important sensory component of food and beverages and it is very important that it is measured reliably and objectively (Taylor, 1994). Electronic noses appeal to both analytical scientists such as those involved in gas chromatography and to sensory scientists. The reasons are that the electronic noses incorporate the objectivity which the analytical chemists appreciate and expect from their results and they function very similarly to the human nose to give rapid results which may be correlated with the way products actually smell, a concept which satisfies the sensory scientist (Marsili, 1995).

In this study we found that bacteriological techniques were inadequate in detecting and conclusively identifying *A. acidoterrestris*. It was also found that, depending on media type used in the enumeration of *A. acidoterrestris* it is also possible for the micro-organism

to go undetected altogether. To elaborate, while BAM was still showing positive growth on plates, the other two media were not - a clear reason for concern in using bacteriological techniques in trying to isolate and/or enumerate *A. acidoterrestris*. Sensory evaluation revealed that panellists vary in their responses to the same stimulus (in this case odour emanating from the juices). Considering that GC is often only called into play when sensory attributes are compromised as by the production of off-flavours and odours detected by consumers (an equivalent to panellists), the question is raised as to how reliable the panellists or consumer's perception is. During this study, it was found that electronic nose technology produced good repeatability in indicating statistically significant differences between and amongst compared samples

While the author acknowledges the value of and results obtained by using the electronic nose, it must again be pointed out that these instruments are not superior to, nor are they meant to replace techniques such as sensory evaluation and gas chromatography. Rather they provide a potentially valuable tool that finds its application somewhere between the two disciplines complementing the sensory testing which scientists have traditionally used for studying aroma (and taste) components of foods and beverages as well as the analytical instrument analysis of foods, namely GC.

In light of inconsistencies in results from the widely accepted sensory evaluation and gas chromatography methods used in the detection of the taint compounds produced by *A. acidoterrestris*, the results obtained from the AromaScan electronic nose cannot be dismissed altogether. It appears that the AromaScan is capable of discriminating differences in levels of odours arising from different *A. acidoterrestris* loads in a similar manner to that described by Korel and Balaban (2002) in their work with other odour-producing microbes. Essentially, the electronic nose can be used to monitor the aroma of products and by pointing to deviations of products from the control can be used as a quality control tool (Taylor, 1994). Overall the advent of electronic noses should have a profound impact on future odour monitoring, not only in the food and beverage industry. Many industries involved in food and beverage processing which rely on human noses in order to maintain product quality, are now presented with an alternative means for automated monitoring of products by means of electronic noses (Taylor, 1994) and recording such data for future records.

Within the juice industry, *A. acidoterrestris* can be detected by electronic noses in juices which have been subjected to conditions under which spoilage is known to occur by detecting deviations when compared to a control. Sensory evaluation and GC can be called into play if further characterisation of the problem is needed once the electronic nose has detected products which are different from control. This would save a considerable amount of analysis time, cost and energy. Furthermore, the risk of deterioration of products already at commercial outlets (shop-shelves) can be minimised if a sample of juice from a batch has been found to spoil under simulated conditions. This can be accomplished through the use of adequate refrigeration temperatures at the outlets (Eiroa *et al*., 1999). If the batch has not left the premises, it can be reworked or diverted to more robust products, for example UHT products (Pettipher *et al*., 1997).

The application of electronic noses such as the AromaScan as an analytical instrument is relatively simple and easy, as is the preparation of samples to be analysed by this instrument. In addition, the results are easily understandable. The prospects are reasonable and growth in the use of this technology can clearly be anticipated. It is hoped that this analytical instrument will soon be as readily accepted for use in the detection of microbes as techniques such as GC and sensory evaluation. If one considers that GC is used as an analytical control tool, it should be apparent that the electronic nose also has potential in the area. It took about 30 years for GC to reach the status that it enjoys for its routine use (Mielle, 1996). It is hoped that the value of electronic nose technology becomes apparent in a shorter period of time.

