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Production of pyrazine flavours by mycelial fungi

by

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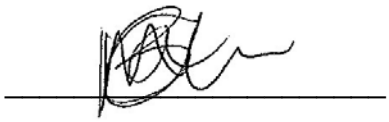
I, Aisha Bibi Mahomed Ali, declare that the thesis/dissertation, which I hereby submit for the degree, MSc Microbiology, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: 

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PREFACE

Pyrazines are heterocyclic nitrogen containing compounds responsible for many of the nutty, roasted and green tonalities in the flavour and fragrance industry. These aromas can be synthesized by various methods that include chemical, natural and microbial synthesis. Trends in the flavour and fragrance industry are, however, to move away from chemically synthesized compounds and more towards the synthesis of natural products. As South Africa is known to be rich in its fungal biodiversity, as well as the availability of a large number of locally isolated cultures in the CSIR and UP culture collections, it was decided to explore the possibility to utilise these fungi as a way to produce natural pyrazines. Further information regarding the uses of pyrazine compounds and their market value is presented in chapter 1. Information regarding the synthesis of pyrazines, including the biotechnological approaches through fermentation processes, is also provided and discussed. Submerged and solid substrate fermentation processes are compared, and their advantages and disadvantages discussed.

A sensory evaluation panel was set up and trained for the detection of pyrazine-like aromas. In total, 280 fungi were randomly selected from the CSIR and UP culture collections, with the only prerequisite that these isolates should represent fungi that were isolated from South African environments. The results obtained from the sensory evaluation study are presented in chapter 2. Two different media, including modified Czapeck Dox medium and tryptic soy broth, were evaluated for pyrazine production. This chapter shows the comparison of flavour production on the two media. It also shows the comparison of the flavours produced by the fungi in the different culture collections and which taxonomic fungal groups can be targeted to produce certain flavours.

From the initial screening test, fungi based on the aromas noted, as well as the market values of the possible pyrazines produced, were selected for analytical confirmation of pyrazine production as discussed in chapter 3. The purge and trap extraction method was employed for the extraction of pyrazines, and pyrazine production was confirmed using GC-TOFMS, as well as sniffing at the end of the GC column. Thereafter, the fungi, ***Penicillium rubrum*** (MRC1723) and ***Penicillium purpurogenum*** (MRC 181), were selected for further studies due to their ability to produce methoxypyrazines. These compounds are high impact aroma chemicals and have a high market value. For the extraction of these pyrazines from the fungal broth a liquid-liquid extraction method was developed and quantified using GC-TOFMS. Results from this study are presented in chapter 4.

Soy press cake, a by-product from the extraction of oils in a biodiesel project based at the CSIR, was evaluated as substrate for the growth and production of pyrazines in a solid state fermentation process by ***Penicillium rubrum*** and ***Penicillium purpurogenum***. These results are presented in the research note. A solid-liquid extraction method was developed together with the liquid-liquid extraction method in chapter 3.

A final summary is included that discuss the overall conclusions of the study and future work needed to develop a process for the production of methoxypyrazines by fungi. The ultimate challenge is to find a way of producing methoxypyrazines in a fermentation process that is commercially viable for the flavour and fragrance industry.

LIST OF ABBREVIATIONS

aHVP	acid Hydrolysed Vegetable Protein
CRMs	Certified Reference Materials
cm	centimetres
CO_2	Carbon dioxide
CIS	Cooled Injection System
CSIR	Council for Scientific and Industrial Research
Cz-Meduim	Czapek Dox Medium
DCM	Dichloromethane
DST	Department of Science and Technology
EI	Electron Ionisation
EFSA	European Food Safety Authority
eV	electron Volt
FABI	Forestry and Agricultural Biotechnology Institute
FAO	Food and Agriculture Organization
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
GC-MS-SIM	Gas Chromatography – Mass Spectrometry – Selected Ion Monitoring
GC-O	GC-Olfactometry
GC-TOFMS	Gas Chromatography-Time of Flight Mass Spectrometry
GMO	Genetically Modified Organisms
HCl	Hydrochloric Acid
HRGC-IRMS	High Resolution Gas Chromatography-Isotope Ratio Mass Spectrometric
hrs	hours
i.d.	inner diameter
JECFA	Joint FAO/WHO Expert Committee on Food Additives

kg	kilogram
kPa	kilopascal
m/z	mass:charge
MEA	malt extract agar
MIBP	2-methoxy-3-isobutylpyrazine
µm	micrometer
min	minute
MIPP	2-methoxy-3-5,6-isopropylpyrazine
mL	millilitre
mm	millimeter
MDGC	Multi-Dimensional capillary Gas Chromatography
NaCl	Sodium chloride
NIST	National Institute of Standards and Technology
nm	nanometer
O_2	Oxygen
^oC	degrees celcius
o.d.	outer diameter
PDA	potato dextrose agar
ppm	parts per million
QMC	Qualitative Monitoring Compound (2-methoxy-3-(1-methylpropyl)pyrazine)
rpm	revolutions per minute
RSD	Relative Standard Deviation
s or sec	seconds
SIM	Single Ion Monitoring
SmF	Submerged Fermentation
SPME	Solid Phase Micro-extraction
SPI	Soy Protein Isolate
sp.	Single species
spp.	More than one species
SSF	Solid Substrate Fermentation

TDS	Thermal Desorption System
TSB	Tryptic Soy Broth
µA	micro Amperes
µg	micrograms
µL	microlitres
UP	University of Pretoria
USA	United States of America
VVM	Volume per Volume per Minute (gas volume flow per unit of liquid volume per minute)
w/v	weight per volume
WHO	World Health Organisation

CHAPTER 1

Pyrazine production in the flavour industry: A Review

1.1. INTRODUCTION

Throughout history, humans have been fascinated by the food they eat and are always looking for new ways to make this prominent activity in their lives more appealing and appetizing. Historically, humans first used herbs and spices as flavourants. Later, with the advent of organic chemistry, people have started to use chemically synthesized compounds with enhanced and unusual characteristics. However, in recent years, through the study of microbiology, biochemistry and genetics, man started to apply fermentation technology for the production of flavours, fragrances and colour ingredients (Manley, **1994)**. With the increased production of processed foods, additives such as flavours have become crucial to especially the fast food industries. It is estimated that approximately 80% of the flavours and fragrances produced globally are chemically synthesized (Krings & Berger, **1998**). However, the trend today is that consumers are becoming increasingly aware of their health and attempt to be more conscious regarding their health and nutrition. There has thus been a major shift in the way consumers are buying food and, therefore, pressure is placed on food companies to produce food products that can be labeled as natural (Krings & Berger, **1998**). In addition, people with different religious beliefs are also aware of the food that they consume. Therefore, there is also a great demand that products should have the halaal and kosher status (Manley, **1994**; Chaudry & Regenstein, **1994**).

Natural flavours and fragrances that comply with the demands of consumers are mostly obtained from plants. Companies involved in this industry also have to take special care that the extraction and formulation methods used should be regarded as natural. However, this is not always economically viable as companies are affected by the availability of plant material, which is sometimes out of season or natural disasters that influence harvests. An alternative method that is not yet fully exploited for the production of aroma compounds is microbial biotechnology. Through the use of biotechnology, microorganisms can be

utilized to produce these compounds from natural substrates, using biochemical pathways found in nature (Aguedo ***et al.***, **2004**).

Aromas include various classes of chemical molecules. Acids, alcohols, lactones, esters, ketones, pyrazines and terpenoids are the chemical groups of most importance to the flavour industry (Manley, **1990**). The flavour wheel (Figure 1) is an indication of the various kinds of aromas that are used in the flavour industry, but similar compounds can have diverse aromatic characteristics. Pyrazines, for example, are mainly grouped into the nutty; caramel catergories but can also be found in burnt, roasted, beefy, pork, lamb, chicken, savoury bouillon, mushroom, earthy and vegetable sections of the flavour wheel (Rowe, **2002**).

1.1.1. Pyrazines

Pyrazines are aromatic heterocyclic nitrogen containing compounds (Figure 2) that are important flavouring agents in many raw and roasted food products. The main pyrazine compounds responsible for these flavours are alkylated and methoxylated pyrazines as they have strong odorous properties. Pyrazines produce a wide variety of flavours that can be synthesized by chemical methods or by certain microorganisms. They can also be found naturally in various food types such as coffee beans, cocoa beans, nuts and vegetables (Maga, **1982**).

Pyrazines are also found to be produced by a wide variety of insects and play a role as pheromones, which function as deterrents or attractants (Rizzi, **1988**; Woolfson & Rothschild, **1990**). For example, certain fungi mimic flowers to attract insects that serve as vectors for fungal dispersal (Ngugi & Scherm, **2006**). Another example is that the pyrazine, 2,5-bis-1- (methyl)pyrazine is an attractant of ***Carpophilus*** beetles to oranges, and is believed to be of microbial origin (Schulz, Fuhlendorff & Reichenbach, **2004**). In addition, insects such as the ladybug beetle in North America even secrete pyrazines as defense pheromones (Cudjoe, Wiederkehr & Brindle, **2005**).

Due to the aromatic properties of pyrazines, they have many uses in the flavour and fragrance industry. Pyrazines are commonly used in the food industry as flavour compounds. For example 2,3-dimethylpyrazine, which naturally occurs in asparagus, potato, peanuts and coffee, is used in gravies, beverages and sweets. The compound, 2,5-dimethylpyrazine, is used in breakfast cereals and 2,6-dimethylpyrazine is used for its ability to produce the coffee, cocoa, meat or potato flavours (Endredi, Billes & Keresztury**, 2004**). It is often found that one pyrazine compound can have different odour characteristics depending on the dilution used. In the fragrance industry, the addition of an alkoxy-alkylpyrazine, such as 2-methoxy-3-butylpyrazine, can be used as an ingredient in various perfumes. It can also be used to enhance the odour of cosmetics and toiletries (Bramwell ***et al.***, **1975**).

Also, due to the demand from consumers for convenient foods, microwave cooking has become an increasingly popular method of preparing meals. However, microwave temperatures only reach 100^{o}C, preventing the development of the typical roasty, savoury flavours in meat that is achieved in oven roasting at temperatures above 200^{o}C. Therefore, the savoury flavours of foods are decreased and hence the addition of pyrazines to microwave foods is seen as a possible solution (Rowe, **1998**).

Pyrazines are not only used as flavouring agents, but have various other uses in pesticides, insecticides, dyes and pharmaceutical compounds. For example, aspergillic acid, phenazine and benzopyrazine are used as antimicrobials (Girija, **2002**). Some soil microorganisms produce pyrazines that have fungistatic properties (Chuankun ***et al***., **2004**). Pyrazinamide and 2-allylthiopyrazine is used in pharmaceutical compounds to treat tuberculosis and as a chemoprotective agent respectively (Girija, **2002**). A United States patent (Bakthavatchalam, Wilde & Gilligan, **2003**) claimed that pyrazines can be used for the treatment of neurological disorders. Pyrazines have also been used in bleaches (Busch ***et al***.,

2002). Pulcherriminic acid, an antimicrobial, and Folic acid, a source of Vitamin B_{10}, are also important pyrazines (Girija, **2002**).

In 1994, Manley reported that there were approximately 1500 chemically synthesized compounds used in the flavour industry in the USA. Of those, only about 20 flavours were commercially produced through means of fermentation processes, and it is possible that the numbers have not increased significantly since then. This is evidence that the full potential of using microorganisms as producers of flavours and fragrances has not yet been realised. In the past, most studies have focussed on bacteria as sources of pyrazine production, although fungi also have this ability (Seitz, **1994**)**.** This apparent lack of data on fungi does not necessarily mean that fungi should not be seen as good candidates for pyrazine production, but rather that there is a need to investigate their abilities. One of the benefits of using fungi as sources of flavour compounds is that these organisms tend to produce volatiles that are closest to those of plants (Krings & Berger, **1998**).

South Africa is known for its unique plant and animal life and the land is represented by various biomes and centers of diversity. Included is the Cape Floristic Region that is geographically the smallest yet most diverse biome known. Associated with these regions are a unique mycoflora (Crous ***et al.***, **2006**) that resembles a unique diversity in its ability to produce flavours. Crous ***et al.*** **(2006)** estimated that there could be as many as 171 500 fungal species in South Africa and further indicated that a vast amount of South Africa's fungal content is still unknown.

1.2. MARKET VALUE OF PYRAZINES

An IAL consultants study was done in 2002 describing the flavours and fragrance market globally. At that stage, the global market was estimated at US$ 11 billion in 2001 with flavours accounting for 49.6% of the market at US$ 5,5 billion. The expectation was that the market would grow by 3.3% per annum up to 2006 (IAL

consultants, **2002**). According to a later report by the IAL Consultants in 2007, the global flavour and fragrance market value increased to US$ 12.6 billion in 2006 and is expected to grow at a rate of 3.5% per annum (Gristwood, **2007**). The beverages sector was the largest global end-use market for flavours in 2001, with trends for gourmet hot beverages, such as luxury-flavoured coffees, in North America. Flavour companies such as Symrise also state that flavoured coffee and coffee-based beverages are a thriving market in the beverages category of the company (Symrise, **2008**). In the savoury and convenience food sector the highest annual growth rates were forecasted at 4.4% p.a. and in the food sector at 4.2% p.a. This supports the notion that pyrazines are seen as important flavouring agents due to their ability to strongly resemble these flavours.

The total market for flavours in specific parts of the world, such as in Western and Eastern Europe, was US$ 1,358.3 and US$ 261.0 million respectively, and that of the USA was US$ 2,051.1 million in 2001 (IAL Consultants, **2002**). The total annual pyrazine production in Europe was estimated at 2700 kg and in the USA at 2100 kg. According to a Joint FAO/WHO Expert Committee on Food Additives (JECFA) report in 2004, a total of 41 pyrazines were regarded as toxicologically safe for use in the flavour industry (Mattia**,** Renwick and Sipes, **2004**). In 2008 the European Food Safety Authority (EFSA) reevaluated 18 pyrazines, representative of the 41 pyrazines evaluated by JECFA and concluded that 40 out of the 41 pyrazines tested showed no safety concerns (EFSA, **2008**).

The ten most abundantly produced pyrazines in Europe and the USA are described in table 1 (Mattia ***et al***., **2004**). Information regarding the natural occurrence of pyrazines in food is also given in this table. Mattia ***et al.*** (**2004**) reported that pyrazines such as 2,3,5-trimethylpyrazine, 2-ethyl-3-methylpyrazine and 2-ethyl-3,(5,6)-dimethylpyrazine make up approximately 64% of the total annual production in Europe with the production of each pyrazine being 840 kg, 590 kg and 310 kg respectively. These authors also reported that 66% of

pyrazines used in the USA comprised of mainly 2-acetylpyrazine (920 kg), 2,3,5-trimethylpyrazine (350 kg) and 2,3,5,6-tetramethylpyrazine (140 kg).

A market survey indicated that companies selling pyrazines include Advanced Biotech, Sigma-Aldrich, Shanghai M&U International Trade co, Frutarom, Fleurchem, Pyrazine Specialities, Axxence and Givaudan. The selling prices of pyrazines can differ significantly. An example is the prices of pyrazines that are sold by the companies, Advanced Biotech, Sigma-Aldrich and Shanghai M&U International Trade co (see table 2). The table gives an indication of the value of the pyrazines, with 2-methoxy-3-isobutylpyrazine costing as much as $ 4700.00.kg^{-1}. There is also a substantial difference in the selling price of natural pyrazines compared to their synthetic counterparts. For example, the synthetically produced product of 2,5-dimethylpyrazine is sold at $ 200.00.kg^{-1}, whereas the natural pyrazine is sold at $ 3500.00.kg^{-1}. Amongst the pyrazines sold by these companies only 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,5-dimethylpyrazine and 2,6-dimethylpyrazine and the compound pyrazine had the natural status. There is thus ample opportunity to further expand the list of available natural pyrazines should sources for these compounds be found.

1.3. SYNTHESIS OF PYRAZINES

Depending on the substrates used during manufacturing, different pyrazines can be produced. This can be accomplished through processes such as chemical production, extraction from natural foods such as vegetables, or through microbial fermentation processes.

1.3.1. Chemical Synthesis of Pyrazines

The most generally used method of synthesizing 2,5-disubstituted and 2,3,5,6-tetrasubstituted pyrazines is by the self condensation of α-(primary amino) carbonyl compounds to form dihydropyrazines, which are then oxidized with mild oxidizing agents such as air, hydrogen peroxide, cupric or ferric irons or aqueous

nitric acid as indicated in figure 3 (Barlin, **1982**). They can also be synthesized by the reduction of α-hydroxyamino carbonyl compounds (Figure 4).

Other chemical methods include the oxidation of α-amino alcohols and reduction of α-amino acids. The oxidation of α-amino alcohols takes place by dehydrogenation of the amino aldehyde, cyclization of the hydropyrazine and oxidation of the pyrazine (Barlin, **1982**). An example of the process involving the reduction of α-amino acids is indicated in figure 5. The synthesis of 2,5-dimethylpyrazine can be accomplished in the same manner as that for the chemical compound, pyrazine, but the amino acid alanine is used instead of glycine.

The hydrolysis of 3-imidazolines also yields pyrazines as products. Pyrazines can also be produced through the heating of 1,2-dicarbonyl compounds with α-amino acids through Strecker degradation and Maillard reactions. The production of many alkyl and arylpyrazines occurs through the process as indicated in figure 6 (Barlin, **1982**).

An example of forming pyrazines through the Maillard reaction is heat-treating potato juice extracts (some treated with proteases) through autoclaving or oven heating to form a concentrated liquid or oven dried powder. Heating resulted in alkyl pyrazines that have a peppery flavour; α-acetylpyrazines with a coffee flavour and piperazinediones that has a deep bitter taste (Davids, Yaylayan & Turcotte, **2003**).

1.3.2. Natural synthesis of pyrazines

Most alkyl pyrazines found in food result from the condensation of aldehydes, such as pyruvalaldehyde with an amino acid. This product then undergoes Strecker degradation that results in amino reductones and, by additional steps

that involve self-condensation and oxidation, dimethylpyrazines are formed (Figure 7) (Maga, **1982**).

Acetylpyrazines can be produced by the condensation of amino acids, glyoxal and other browning reaction products. It has also been proposed that methoxypyrazines have biogenic origins, as they are associated with raw foods (Maga, **1982**). Methoxypyrazines occur in trace quantities, but are extremely important to the flavour industry because of their low sensory threshold (Rizzi, **1988**; Rowe, **2002**). Condensation of amino acids and 1,2-dicarbonyls, and thereafter methylation, leads to the production of methoxypyrazines. In peas, for example, the syntheses of different methoxypyrazines depend on different precursors (Beck, Hansen & Lauritsen**, 2003**). Some examples include the following:

Glyoxal/glyoxylic acid + valine → 2-methoxy -3-isopropylpyrazine
Glyoxal/glyoxylic acid + leucine → 2-methoxy-3-isobutylpyrazine
Glyoxal/glyoxylic acid + isoleucine → 2-methoxy-3-sec-butylpyrazine

In other naturally occurring food, pyruvic aldehyde and pyruvate are the most likely 1,2-dicarbonyl precursors in methoxypyrazines (Maga, **1982**).

Understanding the secondary metabolic pathways that lead to flavour production in plants has allowed for the use of plant cell cultures to improve flavour production. These include different types of cell culture, callus, cell suspensions and immobilized cell cultures. Capsaisin from ***Capsicum*** cells, vanillin from cell suspension cultures of ***Vanilla planifolia,*** the enzyme allinase used for the production of onion flavour from onion cell cultures, have been successfully produced using plant cell culture technologies. Other complex flavours such as the mint and strawberry flavours are more difficult to produce using plant cell cultures where, either the yields were low compared to the parent plant, or not all components of the flavour were produced. However the availability of such tissue culture systems can allow for the improvement of quality and yield of flavour compounds through genetic manipulation (Rhodes ***et al.***, **1992**).

1.3.3. Microbial Synthesis of Pyrazines

The production of pyrazines through the extraction from plants and chemical synthesis can sometimes have drawbacks. For example, most natural aromas are derived from plants in which the compound either occurs in minute quantities or could be difficult to purify. In such cases, a huge amount of plant material is required for the extraction of the aroma compounds. Other problems associated with the extraction from plants is that the quality and availability of the flavour compound may vary. This is due to seasonal variation and climatic factors (Manley, **1994**). Drawbacks associated with the chemical synthesis of pyrazines, apart from the fact that they would not be labeled as natural, is that some processes are not necessarily feasible on an industrial scale. Also in certain processes the final product can be contaminated with other impurities (Kiener, Gameren & Bokel, **1993**).

The use of microorganisms for the production of pyrazines could thus be considered as an attractive alternative with several advantages. Not only do they yield products that could be regarded as natural, but, through controlled fermentation processes, the quality and quantity of the product is uniform. This is achieved by eliminating the influence of external factors such as environmental fluctuations and weather patterns such as those found during the production of agricultural products. In addition, higher yields can be obtained from readily available substrates (Manley, **1994**) and can be specifically formulated for optimal pyrazine production. Another benefit is that microorganisms can easily be genetically or phenotypically manipulated to increase the yields of product production. Alternatively, enzymes such as hydroxylases, produced by certain microorganisms, can be used to catalyze reactions yielding pyrazine products (Peterson & Kiener, **1999**).

Several microorganisms are involved in the synthesis of pyrazines (Table 3). An example is the production of 2.5- dimethylpyrazine and tetramethylpyrazine by ***Bacillus subtilis.*** This is accomplished by using a Solid Substrate Fermentation

process with ground soybeans as the main substrate and L-threonine and acetoin are added to increase the yields (figure 8) (Larroche, Besson & Gros**, 1999**). Studies have shown that pyrazine synthesis normally occurs during the stationary phase. It corresponds to the autolytic phase in ***Bacillus,*** which results in alkalisation of the medium and thus pyrazine synthesis. ***Bacillus*** is also sensitive to aeration rates and for optimal pyrazine production, aeration rates need to be carefully controlled (Larroche ***et al.,*** **1999**). In this study, an optimum aeration rate of 0.005 VVM was noted for pyrazine synthesis. This corresponded to a high oxygen limitation in the bioreactor.

Other bacterial organisms producing pyrazines include ***Pseudomonas perolens*** and ***Pseudomonas taerolens***. ***Pseudomonas perolens*** has the ability to produce 2-methoxy-3-isopropylpyrazine (MIPP) and is synthesized as indicated in figure 9. This bacterium grows well on a medium containing pyruvate as the sole carbon source, as it is a distant precursor of MIPP. Cheng ***et al.*** (**1991**) stated that the formation of MIPP is an enzyme catalysed reaction and not a chemical one. However, the enzymes responsible are not mentioned in this paper.