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APPENDICES

APPENDIX A – Preparation and standardisation of 1.000N sodium hydroxide

40.0978g of sodium hydroxide (NaOH) pellets was weighed and placed into a volumetric flask. The pellets were dissolved and the solution made up to the mark with distilled water. This NaOH solution was standardised in order to determine the actual normality (N). This was done by placing potassium hydrogen phthalate ($KHC_8H_4O_4$) in a 105°C oven for 1 hour, thereafter weighing out 4.0005g thereof into a 250ml Erlenmeyer flask to which 50ml of boiled and cooled distilled water was subsequently added. The flask was swirled to dissolve the $KHC_8H_4O_4$. Using a Pasteur pipette, 3 drops of phenolphthalein indicator were added to the mixture. The NaOH solution was then added to a burette and titrated against. The normality of the NaOH solution was calculated to be 0.980N using the following equation:

$$\text{Normality of NaOH} = \frac{\text{Weight of } KHC_8H_4O_4}{204.22 \times \text{NaOH titre volume}}$$

APPENDIX B – Preparation of *Bacillus acidoterrestris* Medium (BAM) (Silva *et al.*, 1999)

Three solutions, A (macronutrient solution), B (micronutrient solution) and C (bacteriological agar), were individually prepared by adding together the contents for each of the solutions described in Table B1 in separate 1L Schott® bottles. The pH of solution A was adjusted to 4.00 using 0.100N hydrochloric acid. The three solutions, along with a capped, empty 1L Schott® bottle were autoclaved at 121kPa for 15 minutes. Without allowing any of the solutions to cool down after autoclaving, 1ml of solution B was aseptically added to solution C and mixed by shaking. Due to its high temperature, transfer of the 1ml of solution B must be done using a sterile glass pipette and a pumpette. Solution B cannot be used again (Singh, 2001) and was discarded. Half of solution A was then poured into the empty sterile 1L Schott® bottle, followed by the addition of half of solution C to which the 1ml of solution B had been added. This mixture was then capped and shaken well. The remainder of solutions A and C was then added to this bottle and the

mixture was again shaken well. This process facilitates thorough mixing of the *Bacillus acidoterrestris* medium. The medium was allowed to cool to about 50°C, poured into 90 mm diameter petri dishes marked with BAM and the preparation date and allowed to set. Plates were inverted and placed in the refrigerator until use. During this procedure it was very important to be cautious whilst transferring solutions from one bottle into another as solutions were very hot. Oven gloves were used to handle the bottles.

Table B1: Preparation of solutions A, B and C used to make the *Bacillus acidoterrestris* Medium

Solution type	Chemical formula	Common name	Quantity
Solution A	$CaCl_2.2H_2O$	Calcium chloride dihydrate	0.25 g
	$MgSO_4.7H_2O$	Magnesium sulphate	0.50 g
	$(NH_4)_2SO_4$	Ammonium sulphate	0.20 g
	$C_6H_{12}O_6$	Glucose	5.00 g
	KH_2PO_4	Potassium phosphate	3.00 g
	-	Yeast extract	2.00 g
Make up solution A with 500ml distilled water			
Solution B	$ZnSO_4.7H_2O$	Zinc sulphate	0.10 g
	$MnCl_2.4H_2O$	Manganese chloride	0.03 g
	H_3BO_3	Boric acid	0.30 g
	$CoCl_2.6H_2O$	Cobalt chloride	0.20 g
	$CuCl_2.2H_2O$	Copper (II) chloride	0.01 g
	$NiCl_2$.6H2O	Nickel chloride	0.02 g
	$Na_2MoO_4.2H_2O$	Sodium molybdate	0.03 g
Make up solution B with 500ml distilled water			
Solution C	-	Bacteriological agar	15.00 g
Make up solution C with 500ml distilled water			

APPENDIX C – Preparation of solutions for Gram stain

Crystal violet solution

Crystal violet	0.50g
Distilled water	100ml

This stain solution was filtered before use. The stain can be kept indefinitely but should be filtered again before use.

Iodine Solution

Iodine	1.00g
Potassium iodide	2.00g
Distilled water	100ml

Safranin Solution

Safranin O	0.25g
Ethanol	10ml
Distilled water	100ml

The Safranin O was dissolved in the ethanol prior to addition of the water.