Synthesis of pyrazines from ***P***. ***taerolens*** occurs in the late exponential phase. The pyrazines produced by this organism are indicated in table 3. Valine is a precursor of pyrazines produced by this organism. L-leucine and 2-keto-3-methylbutanoic acid are ideal substrates for pyrazine synthesis as 2-keto-3-methylbutanoic acid is a precursor of leucine and valine. Once leucine is formed in excess, feedback inhibition occurs and thereafter only valine can be produced that then leads to the production of pyrazines (Gallois, Kergomad & Adda, **1998**).

Although the production of pyrazines by these bacteria can be of value to the flavour industries, pyrazines can also be regarded as environmental pollutants and as one of the major malodorous classes of compounds in the exhaust gas stream of various food industries (Girija, **2002**; Rappert ***et al.***, **2006**). Removal of the pollutant can be achieved by certain ***Pseudomonas*** species, where

oxygenases that are present may cleave pyrazine rings. Bacteria such as ***Psuedomonas aeruginosa*** can be used in the biodegradation of industrial wastes containing these pyrazines (Girija, **2002**). This results in free amino acids to be metabolised via a number of pathways (Cheng ***et al.***, **1991**). Another microorganism involved in the breakdown of pyrazines is ***Mycobacterium*** sp., where pyrazines are broken down in the presence of oxygen to form ammonia (Rappert ***et al.***, **2006**).

Although bacteria are the more prominent producers of pyrazines, fungi are also able to synthesize these compounds. These include mainly fungi from the genera, ***Aspergillus*** and ***Penicillium***. The fungi usually produce pyrazines during the fermentation of food products. ***Aspergillus sojae*** and ***Aspergillus oryzae*** produce tetramethylpyrazine during the production of soya sauce and sweet wine (Rizzi, **1988**). ***Aspergillus flavus*** produced 2-hydroxy-3-isobutyl-6-sec-butylpyrazine-1-oxide (Aspergillic acid) and 2-hydroxy-3-isobutyl-6-(1-hydroxy-1-methylpropyl)-pyrazine-1-oxide (hydroaspergillic acid) in the presence of a high amino acid and low sugar content (MacDonald, **1973**). Similarly the pyrazine, 2-hydroxy-3,6-disec-butylpyrazine (deoxyaspergillic acid), was produced by ***Aspergillus parasiticus*** in media that was rich in protein but low in carbohydrates (Buchanan & Houston, **1982**). Amongst the ***Penicillium*** species, ***Penicillium vulpinum*** (Larsen & Frisvad, **1994**), as well as other ***Penicillium*** species found naturally in different types of cheese, are known to produce pyrazine compounds (Karahadian, Josephson & Lindsay, **1985**; Suriyaphan ***et al***., **2001**). The fungal phytopathogen, ***Septoria nodorum,*** is also a pyrazine producer (Gallois & Grimont**, 1985**; Rizzi**, 1988**) (see table 3).

Pyrazines are not the only flavour compounds produced by microorganisms. Various other chemical compounds such as terpenes, that have sweet, rose-like aromas, are commonly produced by soil bacteria and fungi such as ***Ceratocystis*** species (Kempler, **1983**; Krings & Berger, **1998**). Vanillin is one of the most important flavour compounds and is produced by the bioconversion of ferulic

acid, phenolic stilbenes, isoeugenol or eugenol. The main vanillin producing strain is a ***Amycolaptopsis*** sp. where it produces as much as 12 $g.L^{-1}$ vanillin in a fed batch process (Schrader ***et al***., **2004**). Vanillin can also be produced from various basidiomycetous fungi during the degradation of lignin (Lomascolo ***et al.***, **1999**). Lactones are usually associated with peachy, coconut, buttery, sweet or nutty aromas (Kempler, **1983**) and the highest producer of these compounds is ***Yarrowia lypolitica*** (Schrader ***et al***., **2004**). Esters are produced by microorganisms through the reaction of organic acids and ethanol, and produce fruity aromas such as pear, pineapple, strawberry, muskmelon and apple (Kempler, **1983**). Amongst the basidiomycetous fungi, ***Ischnoderma benzoinum***, as well as certain bacteria, can produce benzaldehyde, a compound used in cherry and other natural fruit flavours (Krings & Berger, **1998**; Lomascolo ***et al.***, **1999**). Other flavour compounds produced by microorganisms include 2-phenylethanol (a rose-like odour) (Schrader ***et al***., **2004**) and diacetyl (buttery flavour) (Kempler, **1983**).

1.4. FACTORS AFFECTING THE PRODUCTION OF PYRAZINES

1.4.1. Media Composition

Pathways involved in the production of pyrazines indicate that amino acids and sugars are precursors of pyrazine compounds (Beck ***et al.***, **2003**), where different precursors will lead to the formation of different pyrazine compounds (Table 4). The amino acid, valine, is a known precursor of 3-isopropyl-2-methoxypyrazine (Gallois ***et al.***, **1998**; Cheng ***et al.***, **1991**). It can also lead to the formation of other pyrazines such as isobutylpyrazines, sec-butylpyrazines, methylpyrazines and 2-methyl propylpyrazines (Coleman & Steichen, **2006**; Schulz ***et al***., **2004**). The pyrazine, 2,5-dimethylpyrazine, is formed when L-threonine is present in media during the fermentation with ***Bacillus subtilis*** (Besson ***et al***., **1997**). The sugars used as precursors for pyrazine compounds include acetoin. When reacted with ammonia, it forms tetramethylpyrazine (Besson ***et al.***, **1997**) and pyruvate that acts as a precursor to dihydropyrazines, dimethylpyrazines and

tetrahydropyrazines (Kurniadi ***et al.***, **2003**). It also includes acyloin, that, when it reacts with ammonia, it is a precursor to various alkylpyrazines (Rizzi, **1988**). Another example is rhamnose that also leads to the formation of several pyrazines (Coleman & Steichen, **2006**).

Not only will the type of substrate present have an effect on the type of odour produced, but it also will have an effect on the yields of the pyrazines (Table 5) (Gallois ***et al.***, **1998**). The pyrazine, 3-isopropyl-2-methoxypyrazine commonly produces a green pepper/green pea odour. According to a study by Gallois ***et al.*** (**1998**), this odour is noted when ***P. taetrolens*** is grown with glycerol, β-alanine, DL-serine, L-valine or L-leucine as substrates and, additionally, a hazelnut odour is noted when DL-valine is used as a substrate. It is also noted that the highest yields (10 000 $\mu g.L^{-1}$) of 3-isopropyl-2-methoxypyrazine was obtained with the addition of L-leucine to the growth medium. Even though other subtrates can produce the same green pea odour characterstic of this pyrazine, the use of β-alanine only produced 10 $\mu g.L^{-1}$ of 3-isopropyl-2-methoxypyrazine.

It was also noted that the concentration of the substrate affects the yields of the pyrazines produced (Table 6). For example, ***Pseudomonas perolens*** growing on pyruvate as a substrate at a concentration of 0.5% w/v produces 5150 $ng.mL^{-1}$ 2-methoxy-3-isopropylpyrazine and 51 $ng.mL^{-1}$ 2-methoxy-3-secbutylpyrazine. When the concentration of pyruvate is increased to 1% w/v, the yields of each pyrazine increase approximately twofold. However, when the concentration of pyruvate increases to 2% w/v, the yields of each pyrazine decreases (Table 6). The concentration of the substrate is, therefore, important in obtaining optimal yields.

The ratio between the sugar and nitrogen content in the media also has an effect on pyrazine production. For example, ***Aspergillus*** species produce pyrazines in media with a high amino acid content and low carbohydrates, where amino acids serve as precursors of pyrazines. This is important as toxic substances like

aflatoxins are produced in media that are high in carbohydrates and low in amino acids, where sugars form acetate that is the precursor of toxins. In such cases, pyrazines are only produced after aflatoxin production when the carbohydrate source has been depleted (Buchanan & Houston, **1982**; MacDonald, **1973**). Similarly, the sugar:nitrogen content also has an effect on the type of pyrazines produced where low sugar to nitrogen ratios results in the yield of 3-methylbutylpyrazines being higher than 2-methylpropylpyrazines and high sugar to nitrogen ratios results in the yield of 3-methylbutylpyrazines being lower than 2-methylpropylpyrazines (Coleman & Steichen, **2006**).

1.4.2. Temperature

Pyrazines are produced using high temperatures in chemical reactions. The production of high yields of pyrazines from rhamnose, for example, occur at temperatures between 105 and 125^{o}C. However, at a temperature of 90^{o}C a decrease in yield is experienced (Coleman & Steichen, **2006**). Microorganisms, however, produce pyrazines at much lower temperatures. In a fermentation process by ***Bacillus subtilis***, pyrazines were produced at temperatures between 20^{o}C and 27^{o}C. At 20^{o}C lower yields of 2,5-dimethylpyrazine and higher yields of tetramethylpyrazines and other pyrazines were obtained as compared to yields obtained at 27^{o}C. The total pyrazine yield was also higher at 20^{o}C. When temperatures reached above 30^{o}C, pyrazine synthesis was inhibited (Besson ***et al.***, **1997**).

1.4.3. Fermentation time

During the fermentation of cocoa beans a fermentation time of 5 days was shown to be appropriate (Ikrawan, Chaiseri & Vungdeetham, **1997**). Longer fermentation times led to a decrease in pyrazine yields and produced cocoa beans with a weak cocoa aroma. This is due to low amounts of total amino acids present, as well as an absence of glucose. Longer fermentation times may also lead to the breakdown of the produced pyrazine compounds, which ultimately result in the compounds to be used for growth by the microorganisms.

1.4.4. pH

In the Maillard reaction a basic pH favours the production of pyrazines. The effect of pH on pyrazine production in different model systems was evaluated (Bemis-Young, Huang & Bernhard, **1993**). In glucose-glycine model systems, a total of 19 pyrazines were produced in this study where the majority were formed in neutral to very basic pH levels (pH 7-12), where at pH 9.00 and pH 9.64 the greatest variety of pyrazines were formed. The number of pyrazines produced at an acidic pH between 1.00 and 2.34 was significantly less than the number produces at a basic pH. The effect of pH on pyrazine production in meat-related model systems occurred at pH's above 5.00. The aroma of the model systems were also affected by different pH values where a caramel odour was noted at pH 4.5, which later changed to a nutty and roasted aroma as the pH increased (Meynier & Mottram **1995**).

1.4.5. Fermentation media type

Another factor affecting the odour of pyrazines is the type of the medium used e.g. broth or agar. In a study by Gallois & Grimont (**1985**), ***Cedecea davisae***, which served as a control, showed no pyrazine odour when grown on tryptic soy agar, but produced a meaty, slightly nutty odour when grown in tryptic soy broth. ***Serratia*** sp. also produced slightly different odours when grown in Tryptic Soy Broth and on Tryptic Soy Agar (Gallois & Grimont, **1985**).

1.4.6. Use of genetically modified organisms

The use of mutant organisms for the production of pyrazines can also have an influence on the yields of pyrazines obtained. High yields of tetramethylpyrazine have been obtained by mutant strains of ***B. subtilis*** and ***Corynebacterium glutamicum***. Mutations of these organisms were made with the chemical compound nitrosguanidine. This caused a block in the isoleucine-valine pathway of ***C. glutamicum*** that led to a total production of 3 $g.L^{-1}$ tetramethylpyrazine after 5 days (Demain, Jackson & Trenner, **1967**). The ***Bacillus*** mutant produced as much as 4.33 $g.L^{-1}$ of tetramethylpyraznine (Xiao ***et al***., **2006**). A non-emutant

strain of ***Bacillus*** produced only 2.5 $g.L^{-1}$ tetramethylpyrazine compared to the mutant strains (Besson ***et al.***, **1997**).

1.5. PRODUCTION OF PYRAZINES ON AN INDUSTRIAL SCALE

The first step to develop a process to produce pyrazines is to find a suitable organism. Once its ability, including the conditions under which the organism will optimally produce pyrazines, has been established, upscaling of the process can be considered. Industrial scale processes can either include submerged (SmF) or solid substrate fermentation (SSF). The latter is done by growing the microorganism on a solid substrate, mainly organic material, in the absence of free water (Viccini ***et al.*** **2001**; Pérez-Guerra ***et al***., **2003**; Krishna, **2005**; Mazutti, ***et al***., **2006**). It has various practical and economical advantages over SmF. Substrates used are usually agricultural products and by-products. In addition, the fact that less water is used through SSF means that less expensive downstream processing is needed and high product yields can be achieved (Krishna, **2005**; Mazutti, ***et al***., **2006**). Microorganisms such as fungi are ideally adapted to grow on solid substrates. This is due to their ability to produce hyphae that anchor themselves to a solid surface where colonization takes place. Fungi are also especially adapted to grow in extreme environments where low water activities are experienced. These organisms, therefore, can easily colonize solid substrates and utilise the nutrients from these sources (Pérez-Guerra ***et al***., **2003**).

On the other hand, there are also several disadvantages associated with SSF. These include difficulties in the way solid material can be handled, especially where agitation and aeration, heat build-up and control of moisture levels are of concern (Krishna, **2005**). The growth of microorganisms during an SSF process leads to a change in different parameters that can have an effect on growth of microorganisms and product formation. Temperature is one of the parameters that is affected during the fermentation and usually leads to heat build-up caused by the metabolic activities of microorganisms and a slow heat removal. Heat

build-up is also as a result of the low thermal conductivity of substrates and low heat transfer rate from the particle surface to the gas phase (Manpreet ***et al.***, **2005**; Krishna, **2005**).

Another factor that is important during a solid-state fermentation is the control of moisture levels. Fungi generally grow at moisture levels between 20-70% and it is important that optimum moisture levels be maintained. A low moisture content leads to a reduction in the distribution of nutrients, microbial growth, enzyme stability and substrate swelling (Krishna, **2005**). High moisture levels can lead to agglomeration of particles that can result in gas transfer limitations. It can also lead to bacterial contamination (Perez-Guerra, **2003**; Krishna, **2005**; Manpreet ***et al***., **2005**). Variations in the moisture levels can also have an effect on product formation and characteristics such as aroma may be influenced (Manpreet ***et al***., **2005**).

Aeration and agitation are also important factors in SSF, especially in aerobic processes. Aeration plays a role in providing oxygen and in the removal of carbon dioxide, heat and other volatile metabolites produced during metabolism. Factors that influence aeration during the fermentation include the porosity of the substrate where gaseous diffusion increases with a larger pore size (Perez-Guerra, **2003**; Krishna, **2005)**. Agitation during fermentation plays a role in ensuring homogeneity and enhances heat transfer and diffusion of gases. Furthermore agitation should not have a significant influence on the solid support and both the microorganism and substrate should be able to withstand the shear forces caused by agitation (Krishna, **2005**)

When producing pyrazine compounds on an industrial scale one has to look for inexpensive substrates. Soybeans have previously been used for the production of pyrazines, where soybean-based fermentations usually produce 2,5-dimethylpyrazine and tetramethylpyrazine (Besson ***et al.***, **1997**; Larroche ***et al.***, **1999**). Soybeans can thus be used as a substrate as they contain some of the

necessary amino acids that act as precursors for pyrazine production (Table 7). However, they do not contain glycine, serine and alanine that are also known precursors (Iowa Soybean Association, **2001**). Other soybean-based products such as soy protein isolate (SPI) and acid hydrolysed vegetable protein (aHVP) can also be used as substrates as they contain a wide variety of amino acids (Table 8). In foods, the addition of SPI increases the protein content whereas aHVP enhances the cooked and roasted aromas that result from the Maillard reaction (Solina ***et al***., **2005**). Soytone, an enzymatic digest of soybean meal, is another cost effective substrate. Together with a vitamin supplement, as much as 4.33 g.L-1 of tetramethylpyrazine can be produced (Xiao ***et al.***, **2006**).

Yeast extract and casein can also be used as substrates as they contain a wide variety of amino acids that may serve as precursors in pyrazine synthesis. Yeast extract is produced when yeast cells are inactivated. This leads to autolysis that causes the release of peptides, amino acids, vitamins and other yeast cell components. The water soluble fraction is retained and can be used as a natural aromatic ingredient in savoury food or in growth media for the cultivation of microorganisms. Yeast extract contains a total nitrogen content of 8-12%, corresponding to a protein content of 50-75%, an amino nitrogen content of 3-5.2% and a total carbohydrate content of 4-13% (Eurasyp, **2009**). Casein is a mixture of proteins precipitated from milk by rennin. It contains a wide variety of amino acids and is used in the manufacturing of cheese, plastics, adhesives, paints, and other food products (The Free Dictionary, **2009**). The high amino acid content in these substrates makes them ideal candidates for pyrazine production.

Since sugars are also required in the synthesis of some pyrazines, a carbohydrate source is also needed in the substrate. Complex sources such as bagasse (a by-product of sugar production) can be used for this purpose. For example, 2,3-pentanedione that is produced as a by-product in the manufacture of furfural from bagasse can be used as an intermediate in the manufacture of

pyrazines (Illovo Sugar, **2004**). In Morocco a mixture of olive cakes (residues after the extraction of olive oil) and bagasse was used as a substrate for SSF as they are the two main by-products of Morocco's agricultural and food industry (Kademi, Ismaili-Alaoui & Houde, **2003**).

In South Africa, most of the farmland is planted with maize, followed by wheat, sugar cane and sunflower. Compared to the rest of the world, South Africa ranks as the 13th largest producer of sugar cane and the 10th largest producer of sunflower seeds. It is also the world's 9th largest wine producer (SAinfo reporter, **2008**). By-products from these industries will make ideal substrates for products such as flavours, enzymes, antibiotics, organic acids and other value added products (Pandey, Soccol & Mitchell, **2000**). The low cost of these agricultural by-products in an SSF process is an advantage. In addition, other advantages of SSF make such processes economically viable. There is a strong need to develop biotechnology in South Africa and the Department of Science and Technology (DST) has recently proposed a ten-year innovation plan to help drive the economy more towards a knowledge-based one. The production of flavours, such as pyrazines from fungi growing on these natural resources can, therefore, contribute to the strengthening of the bio-economy in South Africa and assisting its goal of positioning itself as a major producer in the pharmaceutical, nutraceutical, flavour, fragrance and bio-pesticide industries (DST, **2009**).

1.6. CHEMICAL ANALYSIS OF PYRAZINES

Flavours are formed as a result of the combination and interaction between certain chemicals present in food. The main purpose for analysing food flavours is to determine qualitative properties to which humans respond to in a favourable or unfavourable manner (Teranishi & Kint, **1993**). In order to understand the way flavours are affecting the taste and smell properties of food one needs to be able to identify the physical and chemical make up of food (Knights, **1992**). Advances in chemical sciences have led to developments in the flavour and fragrance industry where techniques such as Gas Chromatography – Mass Spectrometry

(GC-MS) have permitted flavour chemists to identify important flavour compounds (Rowe, **2002**). Together with sensory evaluation, valuable aroma compounds can also be identified.

1.6.1. Extraction Methods

To be able to study and confirm the pyrazines produced by microorganisms they need to be first extracted and then analysed on a GC-MS. This is the first objective in flavour research, to isolate the flavour molecule from the complex matrix. Commonly used methods for extraction include steam distillation, solvent extraction and adsorption on solid adsorbents (Godefroot, Sandra & Verzele **1981**).

1.6.1.1. Solvent Extraction

Solvent extraction is the most common method used (Cai & Ho, **2001**) where compounds are extracted from an aqueous solution by an organic solvent (Takken, Groesbeek and Roos, **1992**). However there are several disadvantages associated with this method. These include severe losses of volatiles as extracts need to be concentrated by evaporation, as well as contamination of the final product due to non-volatiles that are also extracted (Godefroot ***et al***., **1981**).

1.6.1.2. Supercritical gas (CO_2) Extraction

Supercritical carbon dioxide (CO_2) has unique properties as it can act as a gas or liquid, depending on temperature and pressure. It has thus become important as an industrial solvent for chemical extraction. The use of this method has several advantages that include no chemical residues, a low temperature is used, it is non-toxic, non-flammable and odourless (Takken ***et al.***, **1992**). Due to these advantages, companies such as Evonik Industries are employing this method for the extraction of aroma compounds, ensuring that their flavour products are still regarded as natural (Evonik, **2009**). It, however, has a disadvantage due to the high pressure that is required, making it a costly process (Takken ***et al.***, **1992**)

1.6.1.3. Steam Distillation

Steam distillation is one of the oldest methods used for flavour extraction (Reineccius, **1998**) and is commonly used in extraction of essential oils from plant material (Boelens, **1997**). Steam distillation involves the use of steam to distill volatile components from food, which are then condensed in cold traps. The resulting distillate is a dilute solution of water and the flavour compound. The flavour needs to be then isolated from the aqueous solution by means of solvent extraction. A Simultaneous Distillation-Extraction method has been developed using a Likens Nickerson apparatus or various modifications of it. This results in a more efficient flavour extraction compared to steam distillation (Reineccius, **1998**). Distillation techniques are, however, more adapted to the study of low-volatile compounds and the production of artefacts, as well as incomplete removal or loss of analytes, may be a problem (Cai & Ho, **2001**).

1.6.1.4. Pervaporation

Pervaporation is a method used for the separation of liquid mixtures and is based on a selective transport through a dense membrane (Baudot, Marin & Spinnler**,** **1996**). It finds many applications in wine or beer dealcoholisation, juice concentration and the extraction of aromas from dilute media such as effluents from the food industry or fermentation broth (Souchon, Fontanini & Voilley, **1996**). It is driven by the evaporation of the permeate on the downstream side of the membrane. An advantage of this process is that it can be coupled with a fermenter that results in an increase of the productivity of the bioreactor.

1.6.1.5. Adsorption

The use of adsorbents for the extraction of flavours goes back as far as 1960 (Teranishi & Kint, **1993**). Adsorbents include charcoal, silica gel, alumina and porous polymers. Charcoal is very efficient for trapping volatile compounds as it is not deactivated by water. However, the disadvantage to the use of charcoal is the decomposition of the volatiles when they are thermally desorbed. Thus,

chemists have resorted to the use of porous polymers. The disadvantages associated with these materials are artefact formation and irreversible binding.

Extraction methods that involve the use of adsorbents are Purge and Trap, and Solid Phase Micro-extraction (SPME). In the Purge and Trap method, volatiles are trapped by purging the sample with a carrier gas, for example nitrogen. The volatiles are first adsorbed on an inert support (usually a Tenax column), then thermally desorbed and concentrated again by crystallization in a cold trap (cryofocusing), before thermic injection into a GC column (Marilley and Casey, **2004**). This method is sensitive and, therefore, subjected to severe contamination from the culture medium, chemicals and water (Marilley and Casey, **2004**).

SPME involves the concentration of anylates through adsorption onto a coated fibre (Frank, Owen & Patterson, **2004**). The coated fibre can then be directly immersed into the aqueous sample (direct extraction mode). It can also be done in headspace mode whereby the anylates diffuse into the air and then onto the coated fibre (Pillonel, Bosset & Tabacchie, **2002**). An advantage of this method is that it is effective in concentrating low molecular weight odorous compounds and a disadvantage is that the aging of fibres affects reproducibility of the technique (Marilley & Casey, **2004**). However SPME is more effective than Dynamic headspace techniques, such as the Purge and Trap method, as it is rapid, cheap and easy to automate (Pillonel ***et al.***, **2002**).

1.6.2. Concentration Methods

In certain cases some methods for the extraction of volatiles produce a dilute extract that needs to be concentrated prior to GC analysis. Concentration of volatiles depends on differences in boiling point, freezing point and polarity between the solvent and volatile compounds (Reineccius, **1998**).

1.6.2.1 Evaporation

Evaporation is used to concentrate flavours isolated in organic solvents. The method involved takes advantage of differences in the boiling point between the solvent and the flavour, where the solvent should be thermally stable, have a low boiling point and be of a high purity (Reineccius, **1998**).

1.6.2.2. Freeze concentration

In this method, solutes are concentrated in the unfrozen matrix as water freezes into a solid crystal. High concentration factors cannot be obtained and this process is not ideal for flavour concentration. It is thus rarely used in the flavour industry (Reineccius, **1998**).

1.6.3. Analysis of Flavour Compounds

Chromatography is a common technique used in chemistry for the analysis of pharmaceuticals, environmental pollutants, food and food products, as well as various other organic and inorganic compounds (Cserhati, **2002**). It is a separation technique in which the compounds to be separated are distributed between a mobile and stationary phase. An inert gas (e.g. He, H_2 or N_2) is used as the mobile phase in gas chromatography (GC). As the compounds passes through the column, they interact with the stationary phase. Those that bind strongly are retained for a longer period than those that bind weakly. Thus, the time it takes for a compound from injection to when it elutes from the column is known as the retention time. The retention time of a peak can be used for identification purposes (Scott, **2003**). Quantitative information can also be obtained from the gas chromatogram using the peak area (Karasek & Clement, **1988**).

A common technique used for the analysis of flavour compounds in research is GC-Olfactometry (GC-O), where the odour is sniffed as it elutes from the chromatograph. Aroma Extraction Dilution Analysis and Charm Analysis are methods that have been developed to ensure that results from each run can be

comparable, a shortcoming of GC-O. Quantitation of aroma compounds using these techniques is based on dilution analysis or the determination of odour threshold (Acree, **1993**).

Although a GC is effective in separating compounds to their various components, it cannot be used for the reliable identification of a specific compound. Thus, it is coupled to a mass spectrometer (MS) (Douglas, **2007**). An MS identifies compounds by ionizing and fragmenting molecules and accelerating the resilting ions towards a mass analyser (Douglas, **2007**). Ions are then separated according to their mass to charge ratios, and are recorded as a mass spectrum, which can be used for the identification of the compound (Karasek and Clement, **1988**).

Although MS can be used to confirm the presence of certain compounds, the low concentration, as well as associated complex matrices, sometimes prevent identification of target compounds by full scan MS. The use of Single Ion Monitoring (SIM) mode enhances sensitivity but decreases the amount of qualitative information. Combined with GC retention data, SIM can, however, detect target analytes at low levels of concentration. Trends nowadays are to use MS-MS as it has higher discriminating power to verify the presence of target compounds in complex mixtures. The widespread use of MS-MS, combined with new or traditional chromatographic methods, can be expected (Cserháti, **2002**).

1.7. CHEMICAL AND NATURAL FLAVOURS

Flavour substances currently available on the market include those that can be classified as natural flavours, nature-identical flavours and artificial flavours. Natural flavours are obtained from natural resources (plant or animal) by physical, microbial or enzymatic methods whereas nature-identical flavours are chemically synthesized but structurally identical to natural flavours (Serra, Fuganti & Brenna **2006** and Food Manufacture Market Report, **2008**). Artificial flavours are flavours that are not present in natural products (Food Manufacture

Market Report, **2008**). Consumers demand natural products and it is important to distinguish between natural and nature-identical flavours. One method for doing this is by determining the chiral purity of the product where natural flavours will contain primarily one enantiomer (Mussinan, **1993**)**.** Chirality can be determined using techniques such as chiro-specific Multi-Dimensional capillary Gas Chromatography (MDGC) and MDGC-MS as well as on-line High Resolution Gas Chromatography-Isotope Ratio Mass Spectrometric (HRGC-IRMS) analysis (Schreier, **1992**). ^{14}C analysis is another method that can be used to distinguish between natural and nature identical products. The presence of minute quantities of ^{14}C indicates that the product is natural and is derived from plant material via photosynthesis whereas flavours derived from chemicals lack this isotope **(**Mussinan**, 1993).** This method however only detects natural flavours obtained from plant sources.

1.8. PATENTS IN THE FLAVOUR INDUSTRY

The technologies involved in the discovery of these new compounds can be protected by patenting. A keyword patent search has indicated that with regards to the microbial production of pyrazine compounds, no patents have been found. Patents, however, have been issued for the production of other flavours by fungi. These include processes for the production of methylketones, a blue cheese flavour, by ***Penicillium roquefortii***, ***Penicillium caseicolum***, ***Aspergillus niger***, ***Trichoderma koningii*** (Cruely, Gros & Larocche, **1990**) and ***Aureobasidium pullulans*** (Van Grinsven ***et al***., **1994**). Processes for the production of lactones by the yeast ***Saccharomyces cerevisiae*** (Maria de Laat & van der Schaft, **1992**) and various fungal species of the genus ***Mucor*** (Page & Eilerman, **1991**) have also been patented. Although patents do exist for the discovery of new flavours, the flavour industry relies more on trade secrets rather than patent protection. This is due to patent infringement being more difficult to prove in the flavour industry compared to other industries (Reineccius, **1998**).

1.9. CONCLUSION

There is an ever increasing demand for existing flavours and fragrances due to the growth in the world population, but also due to the advancements in the developing world. Therefore, new and novel ways need to be found to address these demands in order to expand the flavour resources on a global scale. The expectations are thus that the developing world will place an increasing pressure on suppliers of flavours and fragrances worldwide. An example of this trend is the South African flavour market. According to estimates in 2002 the market for flavours in South Africa had the value of US$ 60.7 million, which is small compared to the markets in Europe and the USA (IAL Consultants, **2002**). However, expectations are that the industry is growing at a faster rate of 3.6% p.a. compared to that of Europe and USA which is growing at a rate of 2.5% p.a.

Food industries are market driven and are more receptive to trends such as the demand for high value products. According to Rowe (**2002**) there are several pyrazines that are high impact aroma chemicals, such as 2,3,5-trimethylpyrazine and acetylpyrazine, which have a unique popcorn odour and 2-isobutyl-3-methoxypyrazine that is responsible for the characteristic bell pepper odour. He also stated that there is a great demand for high impact aroma chemicals and that they are fairly expensive. These pyrazines are commonly produced via the Maillard reaction or extracted from natural plant sources.

Other important trends in the flavour industry are the increasing demand for products to have the natural and/or halaal and kosher status. Microbial production is an alternative method for the production of pyrazines that comply with such status. Also, the lack of use of animal products makes it easier to comply with halaal and kosher regulations. The use of microorganisms in flavour production could also be an economically viable process as cheap substrates (which are generally by-products from especially the food and agricultural industries) can be used in the fermentation media.

Advances in biotechnology allow the manipulation of microorganisms on a genetic level and open new possibilities for the flavour industry. Even though this technique has not been developed and utilised at the same level as by the pharmaceutical industries, it forms an important part of research activities in many flavour and fragrance companies (Muheim ***et al***., **1997**). There are, however, disadvantages associated with the use of recombinant biotechnology in the food industry as consumers generally do not accept genetically modified organisms (GMO) in their food products. Therefore, GMO containing foods are required to be labelled. However, food additives and flavourings are not required to be labelled, provided that they are not produced using genetically modified crops, and the genetically modified microorganism used is absent in the final product (von Wright & Bruce, **2003**). Also, a flavour produced from a genetically modified microorganism may not be regarded as natural and would thus not readily be accepted by consumers. The advantage of using recombinant biotechnology could be realised when specific flavour ingredients need to be produced that are either not available or uneconomic to produce when compared to conventional chemical procedures (Knights, **1992**)

South Africa is endowed with seven plant biomes that contain a unique diversity of plant and animal life. Amongst them is the Fynbos Biome that contains the largest number of endemic plants to the region. Associated with these biomes are also a unique variety of mycoflora that remains vastly unexploited. There is thus substantial potential of utilising South African mycoflora to find new and interesting flavour compounds due to the basically undiscovered state of these organisms compared to the rest of the world.

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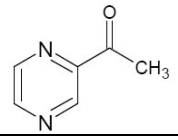
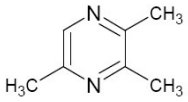
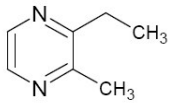
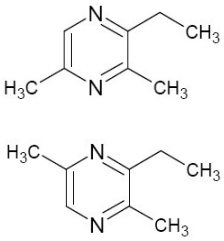
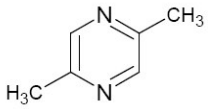
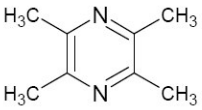
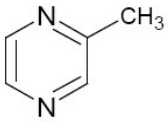
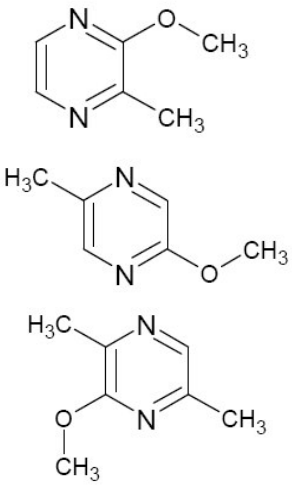
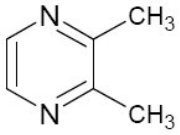
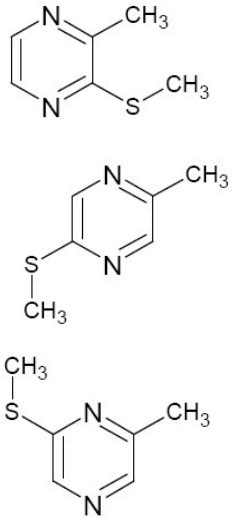
Table 1: Annual volumes of usage of the top ten pyrazines as flavouring agents in Europe and the USA (Mattia ***et al.***, **2004**).

Rank	Name of pyrazine	Structure	*Annual volume in Europe (kg)	*Annual volume in the USA (kg)	Annual volume in naturally occurring foods (kg) Europe
1	Acetylpyrazine		96	923	1 900
2	2,3,5-Trimethylpyrazine		843	347	23 000
3	2-Ethyl-3-methylpyrazine		589	72	18 000
4	2-Ethyl-3, (5 or 6)-dimethylpyrazine		309	72	7 100
5	2,5-Dimethylpyrazine		156	59	37 000
6	2,3,5,6-Tetramethylpyrazine		55	144	7 700
7	2-Methylpyrazine		139	50	114 000

*Synthetic pyrazine production

Table 1 (Continued): Annual volumes of usage of the top ten pyrazines as flavouring agents in Europe and the USA (Mattia ***et al.***, **2004**).

Rank	Name of pyrazine	Structure	*Annual volume in Europe (kg)	*Annual volume in the USA (kg)	Annual volume in naturally occurring foods (kg) Europe
8	(2,5 or 6)-Methoxy-3-methylpyrazine		Not known	113	Not known
9	2,3-Dimethylpyrazine		112	27	7 700
10	(3,5 or 6)-(Methylthio)-2-methylpyrazine		52	99	-

*Sythetic pyrazine production

Table 2: Comparison of current pyrazine prices supplied by some companies.

	Advanced Biotech		Sigma		M&U	
	Pyrazine Name	Price (US$/kg)	Pyrazine Name	Price (R/kg)	Pyrazine Name	Price (US$/kg)
1	2-Methoxy-3-isobutylpyrazine	4700	2-Methoxy-3-isopropylpyrazine	8414,48/100g	2,3-diethyl-5-methylpyrazine	3200
2	2-Methoxy-3-secbutylpyrazine	3800	2-Isobutyl-3-methylpyrazine	42072,41	2,3-dimethyl-5-(2-methylbutyl)pyrazine	3100
3	2-Methoxy-3-isopropylpyrazine	2200	2-Methoxy-3- (1-Methylpropyl)pyrazine	42072,41	2,3-dimethyl-5-sec butylpyrazine	2500
4	5(H)-5-Methyl-6, 7-dihydrochloropenta (b)pyrazine	1800	2-Acetyl-3-ethylpyrazine	38566,38	2-Methoxy-3-isopropylpyrazine	2500
5	2,3-Dimethyl-5-methylpyrazine	1500	2-Methoxy-3-isobutylpyrazine	28048,27	2-Methoxy-3-secbutylpyrazine	2500
6	2-Acetylpyrazine	650	2-Ethyl-3-methoxypyrazine	28048,27	2-Methoxy-3-isobutylpyrazine	2300
7	2-Ethylpyrazine	600	2-Acetyl-3, 5(or 6)-dimethylpyrazine	26645,85	2,3-Diethylpyrazine	2000
8	2-Methoxy-3 (or 6)-methylpyrazine	600	2-Acetyl-3-methylpyrazine	21737,41	2,5-Diethylpyrazine	2000
9	2-Methoxypyrazine	575	Pyrazineethanethiol	21036,20	2,3-Dimethyl-5-isobutylpyrazine	1600
10	2,6-Dimethylpyrazine	550	2-Acetylpyrazine	18231,38	2,3-Dimethyl-5-ethylpyrazine	1500

Table 3: Microorganisms that are known to produce pyrazines.

Name of organism	Source	Maintenance	Substrates	Pyrazine produced	Yields	Reference
Fungi						
Aspergillus flavus	ATTC[1] 15546 and PRL 932	Czapeck Dox Agar	2% Yeast Extract + amino acids, dH2O and barley or peas or wheat	2-hydroxy-3-isobutyl-6-sec-butylpyrazine-1-oxide (Aspergillic acid) 2-hydroxy-3-isobutyl-6-(1-hydroxy-1-methylpropyl)-pyrazine-1-oxide (hydroaspergillic acid)	4.3mg in 0% sucrose 25mg in 0% sucrose	MacDonald, **1973**
Aspergillus parasiticus	NRRL 2999	PDA slants at 4°C	Peptone Mineral Salts Medium	2-hydroxy-3,6-disec-butylpyrazine (deoxyaspergillic acid)	Not known	Buchanan & Houston, **1982**
				3-isopropyl-2-methoxypyrazine	Not known	
Aspergillus sojae StrainX-1	Not known	Not known	Not known	2-hydroxy-3,6-disec-butylpyrazine (deoxyaspergillic acid)	Not known	Seitz, **1994**
				2-Hydroxy-3-isobutyl-6-(1-hydroxy-1-methylethyl)pyrazine		
				2-Hydroxy-3-sec-butyl-6-(1-hydroxy-1-methylpropyl)pyrazine		
Aspergillus sojae	Not known	Not known	Not known	3-Isobutyl-6-s-butyl-2-hydroxyprazine	Not known	Rizzi, **1988**
Aspergillus oryzae	Not known	Not known	Not known	3-isopropyl-2-methoxypyrazine	Not known	Gallois & Grimont**,** **1985**
Candida pulcherrima (yeast)	Not known	Not known	Not known	2-hydroxy-diisobutylpyrazine-1,4-oxide(pulcherriminic acid)	Not known	Beck ***et al.***, **2003**

Notes:
[1] ATCC- American Type Culture Collection
[2] IBT-Fungal culture collection at the Department of Biotechnology, The Technical University of Denmark.
[3] Broth A is made up of Yeast Extract, glucose, Bacto-tryptone and KPO_4
[4] Broth B is made up of $NaPO_4$, KPO_4, $MgSO_4$, amino acid or carbon compound + Ammonium nitrate
[5] Broth C is made up of broth B and leucine
[6] CDC- Centres for Disease Control

Table 3 (Continued): Microorganisms that are known to produce pyrazines.

Name of organism	Source	Maintenance	Substrates	Pyrazine produced	Yields	Reference
Fungi						
Penicillium vulpinum	IBT[2] 3228, 3229, 6311,6433, 10606	Malt extract agar	Czapek Yeast Autolysate agar	2-methoxy-3-isopropylpyrazine (earthy, bell pepper, vegetable potato, green pea)	Not Known	Larsen & Frisvad, **1994**
				2-methoxy-3-secbutylpyrazine		
Penicillium clavigerum	IBT[2] 5524			Earthy, musty off-like odour		
Septoria nodorum	Not known	Not known	Not known	3-isopropyl-2-methoxypyrazine (earthy, bell pepper, vegetable, potato)	Not known	Gallois & Grimont, **1985**
				3-s-butyl-6-(4-hydroxybenzoyl)-2,5-dihydroxypyrazine	Not known	Rizzi, **1988**
Bacteria						
Bacillus cereus #147	Not known	Not known	4% glucose and 0.2% yeast autolysate	Tetramethylpyrazine	0.3 $g.L^{-1}$	Seitz, **1994**
Corynebacterium glutanicum	Not known	Not known	Leucine, isoleucine, valine and pantothenate	Tetramethylpyrazine	3 $g.L^{-1}$	Demain ***et al.***, **1967**

Notes:
[1] ATCC- American Type Culture Collection
[2] IBT-Fungal culture collection at the Department of Biotechnology, The Technical University of Denmark.
[3] Broth A is made up of Yeast Extract, glucose, Bacto-tryptone and KPO_4
[4] Broth B is made up of $NaPO_4$, KPO_4, $MgSO_4$, amino acid or carbon compound + Ammonium nitrate
[5] Broth C is made up of broth B and leucine
[6] CDC- Centres for Disease Control

Table 3 (Continued): Microorganisms that are known to produce pyrazines.

Name of organism	Source	Maintenance	Substrates	Pyrazine produced	Yields	Reference
Bacteria						
Bacillus subtilis	IFO 3013	Tryp/Soy Agar	Dehulled yellow soybeans	2,5-Dimethylpyrazine (85%) (Chocolate, roasted nuts, earthy)	550 $mg.L^{-1}$ + threonine	Larroche ***et al.*, 1999**
				Trimethypyrazine (10%) (nutty, reminiscent of coffee and cocoa, baked potato)	50 $mg.L^{-1}$ + threonine	
				Tetramethylpyrazine (1.5%)(nutty,musty, chocolate)		
				2-ethyl-5-methylpyrazine (nutty, roasted, "grassy")		
				2-methylpyrazine (green, nutty, cocoa, musty, fishy)		
				2-ethylmethylpyrazine		
				2,3-dimethylpyrazine (green, nutty, potato, cocoa, coffee, caramel, meaty)		
				Tetramethylpyrazine and trimethylpyrazines (with L-serine)		
Halomonas venusta	Marine bacterium	-	ZoBell+ artificial sea water	2-methoxy-3-(1-methylpropyl)-pyrazine	9.8 $mg.L^{-1}$ with glycine + leucine	Bungert Jans & Becker, **2001**

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[4] Broth B is made up of $NaPO_4$, KPO_4, $MgSO_4$, amino acid or carbon compound + Ammonium nitrate
[5] Broth C is made up of broth B and leucine
[6] CDC- Centres for Disease Control

Table 3 (Continued): Microorganisms that are known to produce pyrazines.

Name of organism	Source	Maintenance	Substrates	Pyrazine produced	Yields	Reference
Bacteria						
Chrondromyces crocatus	Isolated from soil samples	Not known	VY/2-(yeast) agar or CY-(peptone) agar	2,5-Dimethylpyrazine	Not known	Schulz, et al., **2004**
				2-Methoxy-3-methylpyrazine		
				(1-Methylethyl)pyrazine		
				2-methyl-6-(1-methylethyl)pyrazine		
				3-Methoxy-2, 6-dimethylpyrazine		
				3-Methoxy-2, 5-dimethylpyrazine		
				2-Methyl-5-(1-methylethyl)pyrazine		
				2-Methoxy-3-(1-methylethyl)pyrazine		
				Dimethyl-(1-methylethyl)pyrazine		
				2,6-Bis(1-Methylethyl)pyrazine		
				2-Methoxy-3-(1-methylpropyl)pyrazine		
				2-Methoxy-3-(2-methylpropyl)pyrazine		
				2,5-Bis(1-Methylethyl)pyrazine		
				2-(1-hydoxy-1-methylethyl)-3-methoxypyrazine		
				2-(1-methylethenyl)-6-(1-methylethyl)pyrazine		
				2-(1-methylethenyl)-5-(1-methylethyl)pyrazine		
				2-(1-hydoxy-1-methylpropyl)-3-methoxypyrazine		
				2-(1-hydoxy-2-methylpropyl)-3-methoxypyrazine		
				2,5-Bis(2-Methylpropyl)pyrazine		

Notes: [1] ATCC- American Type Culture Collection
[2] IBT-Fungal culture collection at the Department of Biotechnology, The Technical University of Denmark.
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[4] Broth B is made up of $NaPO_4$, KPO_4, $MgSO_4$, amino acid or carbon compound + Ammonium nitrate
[5] Broth C is made up of broth B and leucine
[6] CDC- Centres for Disease Control

Table 3 (Continued): Microorganisms that are known to produce pyrazines.

Name of organism	Source	Maintenance	Substrates	Pyrazine produced	Yields	Reference
Bacteria						
Paenibacillus polymyxa	ATCC[1] 10401	Tryp Soy Broth (TSB)	TSB and TSB+Valine	2,5-diisopropylpyrazine	Most abundant	Beck ***et al.***, **2003**
				2-isopropylpyrazine	Yields not known	
				2-methyl-6-isopropylpyrazine		
				2-methyl-5-isopropylpyrazine		
				2,3-dimethyl-5-isopropylpyrazine		
				2,5-dimethyl-3-isopropylpyrazine		
				2,6-dimethyl-3-isopropylpyrazine		
				2,6-diisopropylpyrazine		
				2,5-dimethyl-3,6-diisopropylpyrazine		
				Isobutylpyrazine		
				2-methyl-5-isobutylpyrazine		
				2,6-dimethyl-5-isobutylpyrazine		
				2,5-dimethyl-6isobutylpyrazine		
				2,5-diisobutylpyrazine		
				2,6-diisobutylpyrazine		
				2-isopropyl-5-sec-butylpyrazine		
				2-isobutyl-5-sec-butylpyrazine		
				2-isopropyl-5- isobutylpyrazine		
				2-isopropyl-6- isobutylpyrazine		
Pseudomonas perolens	ATCC[1] 10757	Nutrient broth	M-56 Minimal salt medium	2-methoxy-3-isopropylpyrazine (earthy, bell pepper vegetable potato green pea)	12.5 $mg.L^{-1}$	Cheng ***et al.***, **1991**
				2-methoxy-3-sec-butylpyrazine		

Notes: [1] ATCC- American Type Culture Collection
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[3] Broth A is made up of Yeast Extract, glucose, Bacto-tryptone and KPO_4
[4] Broth B is made up of $NaPO_4$, KPO_4, $MgSO_4$, amino acid or carbon compound + Ammonium nitrate
[5] Broth C is made up of broth B and leucine
[6] CDC- Centres for Disease Control

Table 3 (Continued): Microorganisms that are known to produce pyrazines.