APPENDIX D – Preparation of solutions for spore stain

Malachite Green Solution

Malachite green	5.00g
Distilled water	100ml

Safranin O Solution

Safranin O	0.50g
Distilled water	100ml

APPENDIX E – Preparation of K-Medium (Walls and Chuyate, 1998)

KM was prepared by adding 1.25 g yeast extract, 2.50 g dehydrated peptone, 7.50 g bacteriological agar, 0.50 g glucose, 0.5 ml Tween 80 and 500 ml distilled water to a 1L Schott® bottle. A magnetic stirring bar was placed in the bottle and the bottle placed on a heated magnetic stirrer until all the ingredients had dissolved. The cap was loosely fitted over the mouth of the bottle and the medium autoclaved at 121 kPa for 20 minutes. While the media was being autoclaved, 1.25 g malic acid was added to 10 ml of distilled water in a small beaker. The solution was stirred until all the malic acid had dissolved. Once the medium had been autoclaved, the water-malic acid mixture was drawn into a sterile 10 ml plastic disposable syringe, a Cameo 25AS acetate disposable membrane filter steriliser with a pore size of 0.22 μ was aseptically attached to the spout of the plastic syringe. The water–malic acid combination was immediately added through the membrane filter steriliser into the freshly autoclaved medium. The bottle was swirled to facilitate mixing. When cooled to approximately 50°C, the acidified KM was poured into 90 mm diameter petri dishes. The plates were allowed to solidify in a refrigerator (≤5°C). Plates prepared in this way are said to last 60 days.

APPENDIX F – Rules for counting of plates (Swanson *et al.*, 1992)

Only those rules applicable to this study were sourced from the literature

General

Plates were promptly removed from the incubator. Colonies on selected plates containing between 25 to 250 colonies were counted. Colonies were counted with the aid of magnification under uniform and properly controlled artificial illumination.

Computing and reporting

To compute colony counts, the total number of colonies per plate were multiplied by the reciprocal of the dilution used. Counts thus established were reported as colony forming units (cfu) per ml.

The following rules were used to select plates and calculate the cfu per ml as applicable:

One plate with 25 to 250 colonies

Plates with 25 to 250 colonies were selected and counted, and the dilution used and the total number of colonies counted recorded.

Consecutive dilutions with 25 to 250 colonies

If plates from two consecutive decimal dilutions yield 25 to 250 colonies each, compute the count per g or ml for each dilution and report the arithmetic average as the CFU per g or ml, unless the higher computed count per g or ml is more than twice the lower one. In that case report the lower computed count as the cfu per ml.

Plates without colonies

If plates from all dilutions have no colonies, report the estimated count as less than (<) one times the corresponding lowest dilution. For example if the lowest dilution is 1:10, then the count will be reported as <10 estimate (est.) if none of the plates from any of the dilutions have colonies.

APPENDIX G – Minimum number of correct judgements to establish significance at various probability levels for the triangle test (Stone and Sidel, 1993)

Number of trials (n)	Probability levels						
	0.05	0.04	0.03	0.02	0.01	0.005	0.001
5	4	5	5	5	5	5	
6	5	5	5	5	6	6	
7	5	6	6	6	6	7	7
8	6	6	6	6	7	7	8
9	6	7	7	7	7	8	8
10	7	7	7	7	8	8	9
11	7	7	8	8	8	9	10
12	8	8	8	8	9	9	10
13	8	8	9	9	9	10	11
14	9	9	9	9	10	10	11
15	9	9	10	10	10	11	12
16	9	10	10	10	11	11	12
17	10	10	10	11	11	12	13
18	10	11	11	11	12	12	13
19	11	11	11	12	12	13	14
20	11	11	12	12	13	13	14
21	12	12	12	13	13	14	15
22	12	12	13	13	14	14	15
23	12	13	13	13	14	15	16
24	13	13	13	14	15	15	16
25	13	14	14	14	15	16	17
26	14	14	14	15	15	16	17
27	14	14	15	15	16	17	18
28	15	15	15	16	16	17	18
29	15	15	16	16	17	17	19
30	15	16	16	16	17	18	19
31	16	16	16	17	18	18	20
32	16	16	17	17	18	19	20
33	17	17	17	18	18	19	21
34	17	17	18	18	19	20	21
35	17	18	18	19	19	20	22
36	18	18	18	19	20	20	22
37	18	18	19	19	20	21	22
38	19	19	19	20	21	21	23
39	19	19	20	20	21	22	23
40	19	20	20	21	21	22	24
41	20	20	20	21	22	23	24
42	20	20	21	21	22	23	25
43	20	21	21	22	23	24	25
44	21	21	22	22	23	24	26
45	21	22	22	23	24	24	26
46	22	22	22	23	24	25	27
47	22	22	23	23	24	25	27
48	22	23	23	24	25	26	27
49	23	23	24	24	25	26	28
50	23	24	24	25	26	26	28
60	27	27	28	29	30	31	33
70	31	31	32	33	34	35	37
80	35	35	36	36	38	39	41
90	38	39	40	40	42	43	45
100	42	43	43	44	45	47	49