Name of organism	Source	Maintenance	Substrates	Pyrazine produced	Yields	Reference
Bacteria						
Pseudomonas taerolens	Isolated from Munsters cheese similar to ATCC[1] 4683	Broth A[3]	Broth A[3] Broth B[4] Broth C[5]	3-isopropyl-methoxypyrazine	10 µg.L^{-1}	Gallois ***et al*, 1998**
				Methylpyrazine		
				2,5-dimethylpyrazine (chocolate, roasted nuts, earthy)	10 µg.L^{-1}	
				Trimethylpyrazine(nutty, reminiscent of coffee and cocoa, baked potato)		
				2,5-dimethyl-3-ethylpyrazine		
Serratia odifera	ATCC[1] 33077	Tryptic Soy (TS) Broth	TS broth TS agar	3-isopropyl-2-methoxy-5-methylpyrazine	8-12 mg.L^{-1}	Gallois & Grimont**, 1985**
				3-isobutyl-2-methoxy-6-methylpyrazine	0.1-0.5 mg.L^{-1}	
Serratia ficara	ATCC[1] 33105			3-isopropyl-2-methoxy-5-methylpyrazine	8-12 mg.L^{-1}	
				3-secButyl-2-methoxy-5(6)-methylpyrazine	0.1-0.5 mg.L^{-1}	
Serratia rubidae	Not known			2-ethyl-6-methylpyrazine	1-3 mg.L^{-1}	
				2,3,5-trimethylpyrazine(nutty, reminiscent of coffee and cocoa, baked potato)	1-3 mg.L^{-1}	
				3-isopropyl-2-methoxy-5-methylpyrazine	15-20 mg.L^{-1}	
				3-secButyl-2-methoxy-5(6)-methylpyrazine	8-12 mg.L^{-1}	
Cedecea davisae	CDC[6] 2296-76			3-secButyl-2-methoxypyrazine	8-12 mg.L^{-1}	
				3-isopropyl-2-methoxy-pyrazine(earthy, bell pepper, vegetable, potato)	0.1-0.5 mg.L^{-1}	
				2,3,5-trimethylpyrazine(nutty, reminiscent of coffee and cocoa, baked potato)	1-3 mg.L^{-1}	

Notes:
[1] ATCC- American Type Culture Collection
[2] IBT-Fungal culture collection at the Department of Biotechnology, The Technical University of Denmark.
[3] Broth A is made up of Yeast Extract, glucose, Bacto-tryptone and KPO_4
[4] Broth B is made up of $NaPO_4$, KPO_4, $MgSO_4$, amino acid or carbon compound + Ammonium nitrate
[5] Broth C is made up of broth B and leucine
[6] CDC- Centres for Disease Control

Table 4: Precursors of pyrazine production.

Precursors	Pyrazines	Reference
L-Threonine	2,5-dimethylpyrazine	Besson ***et al.***, **1997**
Acetoin and Ammonia	Tetramethylpyrazine	Besson ***et al.***, **1997**
Pyruvate	Dihydropyrazines Dimethylpyrazines Tetrahydropyrazines	Kurniadi ***et al.***, **2003**
Rhamnose	Pyrazines	Coleman & Steichen, **2006**
Leucine	3-methylbutylpyrazines	Coleman & Steichen, **2006**
Valine	2-methylpropylpyrazines	Coleman & Steichen, **2006**
Valine, leucine, isoleucine or alanine	Isopropylpyrazines Isobutylpyrazines Secbutylpyrazines or Methylpyrazines	Schulz ***et al.***, **2004**
Acyloin + Ammonia	Alkylpyrazines	Rizzi, **1988**
Glycine + Valine	3-isopropyl-2-methoxypyrazine	Cheng, **1991**
Valine + Leucine	3-isopropyl-2-methoxypyrazine	Gallois ***et al.***, **1998**

Table 5: Odour and yields of 3 –isopropyl-2-methoxypyrazine production when grown on different substrates (Gallois ***et al,*** **1998**).

Substrate	Odour	Concentration of 3-isopropyl-2-methoxypyrazine µg.L^{-1}
Fructose	Pungent	5
Glycerol	Green peas	50
β-alanine	Very weak green peas	10
DL-α-alanine	Ammonia	10
L-hydroxyproline	Ammonia	10
DL- serine	Green peas	10
DL-valine	Hazelnut	100
L-valine	Green peas	1 000
L-leucine	Green peas	10 000

Table 6: The production of 2-methoxy-3-alkyl pyrazines by a mutant of ***Pseudomonas perolens*** on different substrates and concentrations.

Carbon	***Concentration* (% w/v)**	***Pyrazine (ng.mL^{-1})* 2-Methoxy-3-isopropylpyrazine**	***Pyrazine (ng.mL^{-1})* 2-Methoxy-3-sec-butylpyrazine**
Glucose	**0.5**	**2030**	**21**
	1.0	960	11
	2.0	840	9
Lactate	0.5	6280	68
	1.0	**9890**	**112**
	2.0	4730	48
Pyruvate	0.5	5150	51
	1.0	**11120**	**100**
	2.0	8400	95
Glycerol	0.5	3160	86
	1.0	3170	69
Nutrient Broth	-	1250	145

The highest yields obtained at different concentrations are highlighted in bold.

Table 7: Amino acid composition of dehulled soybeans.

Amino acid	Percentage
Arginine	3.8
Lysine	**3.2**
Methionine	**0.75**
Cystine	**0.74**
Tryptophan	0.7
Histidine	1.3
Leucine	**3.8**
Isoleucine	**2.6**
Phenylalanine	2.7
Threonine	**2.0**
Valine	**2.7**

Amino acids that may serve as precursors for pyrazine production are highlighted in bold.

Table 8: Table of amino acid content of Soy Protein Isolate (SPI) and acid Hydrolased Vegetable Protein (aHVP) (Solina ***et al***., **2005**).

Amino acid	SPI (mg/10g)	aHVP (mg/10g)
Aspartic acid	770	49.0
Glutamic acid	1368	327
Asparginine	-	2.4
Serine	**368**	**125**
Glycine	**287**	**72.8**
Threonine	**297**	**82.2**
Tyrosine	300	39
Cysteine	**95**	**16.8**
Alanine	**280**	**240**
Proline	376	218
Valine	**360**	**90.1**
Methionine	**102**	**33.0**
Isoleucine	**373**	**48.2**
Leucine	**587**	**183**
Phenylalanine	411	117
Tryptophan	-	20
Histidine	207	43.6
Arginine	626	80.6
Lysine	**445**	**47.2**

Amino acids that may serve as precursors for pyrazine production are highlighted in bold.

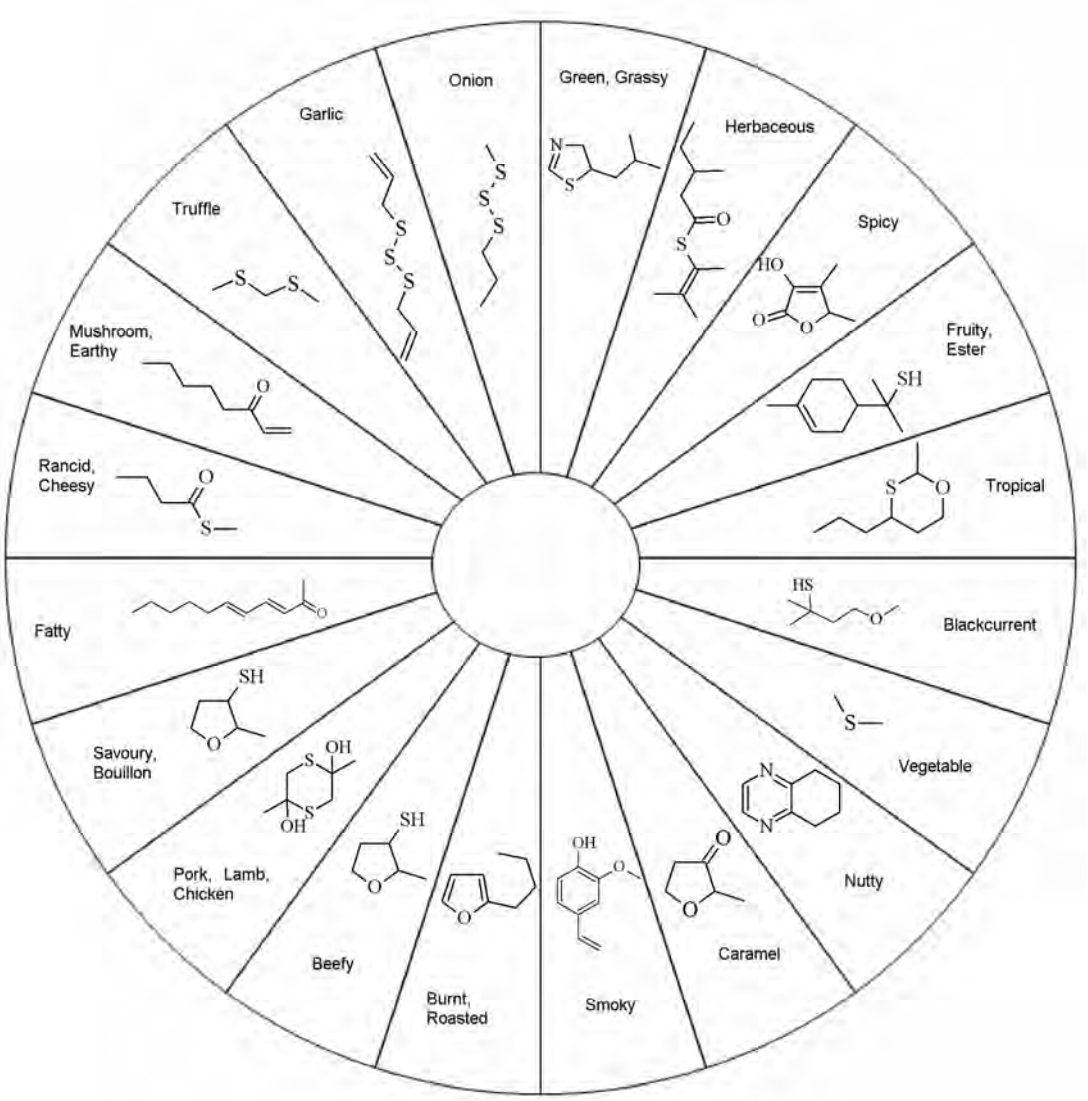
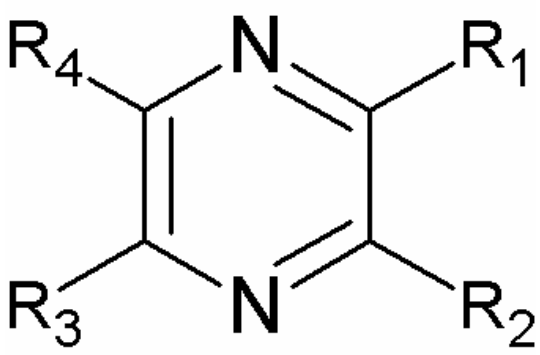


Figure 1: A flavour wheel for high impact aroma chemicals (Rowe, **2002**).



Rn: -H, -alkyl, -OCH3, -OH

Figure 2: The general structure of a pyrazine molecule.

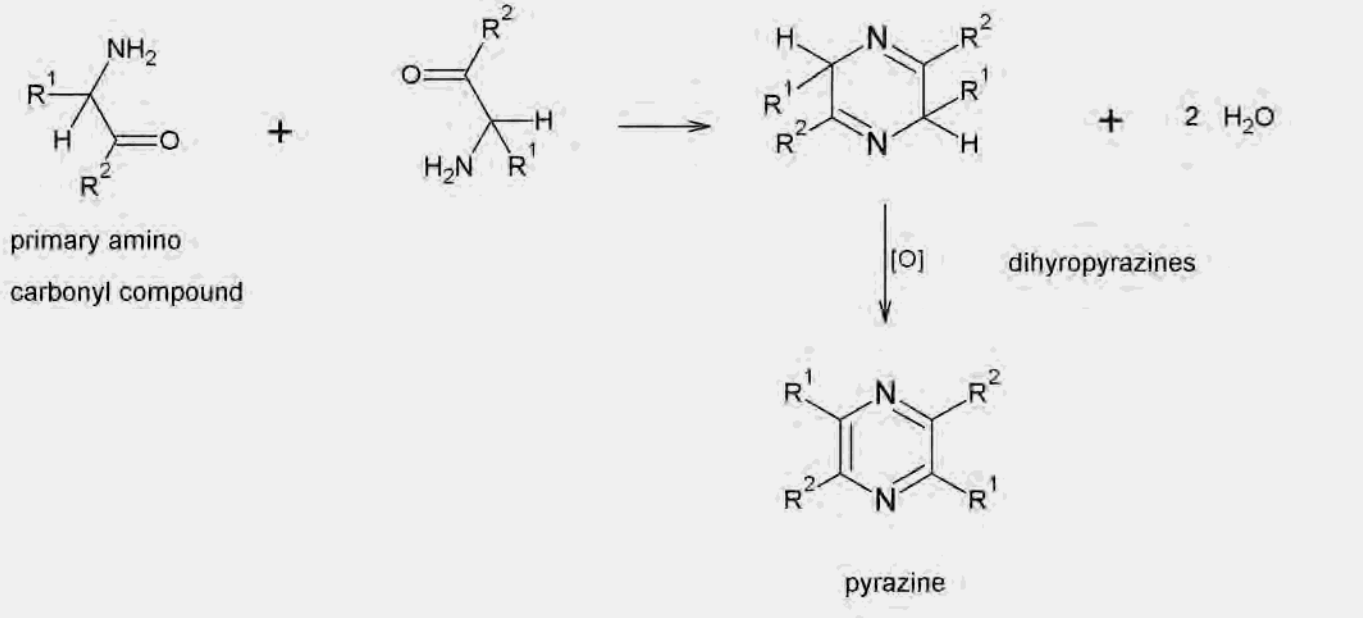


Figure 3: Synthesis of 2,5-disubstituted and 2,3,5,6-tetrasubstituted pyrazines. Source: Barlin (**1982**).

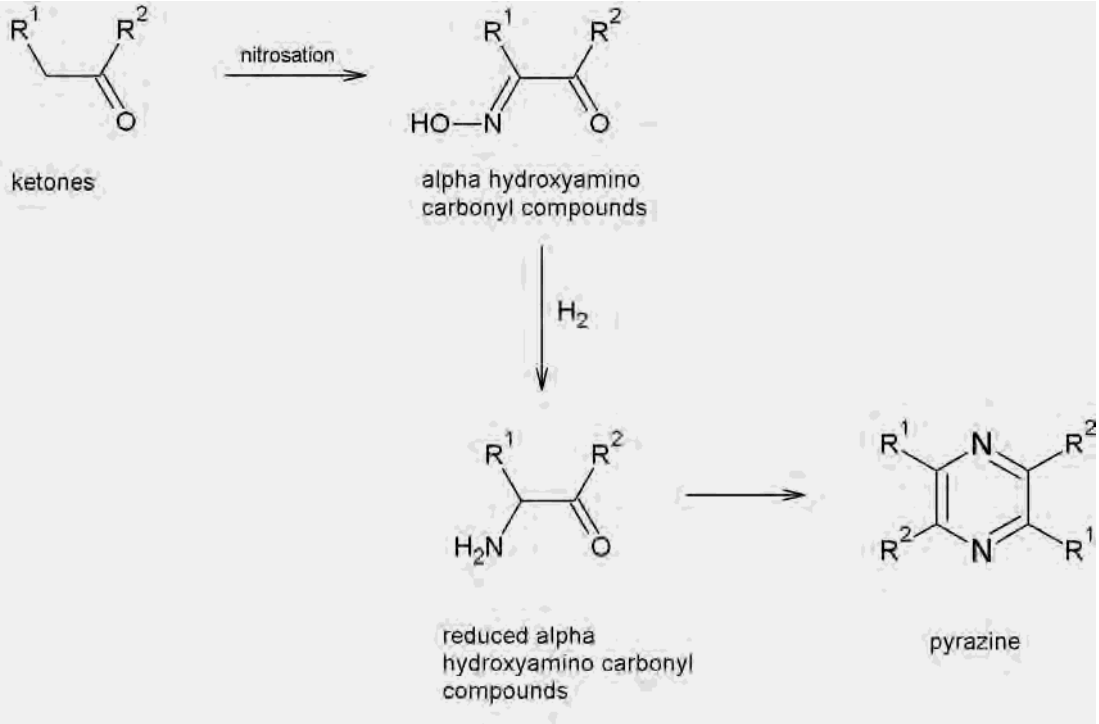


Figure 4: Synthesis of pyrazines through the reduction of α-hydroxyamino carbonyl compounds. Source: Barlin (**1982**).

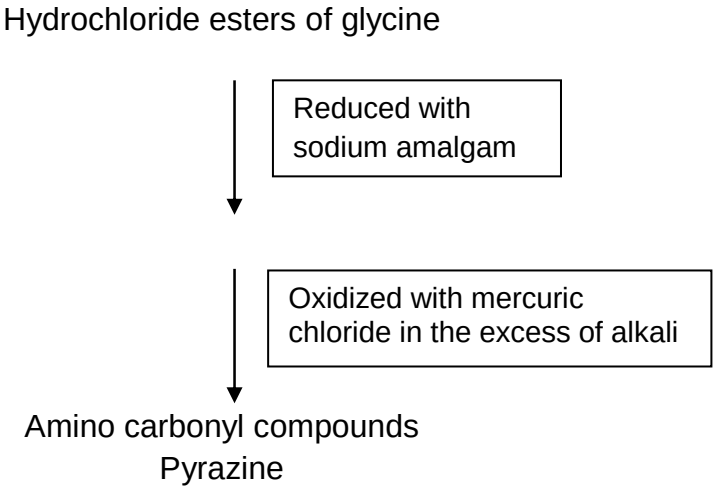


Figure 5: Synthesis of pyrazine using through the reduction of α-amino acids.

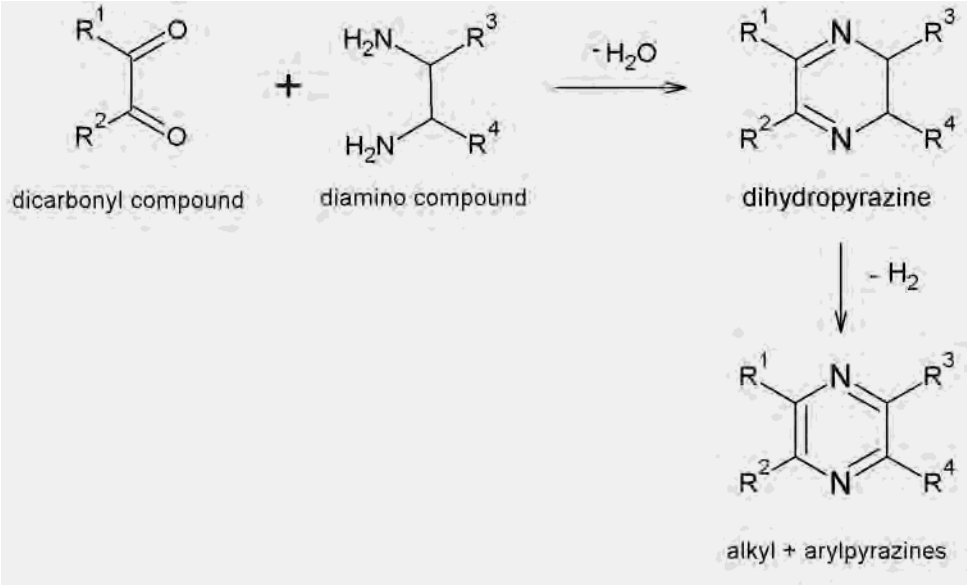


Figure 6: Synthesis of alkyl and arylpyrazines. Source: Barlin (**1982**).

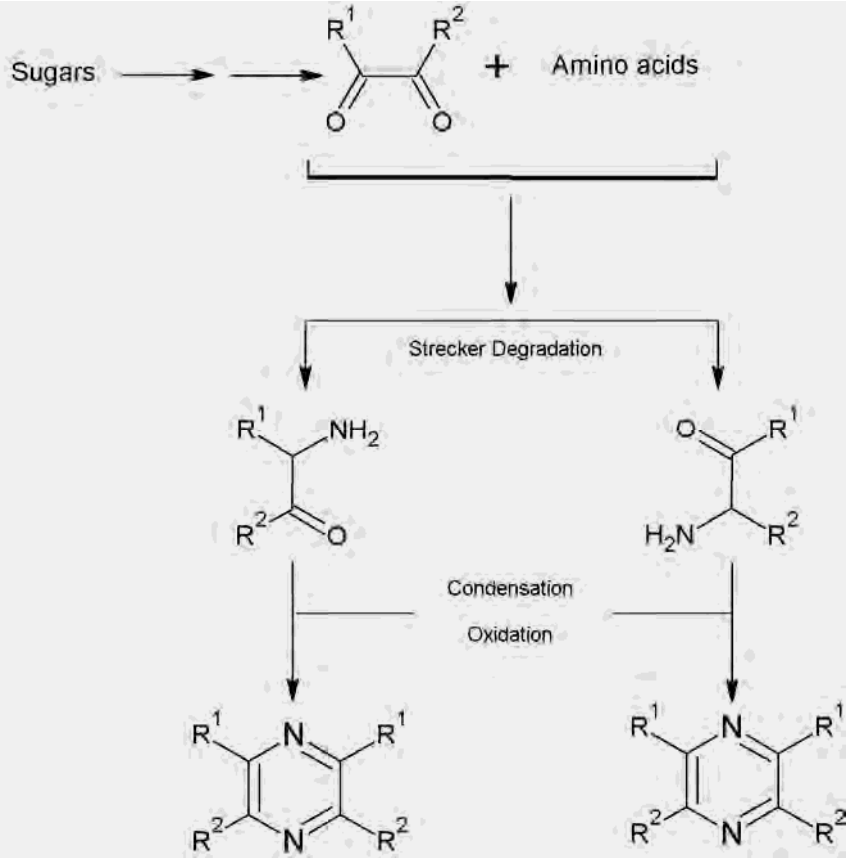


Figure 7: Synthesis of dimethylpyrazines. Source: Maga (**1982**).

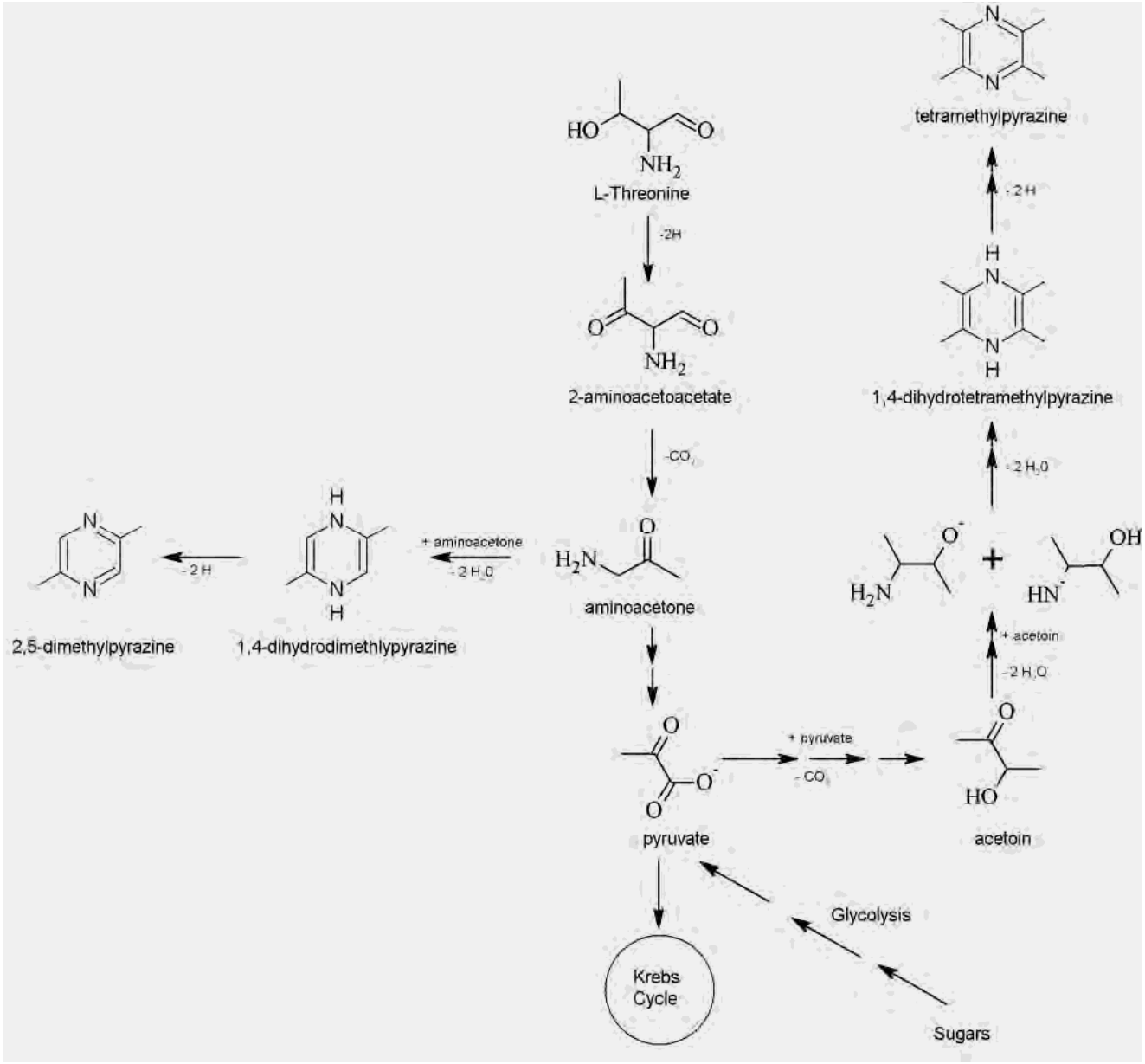


Figure 8: Metabolic pathways for the synthesis of 2,5-dimethylpyrazine and tetramethylpyrazine by ***B. subtilus.*** Source: Larroche ***et al***. (**1999**).

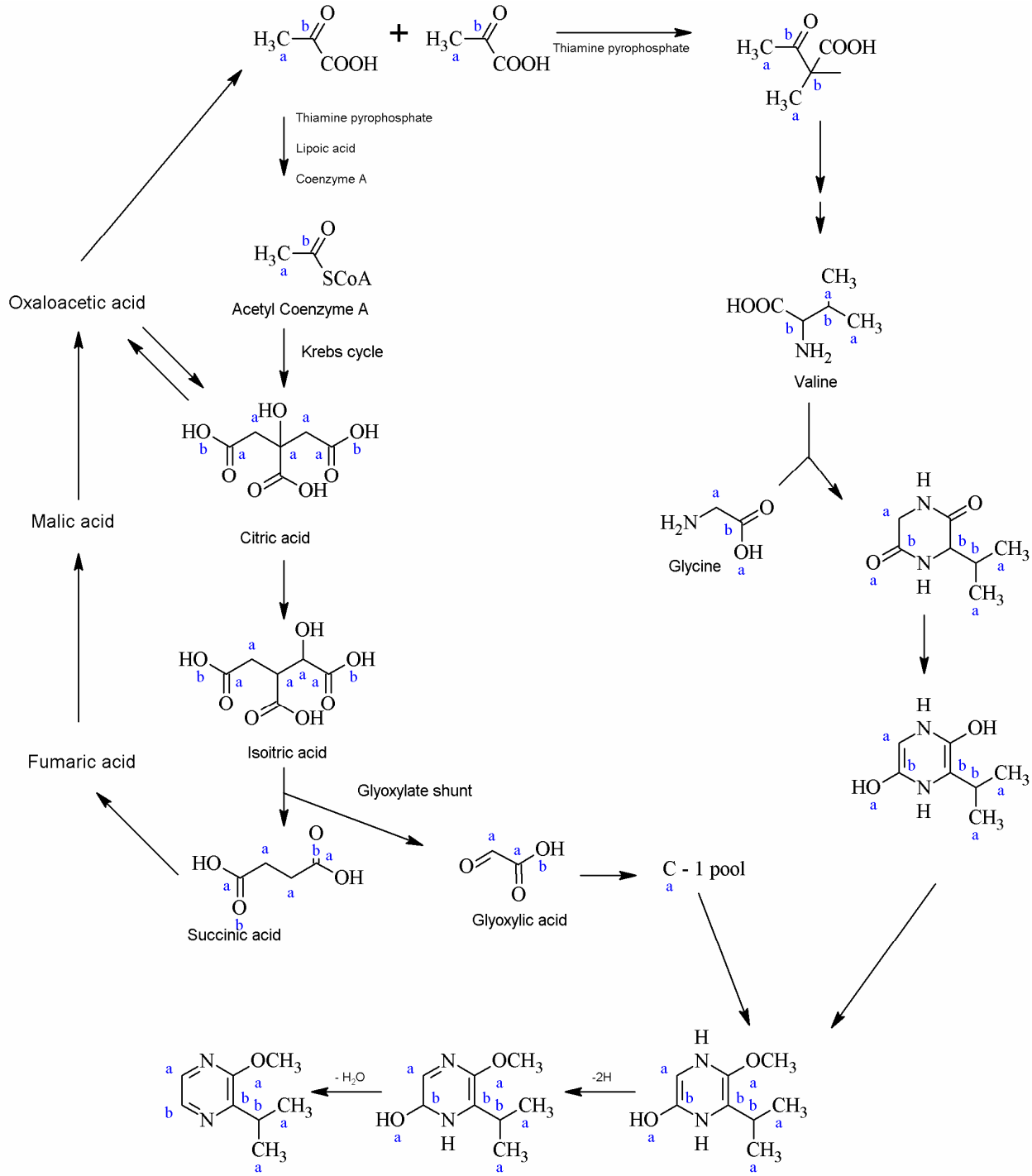


Figure 9: Proposed biosynthesis of MIPP derived from labelled sodium pyruvate feeding experiments. (a) [2-^{13}C] Pyruvate; (b) [3-^{13}C] pyruvate. Source: Cheng ***et al.*** (**1991**).

CHAPTER 2

Production of pyrazine-like flavours by mycelial fungi from South African environments

2.1. ABSTRACT

Pyrazines comprise one of the chemical groups that are of major importance to the flavour industry. Due to the demand for natural products, the purpose of this study was to screen 280 fungi from South African environments for pyrazine flavour production. Fungi were selected from the culture collection at the CSIR and the culture collection at the University of Pretoria. Two different media, including modified Czapeck Dox medium (Cz) and tryptic soy broth (TSB), were selected as substrates. The fungi were screened using sensory evaluation for the presence of nutty, meaty, caramel, chocolate, green, and potato flavours. These are known to be characteristic of pyrazines. Results indicated that 45% of the total fungi screened were flavour producers that resemble typical aromas of pyrazines. It also indicated that members of the 11 fungal genera and groups represented in this study were specific in the type of flavours produced. For example, members of ***Aspergillus***, ***Penicillium***, basidiomycetous fungi, coelomycetous fungi and ophiostomatoid fungi were the most prominent flavour producers amongst the groups of fungi tested. Results have shown that the type of substrate used influenced the range of flavours produced. In addition, mycelial fungi can be considered as good candidates for the production of natural flavours such as pyrazines. However, whether the production of these flavours can be developed into economically viable processes is yet to be demonstrated.

2.2. INTRODUCTION

Aromatic compounds are chemicals that include a wide variety of classes. Acids, alcohols, lactones, esters, ketones, pyrazines and terpenoids are the preferred chemical groups that are extensively used by flavour industries (Manley, **1994**). Among these, pyrazines are regarded as one of the more important groups of flavours due to their applications in a wide variety of products in the food industry (Endredi, Billes & Keresztury, **2004**). These are aromatic, heterocyclic, nitrogen containing compounds and are important flavouring agents in many raw and roasted food products (Beck, Hansen & Lauritsen, **2003;** Larroche Besson & Gros, **1999**). For example 2,3-dimethylpyrazine, naturally found in asparagus, potato, peanuts and coffee, is used in gravies, beverages and sweets, and 2,6-dimethylpyrazine is used as

a component of the coffee, cocoa, meat and potato flavours (Endredi ***et al***., **2004**).

Approximately 80% of the flavours and fragrances produced globally are chemically synthesized. However, food industries are market driven and more receptive to trends such as the demand for natural products (Krings & Berger, **1998**). There is also a considerable demand that products should have the halaal and kosher status (Manley, **1994**; Chaudry & Regenstein, **1994).** In the past, most natural flavours were produced from plants, and often the active sensory compound is either present in minute quantities, or occurs in a chemically bound form that makes it difficult to purify (Kademi, Ismaili-Alaoui & Houde, **2003**). In many cases, this leads to economically non-viable processes. Therefore, new methods are required for the production of natural flavours and numerous researchers have sought to study microorganisms, capable of producing natural compounds, which may be of commercial interest (Serra, Fuganti & Brenna, **2005**; Lomascolo ***et al.***, **1999;** Krings & Berger, **1998**).

Several microorganisms are involved in the synthesis of pyrazine compounds of which bacteria are the main producers. For example, ***Bacillus subtilis*** is able to synthesize 550 $mg.L^{-1}$ of 2,5-dimethylpyrazine when grown on soybeans supplemented with threonine (Larroche ***et al.***, **1999**). Only a small number of fungi have shown to produce pyrazines applicable in the flavour industry (Larsen & Frisvad, **1994**; Gallois & Grimont, **1985**). However, it is known that fungi are capable of producing compounds that closely resemble plant volatiles (Krings & Berger, **1998**). Fungi belonging to the genera, ***Aspergillus*** and ***Penicillium***, have shown to be pyrazine producers, where many ***Penicillium*** spp. are responsible for the earth-like musty notes of cheeses (Karahadian, Josephson & Lindsay, **1985**).

South Africa is known for its unique plant and animal life and it is highly likely that its mycoflora also resembles a unique diversity in its ability to produce flavours (Crous ***et al.***, **2006**). To explore the potential of South Africa's fungal biodiversity, fungi from the culture collection of the Forestry and Agricultural

Biotechnology Institute (FABI) at the University of Pretoria (UP), as well as the CSIR culture collection, were selected to be evaluated for the production of compounds resembling pyrazine flavours.

2.3. MATERIALS AND METHODS

2.3.1. Fungal Strains Screened

In total, 280 fungi were selected from the CSIR and CMW culture collections to be screened for flavours resembling pyrazines (Table 1). The fungi were selected based on their association with natural environments or with food and feed commodities in South Africa. They were first revived from a state of lyophilyzation by adding 1 mL of autoclaved distilled water to vials containing the lyophilized fungi using sterile methods. Thereafter, 100 µL of the fungal solution was plated on potato dextrose agar (PDA) and malt extract agar (MEA) using the spread plate technique.

2.3.2. Cultivation of fungi

PDA (Merk, SA) was prepared according to the manufacturers instructions. MEA was prepared by adding 20 g malt extract (Merk, SA), 1 g peptone (Merk, SA), 20 g glucose (ACE, SA) and 20 g bacteriological agar (Merk, SA) to 1 L distilled water. This was then autoclaved for 20 min at 121^{o}C steam pressure of 100 kPa (Pitt, **1991**).

A piece of actively growing mycelium and spores were taken by cutting a piece of agar (approximately 4 mm^2) from the plates. Each isolate was then inoculated into 5 mL modified Czapeck Dox (Cz) medium and Tryptic soy broth (TSB) respectively. The Cz was prepared by first making up a buffer solution that comprised of a buffer A and B. Buffer A contained 10.2 g KH_2PO_4 (ACE, SA) per liter of dH_2O, and Buffer B contained 13.05 g K_2HPO_4 (ACE, SA) per liter of dH_2O.

In total, 510 mL of Buffer A and 490 mL of Buffer B were added together to prepare 1 L of the buffer solution. The Cz medium was prepared by adding 4 g $NaNO_3$ (ACE, SA), 1 g $MgSO_4.7H_2O$ (ACE, SA), 1 g KCl (ACE, SA), 0.0366

g $FeSO_4.7H_2O$ (ACE, SA), 0.0178 g $ZnSO_4.7H_2O$ (uniLAB, SA) and 0.00782 g $CuSO_4.5H_2O$ (ACE, SA) to the buffer solution.

In addition, amino acids including glycine (Sigma, SA), L-valine (Sigma, SA), L-cysteine (Merk, SA), L-threonine (Fluka, SA) and L-leucine (Sigma-Aldrich, SA) were added at 1 g each and sugars: pyruvic acid (Merk, SA), glycerol (Fluka, SA), lactic acid (Saarchem, SA) and glucose (ACE, SA) were respectively added at 1.25 g each, as these are known precursors for pyrazine production.

TSB (Merk, SA) was included in this study due to the fact that it has been previously used for the production of pyrazines (Beck ***et al.***, **2003**; Gallois & Grimont**, 1985**). It was prepared according to the manufacturer's instructions. The inoculated fungi were then incubated at 25^{o}C for 7 days and pyrazine-like flavours were detected by means of a sniffing panel. The setting up of the sniffing panel is discussed below.

2.3.3. Setting up the sniffing panel

In order to detect the specific odours that resemble pyrazine flavours, a sniffing panel of 5 members was used. The members were first trained at the Department of Food Science and Technology at the University of Pretoria to familiarize themselves with the odours of pyrazines. The detection of the odours was based on the fact that volatiles enter the nasal passage and are perceived by the olfactory system (Meilgaard, Civille & Carr, **1987**). This was done by taking 3 short sniffs to avoid saturation and fatigue of the sensory organ (Maskowitz, **1988**). Before the actual sensory evaluation study, the panel members were exposed to different food samples and chemical standards that corresponded with the caramel, chocolate, nutty, meaty, coffee, potato, green and other odours (Table 2).

During the sensory evaluation, the flavour descriptions of the panel members were recorded and then categorized according to the main flavour groups mentioned above. Interpretation of the results was based on these main flavour groupings.

2.4. RESULTS

2.4.1. Flavour production by the fungi evaluated

The results obtained from the panel members are summarised in table 3. Here, the observations of each member are described, using the descriptors as indicated in table 4, for each fungus grown in the two different media used. The findings were used to compile tables 5 and 6 that represent the main flavour groups. The results for each fungus were grouped into the caramel, meaty, nutty, chocolate, coffee, green and potato odours. To explain how the results in table 3 were interpreted, an example is used where ***Mucor*** sp. (MRC 2430) was analysed. For example, the odours on Cz medium were described by panel member 1 as "strong coffee", by panel member 3 as "caramel" and by panel member 4 as "coffee/chocolate". In addition, panel member 2 indicated a "raw nuts odour" on TSB, Therefore, it is indicated in table 5, that this fungus is capable of producing flavours in the coffee, chocolate, caramel and nutty categories in table 5. In table 6, where Cz medium and TSB are compared with each other, the fungus is indicated to produce the caramel, chocolate and coffee flavours in Cz and a nutty flavour in TSB.

Figure 1, based on the results in table 5, indicates the total number of fungi that produced each flavour. It illustrates that a total of 50 fungi were capable of producing the caramel flavour, 45 the meaty flavour and 53 fungi produced a nutty flavour. The green flavours were produced by 66 fungi and the potato flavours by 12 fungi. Less than 10 fungi produced the remaining pyrazine flavours and no pyrazine odours were observed in 56% of the fungi screened. It was also noted that 66 fungi were able to produce other aromas not resembling pyrazines. Futhermore, it was observed that some pyrazine flavours were produced on both media (as indicated by an **X** in table 5).

2.4.2. Comparison of flavours produced on Cz and TSB

Figure 2, based on the findings in table 6, indicates the number of fungi capable of producing each flavour in the different media used. It was found that 40 of the fungi tested produced the caramel flavour in Cz medium, 46 produced the green flavour. The remaining flavours were produced by less than 20 fungi respectively. In TSB, 45 fungi produced nutty flavours, 35

produced meaty flavours, 40 green flavours, 16 caramel flavours and the remaining flavours were produced by less than 10 fungi respectively. It was also found that in Cz 65% of the fungi did not produce any pyrazine odours, whereas 67% of the fungi did not produce pyrazine odours in TSB.

It is also noted that certain groups are able to produce more pyrazine odours in one medium than in the other. For example, 47% of the basidiomycetous fungi tested produced pyrazine odours in the Cz medium whereas only 21% produced pyrazine odours in the TSB medium, and 54% of the ***Penicillium*** spp. tested produced pyrazine odours in TSB whereas only 32% produced pyrazine odours in Cz medium (Table 6).

2.4.3. Flavour production amongst the groups of fungi

Figure 3 shows the flavour production amongst the different taxonomic groups of the fungi tested. The results are expressed as the percentage of total fungi that produced aromas resembling each flavour category. All groups of fungi screened produced the meaty, nutty and green flavours. Only members of ***Fusarium*** and ***Penicillium*** did not produce caramel flavours. Chocolate flavours were not produced by members of ***Aspergillus***, ***Botryosphaeria***, ***Cladosporium***, ***Fusarium***, and ***Penicillium***. The basidiomycetous fungi, ***Botryosphaeria*** spp., the coelomycetous fungi and ***Penicillium*** spp. were not able to produce any potato flavours. Amongst all the groups screened, only the basidiomycetous fungi, members of the Mucorales, and ophiostomatoid fungi were able to produce coffee flavours.

Based on the results in table 5, 70% of the 33 tested ***Aspergillus*** isolates, 47% of the 19 tested basidiomycetous fungi, 27% of the 22 ***Botryosphaeria*** isolates, 42% of the 19 ***Ceratocystis*** spp., 43% of the 14 tested ***Cladosporium*** spp., 48% of the 31 coelomycetous fungi, 25% of the 20 tested ***Fusarium*** spp., 45% of the 22 fungi in the Mucorales, 39% of the 44 tested ophiostomatoid fungi, 54% of the 28 tested ***Penicillium*** isolates and 46% of other fungi were able to produce aromas resembling pyrazine flavours.

2.4.4. Flavour production by the fungi in the different culture collections

Figure 4 is a comparison of the production of flavours of fungi obtained from the two culture collections. In total, 51% of the 156 fungi tested from the CSIR culture collection were flavour producers whereas only 37% of the 125 fungi screened from the CMW culture collection were flavour producers. In the CSIR culture collection, 33% of the fungi tested produced green flavours and 21% produced nutty flavours. The meaty flavours were produced by 18% of the fungi tested and 15% indicated a caramel flavour. The rest of the flavours were produced by less than 10% of the fungi tested. In comparison, of the 125 fungi screened from the CMW culture collection, 20% were able to produce the caramel flavour. The meaty and nutty flavours were produced by 14% and 15% of the fungi respectively. Less than 20% of the fungi tested produced the remaining flavours.

2.5. DISCUSSION

Overall, fungi from the CSIR and UP culture collections showed to be good flavour producers, as 45% of the total fungi screened showed flavour production (Figure 1). These results only include the production of the caramel, meaty, nutty, chocolate, coffee, green and potato flavours, as these were the target odours for pyrazine flavour compounds. However, confirmations of pyrazine flavours need to be done with chemical analytical methods, as several compounds could be responsible for the same odour. However, the media used in this study should give preference for the production of pyrazine compounds, as it contains precursors that are needed for pyrazine formation. Thus, it is highly likely that the odours noted are due to the formation of pyrazines.

Although fungal isolates from both the CSIR and UP collections were found to produce flavours representing all pyrazine flavour categories, certain tendencies could be observed. For example, the most prominent flavour produced was the green flavour, followed by the nutty flavour and then the caramel and meaty flavours. A much smaller percentage of the fungi were able to produce the chocolate, coffee and potato flavours, indicating that the fungi from the CSIR and UP culture collections are not very good producers of

the latter flavours (Figure 4). Other non-pyrazine flavours were also noted during the screening process. These included the banana flavour produced by some isolates of ***Ceratocystis albifundus,*** as well as cheese and cabbage/cauliflower flavours produced by individual isolates.