APPENDIX H: AromaScan software

1. The AromaScan A32S

Figure H1: The AromaScan A32S electronic nose and accompanying central processing unit. The A (of A32S) represents AromaScan while the 32S represents the 32 sensors present in the instrument.

2. Acquisition parameters

In order to start a sample analysis the icon labelled (a) is pressed and the window illustrated in figure H2 appears.

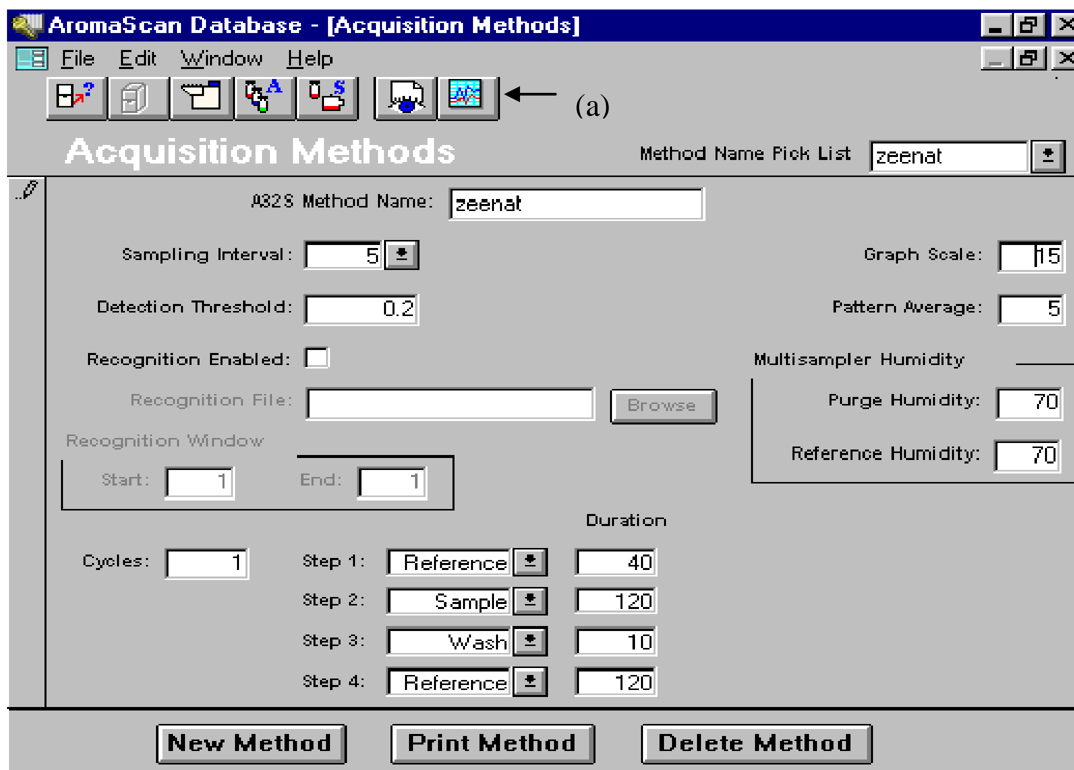

Figure H2: Window of data acquisition parameters of AromaScan illustrating values for sampling interval, humidities, number of cycles, referencing-sampling-washing-referencing steps. Other parameters are as per supplier instructions.

3. Complete profile for a single sample run (data acquisition) using the acquisition parameters indicated in figure H2 (Appendix 2)

In figure H3 a single completed sample analysis corresponding to the acquisition parameters in figure H2 is represented. It can also be seen that a period of 4 minutes and 50 seconds has elapsed (i.e. the entire duration of a single sample run). The 'Time' allows the user to keep track of how much of time has elapsed since the beginning of the analysis. The 'Next Event' registers the time at which switches from referencing, to sampling, to washing and back to referencing will occur and hence the relevant valves (taps) can be opened and closed accordingly. The sample identity can also be seen, in this case the 6th analysis of replicate 1 after 14 days incubation. The histogram in the same figure (bottom, right) represents the corresponding changes in the resistance of the sensors as represented by the line graph. All the sensors at that point are below the baseline as they should be at the end of an analysis.

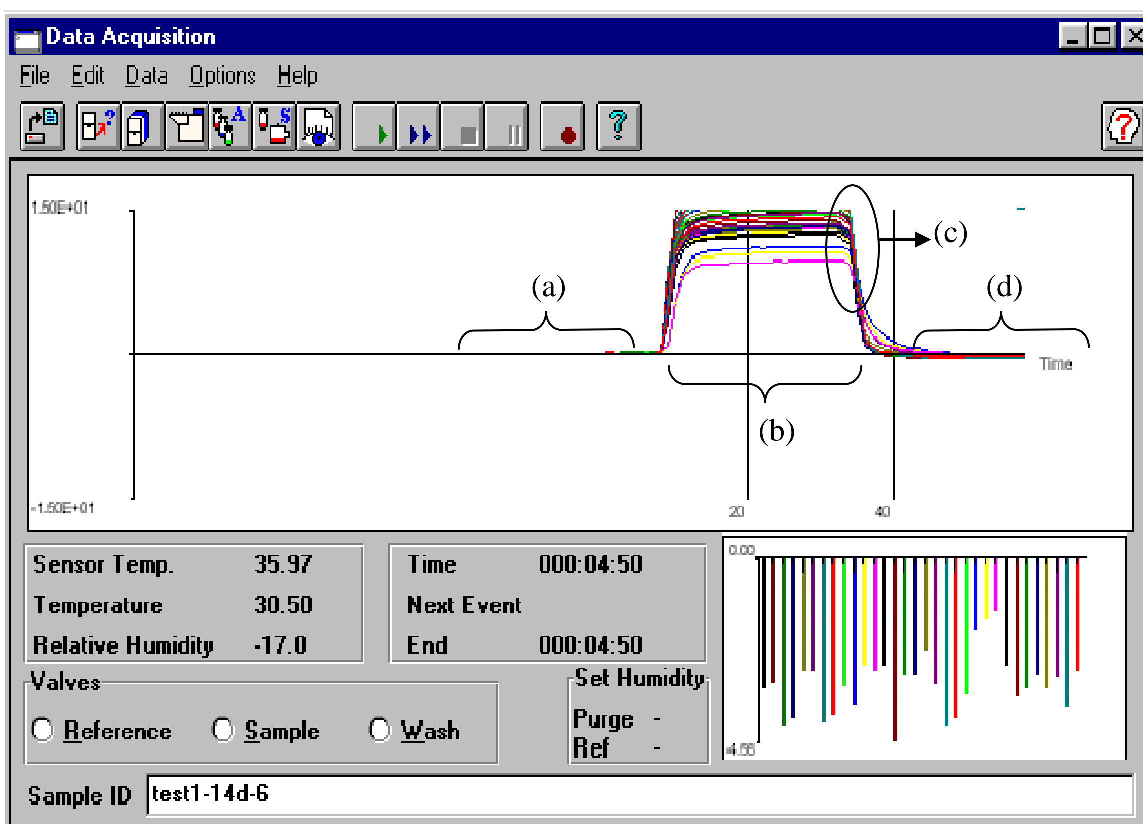

Figure H3: Single completed sample analysis (data aqusition). (a) and (d) Periods corresponding to the 40 seconds and 120 seconds referencing interval; (b) period corresponding to 120 seconds sampling interval; (c) period corresponding to 10 seconds washing interval.