It was evident that the use of different substrates could have a significant difference in the flavours produced as illustrated in figure 2. When fungi are grown in Cz medium, a higher percentage is shown to produce the caramel and chocolate flavours than when grown on TSB. However, when fungi are grown in the TSB medium, a higher percentage is shown to produce the nutty, meaty and potato flavours than when grown in the Cz medium. The green flavour was, however, prominent in both media. This further emphasizes the importance of the composition of substrates. The Cz medium used in this study had a wider range of amino acids and sugars than TSB medium and thus a wider range of pyrazine compounds were expected to be produced in this medium. An example is ***Mucor*** sp. (MRC 2430) that produced caramel, chocolate and coffee flavours in Cz medium compared to a nutty flavour in TSB. Not only did some fungi produce different flavours on each medium but it was also found that certain fungi were able to produce pyrazine odours only in one medium and not in the other. For example ***Rhizopus*** sp. (MRC 4489), produced the caramel, chocolate and coffee pyrazine flavours in the Cz medium and no flavours were observed in TSB (Table 6).

Figure 3 was examined to determine whether certain taxonomic groups could be targeted to produce a certain flavour. It was found that members of ***Penicillium*** and ***Aspergillus*** are more likely to produce the green flavour, and therefore, members of these groups could be regarded as good candidates when these types of flavours are sought after. When the meaty and potato flavours are targeted, ***Cladosporium*** spp. can be used as target organisms as a high percentage in this group produced these flavours. The Mucorales can be targeted for the production of the nutty, chocolate and coffee flavours as many of the fungi in the other groups produced these flavours at a lower percentage, or could not produce them at all. The caramel flavour is produced by a high percentage of basidiomycetous fungi and members of

Ceratocystis, which make these groups the most prominent producers of this flavour. In contrast, members of ***Penicillium*** and ***Fusarium*** were unable to produce this flavour.

When comparing flavour production at species level it was found that in certain cases fungi of the same species were able to produce the same flavour. For example, the common flavour amongst all ***Aspergillus niger*** isolates was the green flavour, and both ***Ceratocystis fimbriata*** isolates produced the caramel flavour (Table 5). It can also be concluded that members of ***Aspergillus*** and ***Penicillium*** contained the highest percentages of fungi able to synthesize flavours, whereas members of ***Fusarium*** and ***Botryosphaeria*** contained the least.

It was found that a higher percentage of fungi from the CSIR culture collection produced flavours than those from the UP culture collection (see figure 4). Results also indicated that the fungi in both culture collections have the affinity to produce the caramel, meaty and nutty flavours, but there is a significant difference in the production of the green flavour. A much higher percentage of fungi in the CSIR culture collection are able to produce the green flavour. This should rather be interpreted as a result of the number of ***Aspergillus*** and ***Penicillium*** isolates used in the screening process, as these fungi are most prominent in the production of the green flavour. However, it should be noted that the fungi screened were representative of the culture collections. Thus, the prominence of the production of particular flavours in certain groups should rather be seen in the context of the culture collections and not necessarily as a character of the taxonomic group in general.

When comparing flavour production by the fungi in the different culture collections, it can be concluded that ***Aspergillus*** spp., ***Penicillium*** spp., and basidiomycetous fungi are the most prominent groups of flavour producers in the CSIR culture collection, whereas the coelomycetous fungi, ***Ceratocystis*** spp. and ophiostomatoid fungi are the most prominent flavour producers in the UP culture collection.

2.6. CONCLUSION

Results from this study indicate that mycelial fungi can be regarded as good candidates to produce flavour compounds such as pyrazines. It is also evident that the CSIR and UP culture collections contain South African fungi that could have applications in the flavour and fragrance industries. Due to the pressure on flavour houses to supply especially the food industry with naturally derived flavour products, the utilisation of these fungi could be of benefit addressing this issue. Using plant derived flavour compounds limits the constant supply due to the seasonal dependence of the plants. In contrast, the use of microorganisms such as mycelial fungi can overcome a number of obstacles such as environmental influences. In addition, control over the production conditions enables the producer to optimise the production process and being able to supply a product that is constant in quality and availability.

This study is only a preliminary indication that South African fungi have the ability to produce aromas representing pyrazine flavours. Whether these fungi have the potential of producing these compounds economically still needs to be proven. It is not always desirable to use a microorganism that produce a number of compounds simultaneously as extraction and purification processes could become quite complex and costly. Further work is now needed to analytically confirm the presence of pyrazine flavours and determine the ability of these fungi to develop economically viable processes for flavour production.

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Table 1: Fungi used in the screening of pyrazine flavours.

Name of Fungi	Culture Collection Number	Name of Fungi	Culture Collection Number	Name of Fungi	Culture Collection Number
***Aspergillus* spp.**		**Basidiomycetous fungi**		***Ceratocystis* spp.**	
Aspergillus avenaceus	MRC 329	***Bjerkandera adusta***	WR 74, WR 195, WR 217	***Ceratocystis eucalyptorum***	CMW 694
Aspergillus candidus	MRC 223, MRC 1164	***Coriolus hirsutus***	WR 105, WR 407	***Ceratocystis fimbriata***	CMW 2473, CMW 10133
Aspergillus clavatus	MRC 1181	***Coriolus versicolor***	WR 164, WR 255, WR 283	***Ceratocystis longrostrum***	CMW 557, CMW 615, CMW 554
Aspergillus flavus	MRC 3952	***Ganoderma applanatum***	WR 150, WR 253	***Ceratocystis occoteae***	CMW 369
Aspergillus fumigatus	MRC 909	***Gloeophyllum sepiarium***	WR 133, WR 210	***Ceratocystis*** spp.	CMW 527, CMW 583, CMW 594
Aspergillus melleus	MRC 1025	***Gloeophyllum trabeum***	WR 68, WR 172	***Chalara elegans***	MRC 3797, MRC 3806, MRC 3810
Aspergillus niger	MRC 898, MRC 1182, MRC 1185, MRC 2572, MRC 3284, MRC 278	***Lenzites betulina***	WR 118, WR 402		
Aspergillus ochraceus	MRC 109, MRC 265	***Phellinus*** sp.	WR 193	***Cladosporium* spp.**	
Aspergillus parasiticus	MRC 11061	***Pycnoporus coccineus***	WR 102	***Cladosporium cladosporioides***	MRC 11388, MRC 10132, MRC 10150, MRC 10260, MRC 10810, MRC 10813
Aspergillus petrakii	MRC 888	***Pycnoporus sanguineus***	WR 114	***Cladosporium cucumerinum***	MRC 10183
Aspergillus nidulans	MRC 1183, MRC 3354			***Cladosporium macrocarpum***	MRC 10210, MRC 10211
		***Botryosphaeria* spp.**		***Cladosporium*** spp.	MRC 3367, MRC 3240, MRC 3366, MRC 3978
Aspergillus rugulosus	MRC 648	***Botryosphaeria dothidea***	CMW 774, CMW 892	***Cladosporium sphaerospermum***	MRC 10263
Aspergillus sydowii	MRC 862	***Botryosphaeria eucalyptorum***	CMW 10125		
				Coelomycetous fungi	
Aspergillus terreus	MRC 1309	***Botryosphaeria obtusa***	CMW 5991	Unknown coelomycetous fungi	CMW 463, CMW 433
Aspergillus umbrosus	MRC 895	***Botryosphaeria parasiticum***	CMW 10123	***Colletotrichum acutatum***	CMW 614
Aspergillus ustus	MRC 277, MRC1163, MRC 1226	***Botryosphaeria parva***	CMW 10122, CMW 10124	***Cytospora eucalypticola***	CMW 914
Aspergillus versicolor	MRC 184, MRC 2720	***Botryosphaeria*** spp.	CMW 197, CMW 328, CMW 568, CMW 903, CMW 947, CMW 954,CMW 7218, CMW 681, CMW 689, CMW 8232, CMW 8233, CMW 8313, CMW 8314	***Cytospora*** spp.	CMW 428, CMW 462, CMW 941
Aspergillus wentii	MRC 2378	Fusicoccum spp.	CMW 795, CMW 822	***Dothiorella*** spp.	CMW 431, CMW 898
Eurotium chevalieri	MRC 429, MRC 3771			***Hainesia lythri***	CMW 965
Eurotium rubrum	MRC 170	***Ceratocystis* spp.**		***Macrophomina phaseolina***	CMW 895
		Ceratocystis albifundus	CMW 4069, CMW 4080, CMW 4092, CMW 4095, CMW 9383		

Table1 (Continued): Fungi used in the screening for pyrazine flavours.

Name of Fungi	Culture Collection Number	Name of Fungi	Culture Collection Number	Name of Fungi	Culture Collection Number
Coelomycetous fungi		**Mucorales**		**Ophiostomatoid fungi**	
Mycosphaerella pini	CMW 686	***Mucor*** spp.	MRC 3173, MRC 4087, MRC 2430, MRC 3243, MRC 3364	***Leptographium*** spp.	CMW 14, CMW 194 CMW 195, CMW 196, CMW 377, CMW 579
Pestalosphaeria sp.	CMW 678	***Rhizopus arrhizus***	MRC 4049, MRC 10225	***Leptographium truncatum***	CMW 406
Pestalotia sp.	CMW 335	***Rhizopus japonicus***	MRC 3185	***Ophiostoma casuarinae***	CMW 780
Phoma betae	MRC 582	***Rhizopus microsporus***	MRC 3935	***Ophiostoma africanum***	CMW 824
Phoma exigua var. foveata	MRC 590	***Rhizopus microsporus*** var ***chinesis***	MRC 10226, MRC 4631	***Ophiostoma cynaroides***	CMW 773
Phoma pomorum	MRC 588	***Rhizopus oryzae***	MRC 2129, MRC 2367, MRC 3319, MRC 3905, MRC 4633	***Ophiostoma ips***	CMW 312
Phoma sorghina	CGM 1265, CGM 1266, CGM 1267, CGM 1268, CGM 1269	***Rhizopus rhizopodiformis***	MRC 1954	***Ophiostoma longrostrum***	CMW 403
Phoma sp.	MRC 3792	***Rhizopus*** spp.	MRC 2770, MRC 4083, MRC 4489	***Ophiostoma macaranga***	CMW 931
Phomopsis sp.	CMW 430	***Rhizopus stolonifer***	MRC 3830, MRC 4050	***Ophiostoma*** ex ***Protea coronata***	CMW 977
Seiridium cardinale	CMW 600, CMW 635, CMW 690	**Ophiostomatoid fungi**		***Ophiostoma quercum***	CMW 865
Seiridium sp.	CMW 606	***Gondwanamyces capense***	CMW 978, CMW 1000	***Ophiostoma*** spp.	CMW 387, CMW 604, CMW 542, CMW 548
Seiridium unicorne	CMW 616	***Gondwanamyces fasciata***	CMW 627	***Ophiostoma splendens***	CMW 924, CMW 985, CMW 897
Staganospora sp.	CMW 685	***Gondwanamyces minuta***	CMW 434	***Sporothrix*** spp.	CMW 398, CMW 400
***Fusarium* spp.**		***Gondwanamyces proteae***	CMW 739, CMW 987	***Thallographium*** sp.	CMW 601, CMW 741
Fusarium anthophilum	MRC 10308	***Graphidium*** sp.	CMW 590	**Penicillium spp.**	
Fusarium chlamydosporum	MRC 3420	***Graphium curvispora***	CMW 934	***Penicillium brevicompactum***	MRC 1731
Fusarium napiforme	MRC 4142, MRC 4267	***Graphium longrostrum***	CMW 628	***Penicillium camembertii***	MRC 1736
Fusarium oxysporum	MRC 4614	***Graphium macaranga***	CMW 935	***Penicillium citrinum***	MRC 224, MRC 304
Fusarium sambucinum	MRC 514	***Graphium*** sp.	CMW 729	***Penicillium commune***	MRC 1729
Fusarium scirpi	MRC 2808, MRC 3774	***Hyalorhinocladiella*** spp.	CMW 593, CMW 538, CMW 539, CMW 540	***Penicillium crustosum***	MRC 316
Fusarium solani	MRC 3767	***Leptographium procerum***	CMW 522	***Penicillium expansum***	MRC 199, MRC 1735
Fusarium subglutinans	MRC 10336	***Leptographium reconditum***	CMW 15	***Penicillium funiculosum***	MRC 281

Fusarium verticillioides	MRC 1171, CGM 1270, MRC 10719, MRC 10723, MRC 10736, MRC 10832, MRC 32, MRC 89, MRC 2338, MRC 10342	***Leptographium serpens***		***Penicillium griseofulvum***	MRC 270, MRC 312, MRC 1741

Table 1 (Continued): Fungi used in the screening for pyrazine flavours.

Name of Fungi	Culture Collection Number	Name of Fungi	Culture Collection Number	Name of Fungi	Culture Collection Number
***Penicillium* spp.**		**Other fungi**		**Other fungi**	
Penicillium islandicum	MRC 397	***Beauveria bassiana***	CMW 391	***Heliconium*** sp.	CMW 339
Penicillium oxalicum	MRC 322, MRC 1740	***Botrytis cinerea***	MRC 3966, MRC 10207	***Hyalodendron*** sp.	CMW 920
Penicillium purpurogenum	MRC 181, MRC 2501	***Byssochlamys nivea***	MRC 10751	***Lophodermium australe***	CMW 683
Penicillium roqueforti	MRC 1697, MRC 1742	***Cenangium ferruginosum***	CMW 680	***Nasmacyclius niveus***	CMW 460
Penicillium rubrum	MRC 1723	***Chaetomium angustisporum***	CGM 1272, MRC 2399	***Phaeoisaria*** sp.	CMW 811
Penicillium spp.	MRC 4607, CGM 1271, MRC 194, MRC 1270	***Chaetopsina*** sp.	CMW 592	***Phialophora*** sp.	CMW 581
Penicillium stoloniferum	MRC 1739	***Curvularia*** spp.	MRC 4566, MRC 1862	***Rhizoctonia*** sp.	CMW 410
Penicillium verrucosum var ***cyclopium***	MRC 1738	***Cyclaneusma minus***	CMW 679	***Stibella*** sp.	CMW 973
Penicillium viridicatum	MRC 292, MRC 10332	***Cylindrocladium*** sp.	CMW 950	***Subulispora*** sp.	CMW 796
Other fungi		***Cylindrocarpon*** sp.	CMW 814		
Ambrosiella sp.	CMW 375	***Geotrichum*** sp.	MRC 1998		
Arthrobotrys spp.	CMW 559, CMW 537	***Gliocladium*** spp.	CMW 599, CMW 640		

Table 2: Samples, descriptors and references used in the sensory evaluation training.

Descriptor	Reference
Caramel	Caramel
	Toffee
	Fudge
	*2,3,5-Trimethylpyrazine
Meaty	Smoked beef chips
	BBQ chips
	Biltong
	Soup powder
Nutty	Roasted peanuts
	Almonds
	Pecan nuts
	Peanut butter
	*2,3,5-Trimethylpyrazine
	2,3-Dimethylpyrazine
	*2-Methoxypyrazine
	2-Acetyl-3,5(6)-dimethylpyrazine
Chocolate	Chocolate
	Cocoa
	*2,3,5-Trimethylpyrazine
	*2-Methoxypyrazine
Coffee	Coffee
Green	Green peppers cubed
	Green beans chopped
	Wet soil
	Green grass
	2-Methoxy-3-methylpyrazine
	2-Methoxy-3,5(6)-dimethylpyazine
Potato	Baked potato
	Plain chips

*Chemical standards at different concentrations demonstrate different odours

Table 3 (Refer to excel document)

Table 4: Description of flavours.

Flavour Group	Description
Caramel	Caramel, toffee, honey
Meaty	Meaty, beefy, savoury, Marmite, salty, soup
Nutty	Roasted nuts, peanuts, old shoes, boiled nuts, any other nutty odour
Chocolate	Chocolate, cocoa
Coffee	Coffee
Green	Earthy, soil, green beans, green peas, celery, parsley, green vegetables
Potato	Potato (baked, fried, mashed), chips
Other	Banana, cheese, strawberry, ammonia, cabbage, cauliflower

Table 5: Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
***Aspergillus* group**											**60**	**58**	**70**
Aspergillus avenaceus	MRC 329									3			
Aspergillus candidus	MRC 223			X						2			
Aspergillus candidus	MRC 1164									1			
Aspergillus clavatus	MRC 1181												
Aspergillus flavus	MRC 3952												
Aspergillus fumigatus	MRC 909												
Aspergillus melleus	MRC 1025									1			
Aspergillus niger	MRC 898						X			1			
Aspergillus niger	MRC 1182						X			1			
Aspergillus niger	MRC 1185									1			
Aspergillus niger	MRC 2752									2			
Aspergillus niger	MRC 3284						X			2			
Aspergillus niger group	MRC 278						X			2			
Aspergillus ochraceus	MRC 109						X			1			
Aspergillus ochraceus	MRC 265												
Aspergillus parasiticus	MRC 11061												
Aspergillus petrakii	MRC 888												
Aspergillus nidulans	MRC 1183									2			
Aspergillus nidulans	MRC 3354												
Aspergillus rugulosus	MRC 648												
Aspergillus sydowii	MRC 862		X				X			3			
Aspergillus terreus	MRC 1309						X			2			
Aspergillus umbrosus	MRC 895												
Aspergillus ustus	MRC 277						X			3			
Aspergillus ustus	MRC 1163						X			1			
Aspergillus ustus	MRC 1226						X			4			
Aspergillus versicolor	MRC 184						X			1			
Aspergillus versicolor	MRC 2720												
Aspergillus wentii	MRC 2378									2			
Eurotium chevalieri	MRC 429									2			
Eurotium chevalieri	MRC 3771									2			
Eurotium rubrum	MRC 170									1			
Eurotium sp.	MRC 2811									1			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus

X = Flavours produced on both media
Colour coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
Basidiomycetous fungi											**47**	**21**	**47**
Bjerkandera adusta	WR 74									2			
Bjerkandera adusta	WR 195												
Bjerkandera adusta	WR 217									2			
Coriolus hirsutus	WR 105												
Coriolus hirsutus	WR 407												
Coriolus versicolor	WR 164												
Coriolus versicolor	WR 255												
Coriolus versicolor	WR 283									2			
Gloeophyllum sepiarium	WR 133									1			
Gloeophyllum sepiarium	WR 210			X						1			
Gloeophyllum trabeum	WR 68												
Gloeophyllum trabeum	WR 172									1			
Ganoderma applanatum	WR 150												
Ganoderma applanatum	WR 253												
Lenzites betulina	WR 118									1			
Lenzites betulina	WR 402									1			
Phellinus sp.	WR 193												
Pycnoporus coccineus	WR 102												
Pycnoporus sanguineus	WR 114	X								3			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Color coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
***Botryosphaeria* spp.**											**18**	**23**	**27**
Botryosphaeria dothidea	CMW 774									1			
Botryosphaeria dothidea	CMW 892									2			
Botryosphaeria eucalyptorum	CMW 10125												
Botryosphaeria obtusa	CMW 5991												
Botryosphaeria parasiticum	CMW 10123									2			
Botryosphaeria parva	CMW 10122												
Botryosphaeria parva	CMW 10124												
***Botryosphaeria* sp.**	CMW 197												
***Botryosphaeria* sp.**	CMW 328												
***Botryosphaeria* sp.**	CMW 568												
***Botryosphaeria* sp.**	CMW 903						X			1			
***Botryosphaeria* sp.**	CMW 947												
***Botryosphaeria* sp.**	CMW 954												
***Botryosphaeria* sp.**	CMW 7218												
***Botryosphaeria* sp.**	CMW 681												
***Botryosphaeria* sp.**	CMW 689												
***Botryosphaeria* sp.**	CMW 8232												
***Botryosphaeria* sp.**	CMW 8233												
***Botryosphaeria* sp.**	CMW 8313												
***Botryosphaeria* sp.**	CMW 8314												
***Fusicoccum* sp.**	CMW 795	X								2			
***Fusicoccum* sp.**	CMW 822									2			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Color coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of fungi	CC #	Pyrazine flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
***Ceratocystis* spp.**											**37**	**21**	**42**
Ceratocystis albifundus	CMW 4069												
Ceratocystis albifundus	CMW 4080												
Ceratocystis albifundus	CMW 4092									2			
Ceratocystis albifundus	CMW 4095									2			
Ceratocystis albifundus	CMW 9383												
Ceratocystis casuarinae	CMW 779												
Ceratocystis eucalyptorum	CMW 694												
Ceratocystis fimbriata	CMW 2473									1			
Ceratocystis fimbriata	CMW 10133	X								2			
Ceratocystis longrostrum	CMW 557												
Ceratocystis longrostrum	CMW 615												
Ceratocystis longrostrum	CMW 554												
Ceratocystis occoteae	CMW 369												
***Ceratocystis* sp.**	CMW 527												
***Ceratocystis* sp.**	CMW 583									3			
***Ceratocystis* sp.**	CMW 594												
Chalara elegans	MRC 3797									2			
Chalara elegans	MRC 3806									1			
Chalara elegans	MRC 3810												
***Cladosporium* spp.**											**14**	**36**	**43**
Cladosporium cladosporioides	MRC 11388			X						3			
Cladosporium cladosporioides	MRC 10132												
Cladosporium cladosporioides	MRC 10150									2			
Cladosporium cladosporioides	MRC 10260												
Cladosporium cladosporioides	MRC 10810												
Cladosporium cladosporioides	MRC 10813												
Cladosporium cucumerinum	MRC 10183									2			
Cladosporium macrocarpum	MRC 10210									2			
Cladosporium macrocarpum	MRC 10211												
***Cladosporium* sp.**	MRC 3367									2			
***Cladosporium* sp.**	MRC 3240												
***Cladosporium* sp.**	MRC 3366												
***Cladosporium* sp.**	MRC 3978												
Cladosporium sphaerospermum	MRC 10263									2			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection

Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Colour coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
Coelomycetous fungi											**39**	**23**	**48**
Coelomycetes	CMW 463												
Coelomycetes	CMW 433												
Colletotrichum acutatum	CMW 614									2			
Cytospora eucalypticola	CMW 914									1			
Cytospora sp.	CMW 428									2			
Cytospora sp.	CMW 462												
Cytospora sp.	CMW 941												
Dothiorella sp.	CMW 431												
Dothiorella sp.	CMW 898									2			
Hainesia lythri	CMW 965									2			
Macrophomina phaseolina	CMW 895												
Mycosphaerella gibsonii	CMW 687									1			
Mycosphaerella pini	CMW 686									1			
Pestalosphaeria sp.	CMW 678									2			
Pestalotia sp.	CMW 335									1			
Phoma betae	MRC 582												
Phoma exigua var. ***foveata***	MRC 590						X			3			
Phoma pomorum	MRC 588												
Phoma sorghina	CGM 1265									1			
Phoma sorghina	CGM 1266												
Phoma sorghina	CGM 1267												
Phoma sorghina	CGM 1268												
Phoma sorghina	CGM 1269									3			
Phoma sp.	MRC 3792			X						3			
Phomopsis sp.	CMW 430									2			
Seiridium cardinale	CMW 600												
Seiridium cardinale	CMW 635												
Seiridium cardinale	CMW 690												
Seiridium sp.	CMW 606												
Seiridium unicorne	CMW 616												
Staganospora sp.	CMW 685									1			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media