4. First step of data reduction – selection of area of aroma profile corresponding to the sampling interval

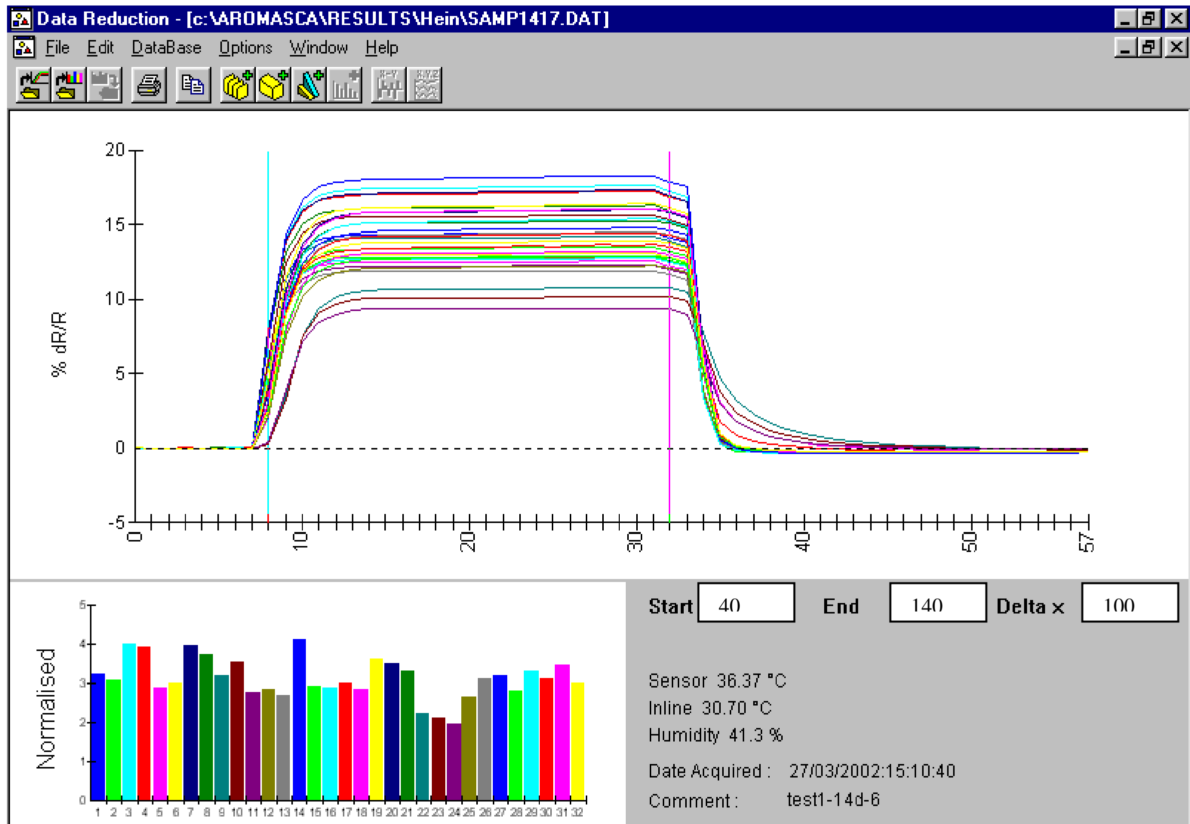

Figure H4: Data reduction by the selection of the 100 second sampling interval (start: 40 seconds; End: 140 seconds as represented by the blue and pink lines respectively) for the sixth run of replicate 1 at the 14 day interval. The histogram on the bottom left corner represents the normalised response for this sampling interval (all above the baseline).