Colour coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
***Fusarium* spp.**											**10**	**25**	**25**
Fusarium anthophilum	MRC 10308												
Fusarium chlamydosporum	MRC 3420												
Fusarium napiforme	MRC 4142			X						3			
Fusarium napiforme	MRC 4267												
Fusarium oxysporum	MRC 4614												
Fusarium sambucinum	MRC 514												
Fusarium scirpi	MRC 2808												
Fusarium scirpi	MRC 3774									2			
Fusarium solani	MRC 3767									1			
Fusarium subglutinans	MRC 10336									1			
Fusarium verticillioides	MRC 1171												
Fusarium verticillioides	CGM 1270												
Fusarium verticillioides	MRC 10719												
Fusarium verticillioides	MRC 10723												
Fusarium verticillioides	MRC 10736												
Fusarium verticillioides	MRC 10832												
Fusarium verticillioides	MRC 32												
Fusarium verticillioides	MRC 89									2			
Fusarium verticillioides	MRC 2338												
Fusarium verticillioides	MRC 10342												

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Colour coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
Mucorales											**45**	**41**	**45**
Mucor sp.	MRC 3173												
Mucor sp.	MRC 4087												
Mucor sp.	MRC 2430									4			
Mucor sp.	MRC 3243												
Mucor sp.	MRC 3364									3			
Rhizopus arrhizus	MRC 4049									2			
Rhizopus arrhizus	MRC 10225												
Rhizopus japonicus	MRC 3185												
Rhizopus microsporus	MRC 3935									3			
Rhizopus microsporus var chinesis	MRC 10226												
Rhizopus microsporus var chinesis	MRC 4631									3			
Rhizopus oryzae	MRC 2129												
Rhizopus oryzae	MRC 2367												
Rhizopus oryzae	MRC 3319												
Rhizopus oryzae	MRC 3905												
Rhizopus oryzae	MRC 4633												
Rhizopus rhizopodiformis	MRC 1954									3			
Rhizopus sp.	MRC 2770									2			
Rhizopus sp.	MRC 4083												
Rhizopus sp.	MRC 4489									3			
Rhizopus stolonifer	MRC 3830									5			
Rhizopus stolonifer	MRC 4050									3			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Color coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
Ophiostomatoid fungi											**32**	**32**	**39**
Gondwanamyces capense	CMW 978									1			
Gondwanamyces capense	CMW 1000									1			
Gondwanamyces fasciata	CMW 627												
Gondwanamyces minuta	CMW 434												
Gondwanamyces proteae	CMW 739												
Gondwanamyces proteae	CMW 987												
Graphidium sp.	CMW 590												
Graphium curvispora	CMW 934									1			
Graphium longrostrum	CMW 628												
Graphium macaranga	CMW 935												
Graphium spp.	CMW 729												
Hyalorhinocladiella sp.	CMW 593												
Hyalorhinocladiella sp.	CMW 538									3			
Hyalorhinocladiella sp.	CMW 539									2			
Hyalorhinocladiella sp.	CMW 540												
Leptographium procerum	CMW 522									2			
Leptographium reconditum	CMW 15	X								2			
Leptographium serpens	CMW 381												
Leptographium sp.	CMW 14												
Leptographium sp.	CMW 194									3			
Leptographium sp.	CMW 195												
Leptographium sp.	CMW 196									2			
Leptographium sp.	CMW 377												
Leptographium sp.	CMW 579												
Leptographium truncatum	CMW 406	X								1			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Colour coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapecks Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
Ophiostomatoid fungi													
Ophiostoma africanum	CMW 824												
Ophiostoma casuarinae	CMW 780									1			
Ophiostoma cynaroides	CMW 773												
Ophiostoma ips	CMW 312												
Ophiostoma longrostrum	CMW 403									4			
Ophiostoma macaranga	CMW 931												
Ophiostoma ex ***Protea coronata***	CMW 977												
Ophiostoma quercum	CMW 865												
Ophiostoma sp.	CMW 387												
Ophiostoma sp.	CMW 604												
Ophiostoma sp.	CMW 542									2			
Ophiostoma sp.	CMW 548		X	X						2			
Ophiostoma splendens	CMW 924									4			
Ophiostoma splendens	CMW 985												
Ophiostoma splendens	CMW 897												
Sporothrix sp.	CMW 398												
Sporothrix sp.	CMW 400									2			
Thallographium sp.	CMW 601									2			
Thallographium sp.	CMW 741												

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Color coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
***Penicillium* spp.**											**32**	**54**	**54**
Penicillium brevicompactum	MRC 1731												
Penicillium camembertii	MRC 1736												
Penicillium citrinum	MRC 224									2			
Penicillium citrinum	MRC 304												
Penicillium commune	MRC 1729									1			
Penicillium crustosum	MRC 316									2			
Penicillium expansum	MRC 199						X			1			
Penicillium expansum	MRC 1735												
Penicillium funiculosum	MRC 281												
Penicillium griseofulvum	MRC 270												
Penicillium griseofulvum	MRC 312						X			3			
Penicillium griseofulvum	MRC 1741												
Penicillium islandicum	MRC 397									1			
Penicillium oxalicum	MRC 322						X			1			
Penicillium oxalicum	MRC 1740									2			
Penicillium purpurogenum	MRC 181									1			
Penicillium purpurogenum	MRC 2501									1			
Penicillium roqueforti	MRC 1697												
Penicillium roqueforti	MRC 1742									1			
Penicillium rubrum	MRC 1723						X			1			
***Penicillium* sp.**	MRC 4607												
***Penicillium* sp.**	CGM 1271									2			
***Penicillium* sp.**	MRC 194												
***Penicillium* sp.**	MRC 1270												
Penicillium stoloniferum	MRC 1739												
Penicillium verrucosum* var *cyclopium	MRC 1738						X			1			
Penicillium viridicatum	MRC 292												
Penicillium viridicatum	MRC 10332						X			1			

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Colour coded = Flavour production observed

Table 5 (continued): Results of pyrazine producers on Czapeck Dox medium and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours								Results			
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	Positive (Total)	Cz %	TSB%	Total%
Other fungi											**32**	**39**	**46**
Ambrosiella sp.	CMW 375												
Arthrobotrys sp.	CMW 559									2			
Arythrobotrys sp.	CMW 537									2			
Beauveria bassiana	CMW 391									2			
Botrytis cinerea	MRC 3966						X			1			
Botrytis cinerea	MRC 10207									1			
Byssochlamys nivea	MRC 10751												
Cenangium ferruginosum	CMW 680												
Chaetomium angustisporum	CGM 1272												
Chaetomium thielavioideum	MRC 2399												
Chaetopsina sp.	CMW 592									2			
Curvularia sp.	MRC 4566									3			
Curvularia sp.	MRC 1862									1			
Cyclaneusma minus	CMW 679									1			
Cylindrocladium sp.	CMW 950												
Cylindrocarpon sp.	CMW 814												
Geotrichum sp.	MRC 1998			X						2			
Gliocladium sp.	CMW 599												
Gliocladium sp.	CMW 640												
Heliconium sp.	CMW 339												
Hyalodendron sp.	CMW 920												
Lophodermium australe	CMW 683									3			
Nasmacyclius niveus	CMW 460			X						3			
Phaeoisaria sp.	CMW 811												
Phialophora sp.	CMW 581												
Rhizoctonia sp.	CMW 410												
Stibella sp.	CMW 973												
Subulispora sp.	CMW 796									2			
Total flavours produced		**50**	**45**	**53**	**7**	**6**	**66**	**12**	**66**				

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Positive (total) =The amount of different aromas detected by the sniffing panel in each fungus
X = Flavours produced on both media
Color coded = Flavour production observed

Table 6: Comparison of results on Czapeck Dox and Tryptic Soy Broth

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz%	Pyrazine Flavours in TSB								TSB%
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
***Aspergillus* spp.**										**60**									**58**
Aspergillus avenaceus	MRC 329																		
Aspergillus candidus	MRC 223																		
Aspergillus candidus	MRC 1164																		
Aspergillus clavatus	MRC 1181																		
Aspergillus flavus	MRC 3952																		
Aspergillus fumigatus	MRC 909																		
Aspergillus melleus	MRC 1025																		
Aspergillus niger	MRC 898																		
Aspergillus niger	MRC 1182																		
Aspergillus niger	MRC 1185																		
Aspergillus niger	MRC 2752																		
Aspergillus niger	MRC 3284																		
Aspergillus niger group	MRC 278																		
Aspergillus ochraceus	MRC 109																		
Aspergillus ochraceus	MRC 265																		
Aspergillus parasiticus	MRC 11061																		
Aspergillus petrakii	MRC 888																		
Aspergillus nidulans	MRC 1183																		
Aspergillus nidulans	MRC 3354																		
Aspergillus rugulosus	MRC 648																		
Aspergillus sydowii	MRC 862																		
Aspergillus terreus	MRC 1309																		
Aspergillus umbrosus	MRC 895																		
Aspergillus ustus	MRC 277																		
Aspergillus ustus	MRC 1163																		
Aspergillus ustus	MRC 1226																		
Aspergillus versicolor	MRC 184																		
Aspergillus versicolor	MRC 2720																		
Aspergillus wentii	MRC 2378																		
Eurotium chevalieri	MRC 429																		
Eurotium chevalieri	MRC 3771																		
Eurotium rubrum	MRC 170																		
Eurotium sp.	MRC 2811																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection

Colour coded = Flavour production observed
CGM fungi obtained from the CAMS culture collection

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz%	Pyrazine Flavours in TSB								TSB%
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
Basidiomycetous fungi										**47**									**21**
Bjerkandera adusta	WR 74																		
Bjerkandera adusta	WR 195																		
Bjerkandera adusta	WR 217																		
Coriolus hirsutus	WR 105																		
Coriolus hirsutus	WR 407																		
Coriolus versicolor	WR 164																		
Coriolus versicolor	WR 255																		
Coriolus versicolor	WR 283																		
Gloeophyllum sepiarium	WR 133																		
Gloeophyllum sepiarium	WR 210																		
Gloeophyllum trabeum	WR 68																		
Gloeophyllum trabeum	WR 172																		
Ganoderma applanatum	WR 150																		
Ganoderma applanatum	WR 253																		
Lenzites betulina	WR 118																		
Lenzites betulina	WR 402																		
Phellinus sp.	WR 193																		
Pycnoporus coccineus	WR 102																		
Pycnoporus sanguineus	WR 114																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection

Color coded = Flavour production observed

Table 6 (continued): Comparison of results on Czapeck Dox and [illegible]

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
***Botryosphaeria* spp.**										**18**									**23**
Botryosphaeria dothidea	CMW 774																		
Botryosphaeria dothidea	CMW 892																		
Botryosphaeria eucalyptorum	CMW 10125																		
Botryosphaeria obtusa	CMW 5991																		
Botryosphaeria parasiticum	CMW 10123																		
Botryosphaeria parva	CMW 10122																		
Botryosphaeria parva	CMW 10124																		
***Botryosphaeria* sp.**	CMW 197																		
***Botryosphaeria* sp.**	CMW 328																		
***Botryosphaeria* sp.**	CMW 568																		
***Botryosphaeria* sp.**	CMW 903																		
***Botryosphaeria* sp.**	CMW 947																		
***Botryosphaeria* sp.**	CMW 954																		
***Botryosphaeria* sp.**	CMW 7218																		
***Botryosphaeria* sp.**	CMW 681																		
***Botryosphaeria* sp.**	CMW 689																		
***Botryosphaeria* sp.**	CMW 8232																		
***Botryosphaeria* sp.**	CMW 8233																		
***Botryosphaeria* sp.**	CMW 8313																		
***Botryosphaeria* sp.**	CMW 8314																		
***Fusicoccum* sp.**	CMW 795																		
***Fusicoccum* sp.**	CMW 822																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained from the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Colour coded = Flavour production observed

Table 6 (continued): Comparison of results on Czapeck Dox

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Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
***Ceratocystis* spp.**										**37**									**21**
Ceratocystis albifundus	CMW 4069																		
Ceratocystis albifundus	CMW 4080																		
Ceratocystis albifundus	CMW 4092																		
Ceratocystis albifundus	CMW 4095																		
Ceratocystis albifundus	CMW 9383																		
Ceratocystis casuarinae	CMW 779																		
Ceratocystis eucalyptorum	CMW 694																		
Ceratocystis fimbriata	CMW 2473																		
Ceratocystis fimbriata	CMW 10133																		
Ceratocystis longrostrum	CMW 557																		
Ceratocystis longrostrum	CMW 615																		
Ceratocystis longrostrum	CMW 554																		
Ceratocystis occoteae	CMW 369																		
Ceratocystis sp.	CMW 527																		
Ceratocystis sp.	CMW 583																		
Ceratocystis sp.	CMW 594																		
Chalara elegans	MRC 3797																		
Chalara elegans	MRC 3806																		
Chalara elegans	MRC 3810																		
***Cladosporium* spp.**										**14**									**36**
Cladosporium cladosporioides	MRC 11388																		
Cladosporium cladosporioides	MRC 10132																		
Cladosporium cladosporioides	MRC 10150																		
Cladosporium cladosporioides	MRC 10260																		
Cladosporium cladosporioides	MRC 10810																		
Cladosporium cladosporioides	MRC 10813																		
Cladosporium cucumerinum	MRC 10183																		
Cladosporium macrocarpum	MRC 10210																		
Cladosporium macrocarpum	MRC 10211																		
Cladosporium sp.	MRC 3367																		
Cladosporium sp.	MRC 3240																		
Cladosporium sp.	MRC 3366																		
Cladosporium sp.	MRC 3978																		
Cladosporium sphaerospermum	MRC 10263																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained from the FABI culture collection

Colour coded = Flavour production observed
CGM fungi obtained from the CAMS culture collection

Table 6 (continued): Comparison of results on Czapeck Dox

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
Coelomycetous fungi										**39**									**23**
Coelomycetes	CMW 463																		
Coelomycetes	CMW 433																		
Colletotrichum acutatum	CMW 614																		
Cytospora eucalypticola	CMW 914																		
Cytospora sp.	CMW 428																		
Cytospora sp.	CMW 462																		
Cytospora sp.	CMW 941																		
Dothiorella sp.	CMW 431																		
Dothiorella sp.	CMW 898																		
Hainesia lythri	CMW 965																		
Macrophomina phaseolina	CMW 895																		
Mycosphaerella gibsonii	CMW 687																		
Mycosphaerella pini	CMW 686																		
Pestalosphaeria sp.	CMW 678																		
Pestalotia sp.	CMW 335																		
Phoma betae	MRC 582																		
Phoma exigua var. foveata	MRC 590																		
Phoma pomorum	MRC 588																		
Phoma sorghina	CGM 1265																		
Phoma sorghina	CGM 1266																		
Phoma sorghina	CGM 1267																		
Phoma sorghina	CGM 1268																		
Phoma sorghina	CGM 1269																		
Phoma sp.	MRC 3792																		
Phomopsis sp.	CMW 430																		
Seiridium cardinale	CMW 600																		
Seiridium cardinale	CMW 635																		
Seiridium cardinale	CMW 690																		
Seiridium sp.	CMW 606																		
Seiridium unicorne	CMW 616																		
Staganospora sp.	CMW 685																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection

Colour coded = Flavour production observed
CGM fungi obtained from the CAMS culture collection

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
***Fusarium* spp.**										**10**									**25**
Fusarium anthophilum	MRC 10308																		
Fusarium chlamydosporum	MRC 3420																		
Fusarium napiforme	MRC 4142																		
Fusarium napiforme	MRC 4267																		
Fusarium oxysporum	MRC 4614																		
Fusarium sambucinum	MRC 514																		
Fusarium scirpi	MRC 2808																		
Fusarium scirpi	MRC 3774																		
Fusarium solani	MRC 3767																		
Fusarium subglutinans	MRC 10336																		
Fusarium verticillioides	MRC 1171																		
Fusarium verticillioides	CGM 1270																		
Fusarium verticillioides	MRC 10719																		
Fusarium verticillioides	MRC 10723																		
Fusarium verticillioides	MRC 10736																		
Fusarium verticillioides	MRC 10832																		
Fusarium verticillioides	MRC 32																		
Fusarium verticillioides	MRC 89																		
Fusarium verticillioides	MRC 2338																		
Fusarium verticillioides	MRC 10342																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection

Colour coded = Flavour production observed
CGM fungi obtained from the CAMS culture collection

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
Mucorales										**45**									**41**
Mucor sp.	MRC 3173																		
Mucor sp.	MRC 4087																		
Mucor sp.	MRC 2430																		
Mucor sp.	MRC 3243																		
Mucor sp.	MRC 3364																		
Rhizopus arrhizus	MRC 4049																		
Rhizopus arrhizus	MRC 10225																		
Rhizopus japonicus	MRC 3185																		
Rhizopus microsporus	MRC 3935																		
Rhizopus microsporus var chinesis	MRC 10226																		
Rhizopus microsporus var chinesis	MRC 4631																		
Rhizopus oryzae	MRC 2129																		
Rhizopus oryzae	MRC 2367																		
Rhizopus oryzae	MRC 3319																		
Rhizopus oryzae	MRC 3905																		
Rhizopus oryzae	MRC 4633																		
Rhizopus rhizopodiformis	MRC 1954																		
Rhizopus sp.	MRC 2770																		
Rhizopus sp.	MRC 4083																		
Rhizopus sp.	MRC 4489																		
Rhizopus stolonifer	MRC 3830																		
Rhizopus stolonifer	MRC 4050																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained from the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Colour coded = Flavour production observed

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
Ophiostomatoid fungi										**32**									**32**
Gondwanamyces capense	CMW 978																		
Gondwanamyces capense	CMW 1000																		
Gondwanamyces fasciata	CMW 627																		
Gondwanamyces minuta	CMW 434																		
Gondwanamyces proteae	CMW 739																		
Gondwanamyces proteae	CMW 987																		
Graphidium sp.	CMW 590																		
Graphium curvispora	CMW 934																		
Graphium longrostrum	CMW 628																		
Graphium macaranga	CMW 935																		
Graphium spp.	CMW 729																		
Hyalorhinocladiella sp.	CMW 593																		
Hyalorhinocladiella sp.	CMW 538																		
Hyalorhinocladiella sp.	CMW 539																		
Hyalorhinocladiella sp.	CMW 540																		
Leptographium procerum	CMW 522																		
Leptographium reconditum	CMW 15																		
Leptographium serpens	CMW 381																		
Leptographium sp.	CMW 14																		
Leptographium sp.	CMW 194																		
Leptographium sp.	CMW 195																		
Leptographium sp.	CMW 196																		
Leptographium sp.	CMW 377																		
Leptographium sp.	CMW 579																		
Leptographium truncatum	CMW 406																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Color coded = Flavour production observed

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
Ophiostomatoid fungi										**31**									**31**
Ophiostoma africanum	CMW 824																		
Ophiostoma casuarinae	CMW 780																		
Ophiostoma cynaroides	CMW 773																		
Ophiostoma ips	CMW 312																		
Ophiostoma longrostrum	CMW 403																		
Ophiostoma macaranga	CMW 931																		
Ophiostoma ex ***Protea coronata***	CMW 977																		
Ophiostoma quercum	CMW 865																		
Ophiostoma sp.	CMW 387																		
Ophiostoma sp.	CMW 604																		
Ophiostoma sp.	CMW 542																		
Ophiostoma sp.	CMW 548																		
Ophiostoma splendens	CMW 924																		
Ophiostoma splendens	CMW 985																		
Ophiostoma splendens	CMW 897																		
Sporothrix sp.	CMW 398																		
Sporothrix sp.	CMW 400																		
Thallographium sp.	CMW 601																		
Thallographium sp.	CMW 741																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained form the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Colour coded = Flavour production observed

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
***Penicillium* spp.**										**32**									**54**
Penicillium brevicompactum	MRC 1731																		
Penicillium camembertii	MRC 1736																		
Penicillium citrinum	MRC 224																		
Penicillium citrinum	MRC 304																		
Penicillium commune	MRC 1729																		
Penicillium crustosum	MRC 316																		
Penicillium expansum	MRC 199																		
Penicillium expansum	MRC 1735																		
Penicillium funiculosum	MRC 281																		
Penicillium griseofulvum	MRC 270																		
Penicillium griseofulvum	MRC 312																		
Penicillium griseofulvum	MRC 1741																		
Penicillium islandicum	MRC 397																		
Penicillium oxalicum	MRC 322																		
Penicillium oxalicum	MRC 1740																		
Penicillium purpurogenum	MRC 181																		
Penicillium purpurogenum	MRC 2501																		
Penicillium roqueforti	MRC 1697																		
Penicillium roqueforti	MRC 1742																		
Penicillium rubrum	MRC 1723																		
***Penicillium* sp.**	MRC 4607																		
***Penicillium* sp.**	CGM 1271																		
***Penicillium* sp.**	MRC 194																		
***Penicillium* sp.**	MRC 1270																		
Penicillium stoloniferum	MRC 1739																		
Penicillium verrucosum var cyclopium	MRC 1738																		
Penicillium viridicatum	MRC 292																		
Penicillium viridicatum	MRC 10332																		

MRC and WR fungi obtained from the CSIR culture collection
CMW fungi obtained from the FABI culture collection
CGM fungi obtained from the CAMS culture collection
Colour coded = Flavour production observed

Table 6 (continued): Comparison of results on Czapeck Dox and Tryptic Soy Broth.