5a. Comparison of grouped data in line graph format

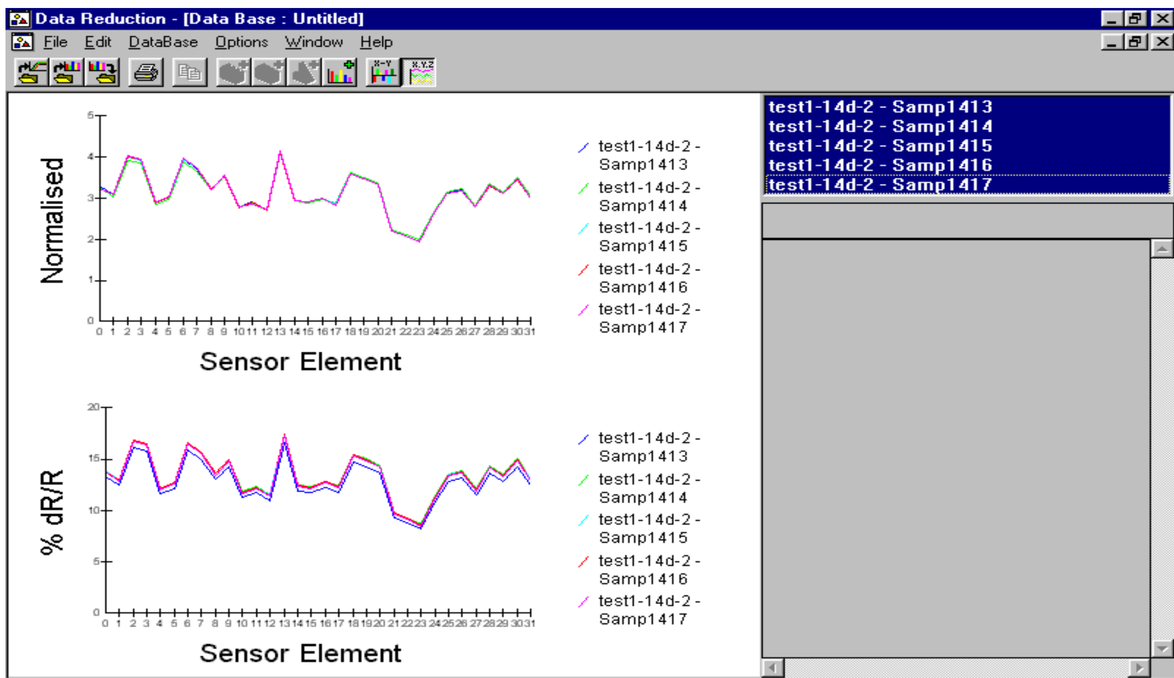

Figure H5a: Comparison of responses of 5 runs of replicate 1 at the 14 day interval in normalised and un-normalised (%dR/R) line graphs.

5b. Comparison of grouped data in histogram format

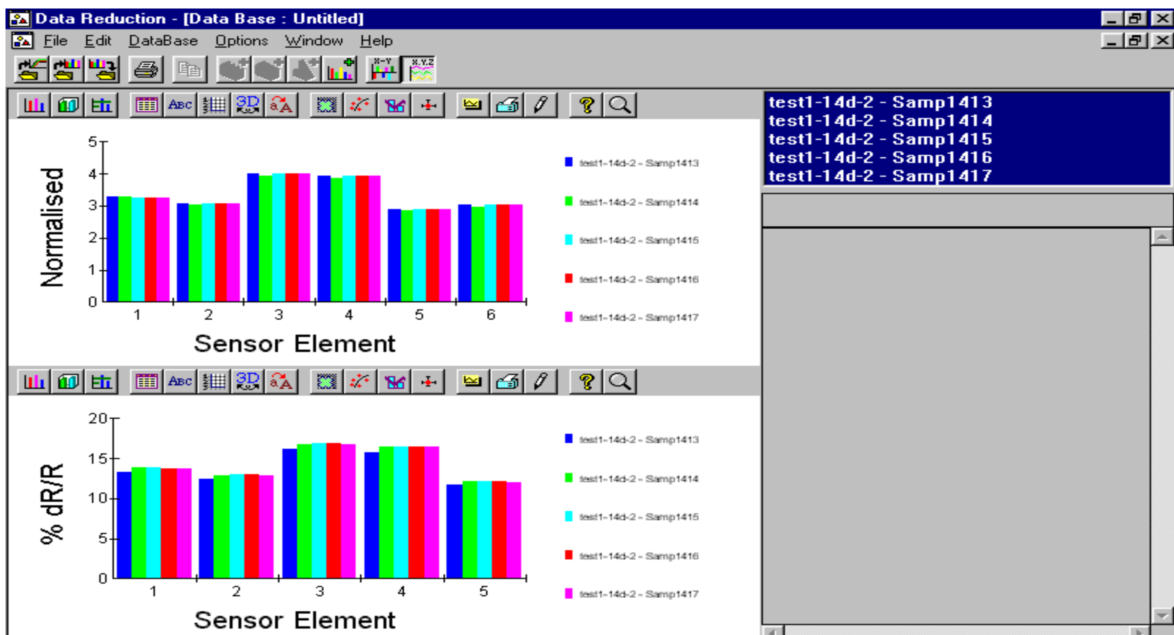

Figure H5b: Comparison of responses of 5 runs of replicate 1 at the 14 day interval in normalised and un-normalised (%dR/R) in bar graph format. For clarity only 5 sensor responses are shown.