Name of Fungi	CC #	Pyrazine Flavours in Czapeck Dox Medium								Cz %	Pyrazine Flavours in TSB								TSB %
		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other		Caramel	Meaty	Nutty	Chocolate	Coffee	Green	Potato	Other	
Other fungi										**32**									**39**
Ambrosiella sp.	CMW 375																		
Arthrobotrys sp.	CMW 559																		
Arythrobotrys sp.	CMW 537																		
Beauveria bassiana	CMW 391																		
Botrytis cinerea	MRC 3966																		
Botrytis cinerea	MRC 10207																		
Byssochlamys nivea	MRC 10751																		
Cenangium ferruginosum	CMW 680																		
Chaetomium angustisporum	CGM 1272																		
Chaetomium thielavioideum	MRC 2399																		
Chaetopsina sp.	CMW 592																		
Curvularia sp.	MRC 4566																		
Curvularia sp.	MRC 1862																		
Cyclaneusma minus	CMW 679																		
Cylindrocladium sp.	CMW 950																		
Cylindrocarpon sp.	CMW 814																		
Geotrichum sp.	MRC 1998																		
Gliocladium sp.	CMW 599																		
Gliocladium sp.	CMW 640																		
Heliconium sp.	CMW 339																		
Hyalodendron sp.	CMW 920																		
Lophodermium australe	CMW 683																		
Nasmacyclius niveus	CMW 460																		
Phaeoisaria sp.	CMW 811																		
Phialophora sp.	CMW 581																		
Rhizoctonia sp.	CMW 410																		
Stibella sp.	CMW 973																		
Subulispora sp.	CMW 796																		
Total flavours produced		**40**	**14**	**16**	**6**	**4**	**46**	**3**	**33**		**16**	**35**	**45**	**2**	**2**	**40**	**9**	**38**	

MRC and WR fungi obtained from the CSIR culture collection
CGM fungi obtained from the CAMS culture collection
CMW fungi obtained from the FABI culture collection
Colour coded = Flavour production observed

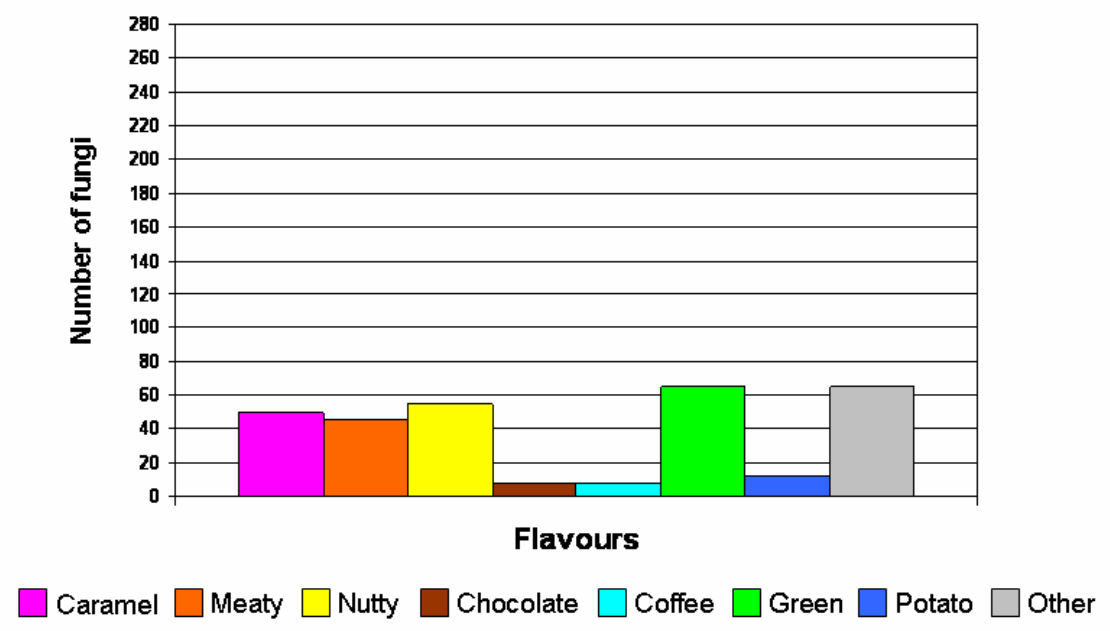

Figure 1: Comparison between the ability of the total number of tested fungi to produce certain flavours.

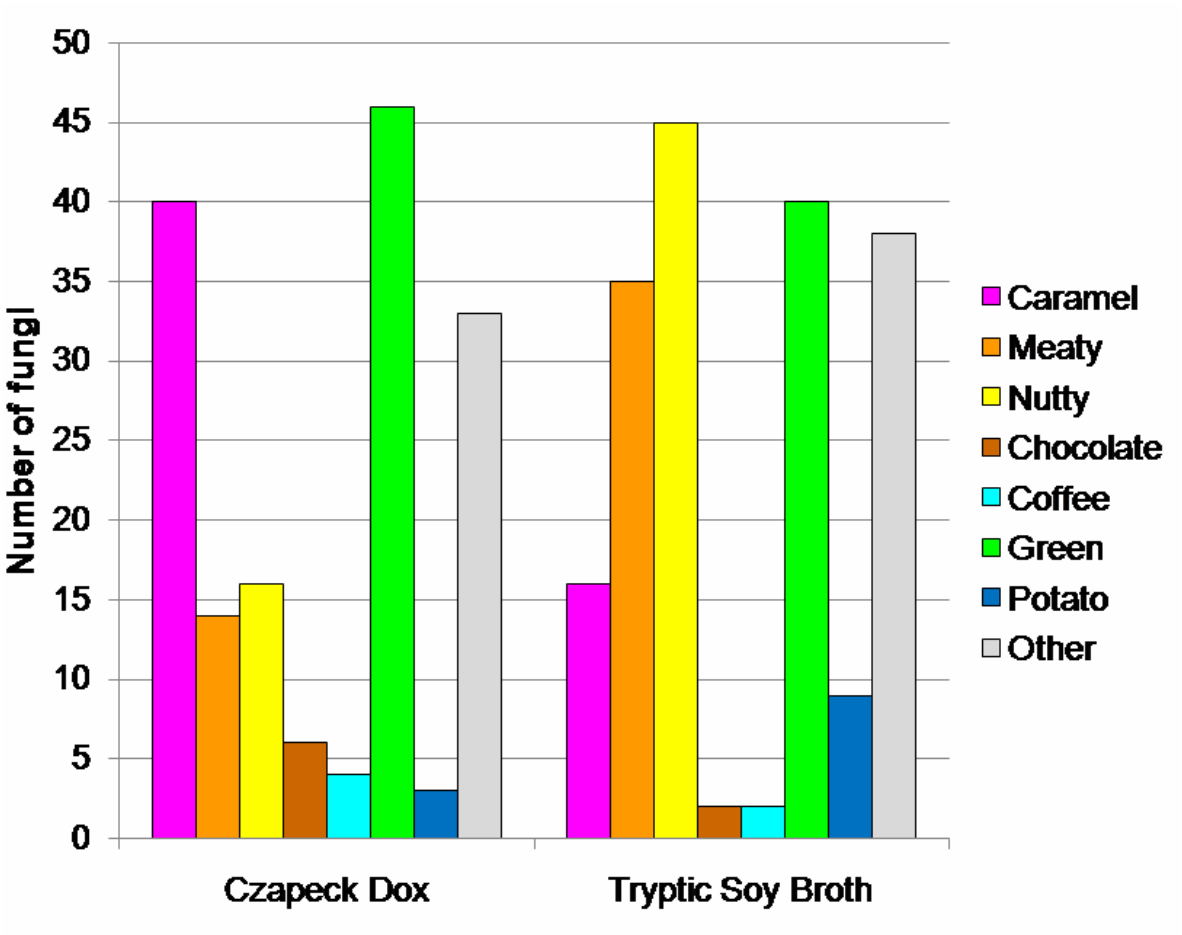

Figure 2: Comparison between Czapeck Dox medium and tryptic soy broth regarding the number of tested fungi that were able to produce certain flavours.

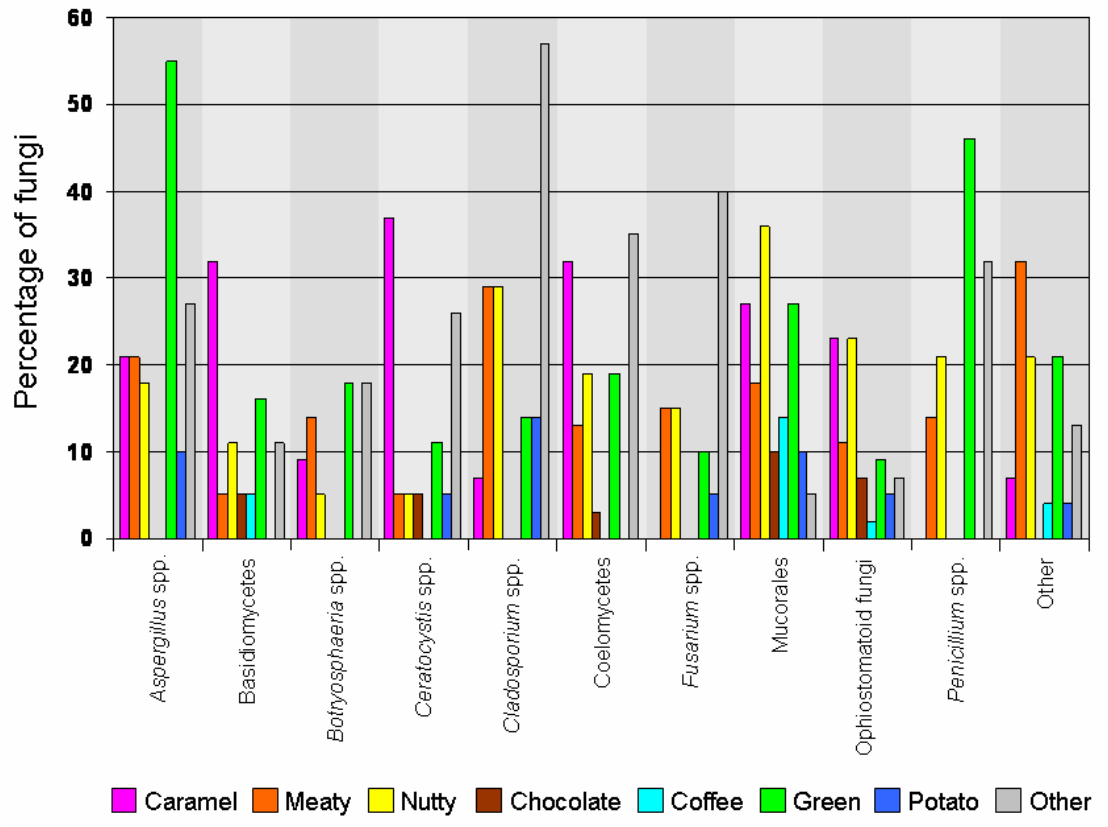

Figure 3: Comparison between the different taxonomic fungal groups and their ability to produce certain flavours.

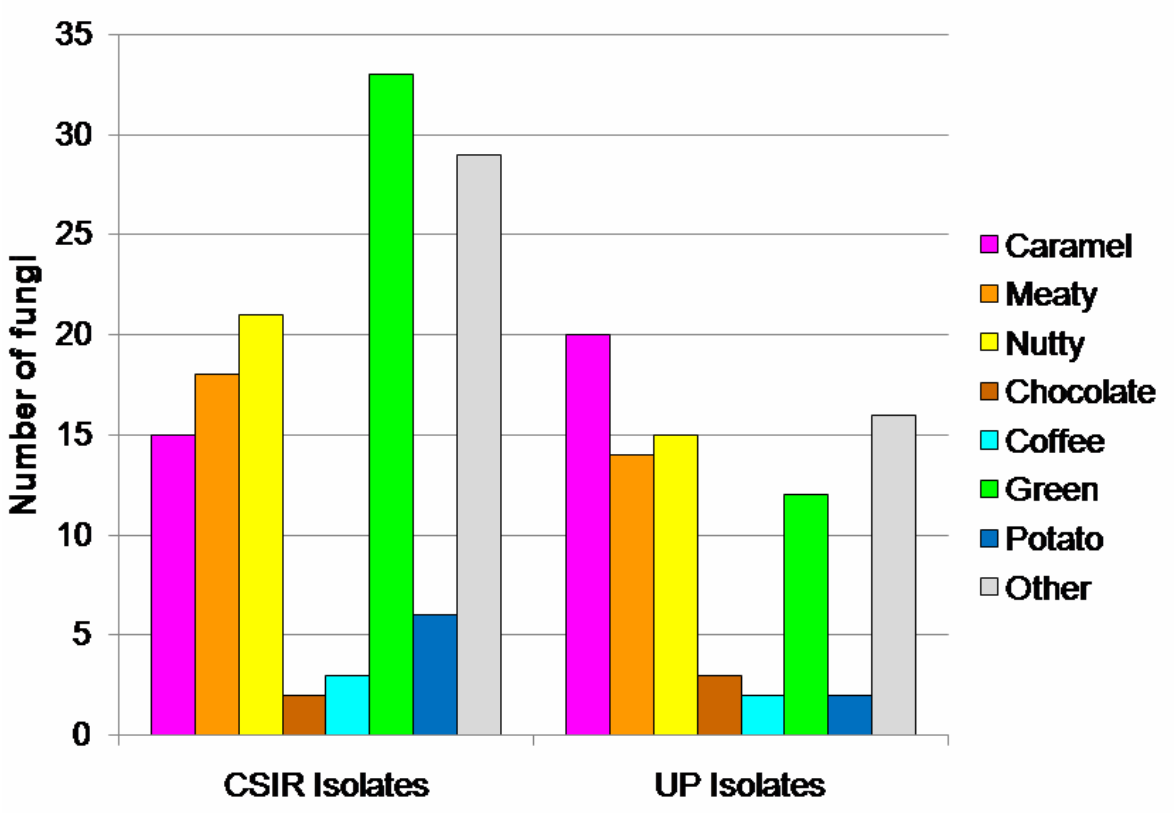

Figure 4: Comparison between the CSIR and UP culture collections regarding the ability of the number of tested fungi to produce certain flavours.

CHAPTER 3

Confirmation of pyrazine production by filamentous fungi via sensory evaluation and chemical analysis

3.1. ABSTRACT

Pyrazines are aromatic heterocyclic nitrogen containing compounds with the alkylated and methoxylated forms exhibiting strong odorous properties. Methoxypyrazines have a characteristic green pepper odour and are of significant value to the flavour industry because of their low sensory thresholds. The purpose of this study was to confirm pyrazine production amongst fungi screened through sensory evaluation and chemical analysis. Confirmation was done by GC-TOFMS and sniffing of pyrazine aromas as they eluted from the end of a GC column. Tentative identification of pyrazines was noted in 7 out the 11 fungi screened by GC-TOFMS. Confirmation of the production of 2-methoxy-3-isobutylpyrazine by ***Penicillium purpurogenum*** (MRC 181) and ***Penicillium rubrum*** (MRC 1723), as well as 2-methoxy-3-isopropylpyrazine by ***Penicillium rubrum*** (MRC 1723) was performed by GC-TOFMS.

3.2. INTRODUCTION

Pyrazines are aromatic heterocyclic nitrogen containing compounds that are used as flavouring agents, but also have an application in the synthesis of various pesticides, insecticides, drugs and dyes (Girija, **2002**). Alkylated and methoxylated pyrazines have strong odorous properties and are thus used as flavour compounds in the food industry (Beck, Hansen & Lauritsen, **2003**). Pyrazines can be found naturally in foods such as coffee beans, cocoa beans, nuts and vegetables (Maga, **1982**), or they can either be chemically synthesized (Barlin, **1982**) or produced via the Maillard reaction (Maga, **1982**). These compounds are also produced during fermentation processes and can be synthesised by microbiological cultures (Rizzi, **1988**; Seitz, **1994**).

Advances in chemical sciences have led to the development of the flavour and fragrance industry where techniques such as GC-MS and GC-olfactometry have permitted flavour chemists to identify the components of natural materials (Rowe, **2002**). Many of these components occur in trace amounts, but contribute significantly to the odour and flavour of the natural product. These compounds are considered as high impact aroma chemicals. Amongst these, pyrazines such as the 2-alkoxy-3-methylpyrazines, certain

methoxypyrazines, as well as acetylpyrazines, have shown to be important to the flavour and fragrance industry (Rowe, **2002**).

Methoxypyrazines, such as 2-isobutyl-3-methoxypyrazine, have characteristic green bell pepper odours and are found in several plants and microorganisms. These pyrazines occur in trace amounts but are known for their low sensory threshold (Rizzi, **1988**; Rowe, **2002**). One example of a microorganism producing methoxypyrazines is ***Pseudomonas taetrolens,*** a bacterium found to produce 3-isopropyl-2-methoxypyrazine (Gallois, Kergomad & Adda, **1998**).

Various mycelial fungi are known to produce pyrazine compounds as well. ***Aspergillus sojae*** and ***A. oryzae*** produce pyrazines during the fermentation of foods (Rizzi, **1988**). Pyrazines can also be produced by other ***Aspergillus*** species, namely, ***A. flavus*** and ***A. parasiticus.*** Although these fungi are known to produce toxic substances such as aflatoxins, they can be manipulated to produce pyrazines by altering their media composition (Buchanan & Houston, **1982;** MacDonald, **1973**). Other fungi shown to produce pyrazines are members of the genera, ***Penicillium*** and ***Septoria*** (Rizzi, **1988**).

Several fungi were found to produce flavour compounds characteristic of pyrazines in a sensory evaluation study that involved a sniffing panel (Chapter 2). The aim of this study was to select 20 isolates that were evaluated by the sniffing panel and obtain confirmatory data that the flavours produced are indeed pyrazines.

3.3. MATERIALS AND METHODS

3.3.1. Preliminary study: Identification of aroma producing fungi by sensory evaluation prior to chemical analyses

Twenty fungi that were screened by a sniffing panel (Chapter 2) were selected for confirmatory analyses. Selection of fungi was based on its potential to produce a pyrazine that is commonly used in industry or one that has a high

market value. A spore suspension of each fungus was made by pipetting 5 mL of sterile distilled water into a colonized petri dish and scraping the spores with a spatula. In total, 2 mL of the spore suspension was inoculated into either 250 mL of modified Czapeck Dox (Cz) medium or tryptic soy broth (TSB) (Table 1), prepared as described previously in chapter 2, section 2.3, page 79. It was then incubated at 25^{o}C on a rotary shaker at 190 revolutions per minute (rpm) for 14 days and evaluated daily by sniffing.

3.3.2. Pyrazine extraction

Volatiles from the fungi were extracted using a method based on the purge and trap method. It employs multi channel silicone rubber traps developed by Potgieter (**2006**). These traps (Figures 1 and 2) were prepared in 17.8 cm long glass tubes of 3.5 mm inner diameter (i.d.), according to the method described by Ortner & Rohwer (**1996**). Each trap contained eleven polydimethylsiloxane channels (0.64 mm outer diameter (o.d.) 0.3 mm i.d., Sil-Tec, Technical Products, USA) and was 55 mm long. In total, 250 mL of the sample was kept in a water bath at 40^{o}C and the multi channel silicone rubber trap was maintained at 50^{o}C. These were warmed in a water-bath for 5 min prior to the flow of gas. Nitrogen gas (Afrox 5.0) was then purged through the sample for 30 min at a flow rate of 25 mL.min^{-1} and an operating pressure of 50 kPa. The purged volatiles were collected on a multi channel silicone rubber trap (Potgieter, **2006**). The same method was used for the extraction of the chemical standards of 2-methoxy-3-isobutylpyrazines (MIBP) and 2-methoxy-3,5/6-isopropylpyrazine (MIPP), where for each standard a volume of 250 mL water was spiked with 1 µL of the chemical standard.

3.3.3. Chemical analyses: TDS-CIS-GC-MSTOF screening

The trapped volatiles were thermally desorbed using the Gerstel Thermal Desorption System (TDS) and then cryogenically focused via a cooled injection system (CIS). The traps were desorbed at 30^{o}C (3 min) to 250^{o}C (10 min) at 60^{o}C.min^{-1} and the TDS sample mode was splitless. The desorption flow rate was 50 mL.min^{-1} (Helium Ultra High Purity, Afrox) with the GC inlet in the solvent vent mode and the vent pressure at 47 kPa. The vent end time was 0 min. The TDS transfer line was at 250^{o}C. The transfer capillary was

uncoated and deactivated with an o.d. 0.70 mm/ i.d. 0.53 mm. Cryo-focusing of the volatiles was achieved using liquid nitrogen at -20°C and then rapidly heating at 5°C.s^{-1} to 250°C (10 min). A baffled and deactivated inlet glass liner was used (Gerstel).

Analytes were then separated using the Agilent 6890A gas chromatograph. A Zebron non-polar column (ZB1, 30 m x 250 µm x 25 µm), obtained from Separations, was used. The GC oven was temperature programmed from 10^{o}C (3 min) with 5^{o}C.min^{-1} increments to 250^{o}C (5 min). The run time was 56 min. A column post-run was done at 280^{o}C (5 min). The column head pressure was 47 kPa in the constant pressure mode resulting in a carrier gas (Helium Ultra High Purity, Afrox) velocity of 37 cm.s^{-1} (1.1 mL.min^{-1}) at 10^{o}C (oven cryogenically cooled with liquid nitrogen). The purge flow to split vent was 50 mL.min^{-1} at 3 min with the GC inlet in the solvent vent mode.

The GC was coupled to a Micromass GCT, TOF accurate mass MS (Microsep, South Africa). The GC-TOFMS transfer line was at 275^{o}C. The MS electron ionisation (EI, positive ion) source temperature was 200^{o}C. The electron energy was 70 eV and the scan rate was 10 scans/sec. The mass scan range was 35-650 atomic mass units. The trap current was 200 µA and the detector voltage 2590 V. There was no MS solvent delay. The instrument was manually tuned using 2,4,6-tris(flouromethyl)-[1,3,5] triazine (TRIS, “metri”). The internal reference mass (a lock mass) was 284.9949 m/z.

3.3.4. Sensory evaluation of the pyrazines eluting from a GC column

The end of the GC column was removed from the TOFMS and inserted through the detector port of the GC into the atmosphere. TDS-CIS-GC conditions remained the same.

3.4. RESULTS

3.4.1. Preliminary study: Identification of aroma producing fungi by sensory evaluation prior to chemical analyses

The results obtained through the preliminary study were based on the sniffing (human nose) of the samples. It illustrates the flavours that were observed by monitoring the aromatic flavours over a period of fourteen days. The type of flavours and their intensities on each day is reflected in table 2. The aromatic flavours noted were the caramel, meaty, nutty, chocolate, coffee, green and potato flavours that are characteristic of pyrazines. Other aromas observed were banana, as well as some unpleasant ones such as ammonia and cabbage/cauliflower aromas. Figure 3 gives a clearer indication as to the different flavours produced by each fungus as well as the intensities of the flavour observed each day.

3.4.2. Chemical analyses: TDS-CIS-GC-TOFMS screening

The samples were screened for their pyrazine content. Table 3 contains the results obtained by the GC-TOFMS analyses. It shows the pyrazines that were detected in each of the eleven fungi analysed. The table also gives the retention times of the pyrazines detected using the deconvolution programme (Chromalynx) and the NIST library, and that of the manual searches using the Wiley library.

The target pyrazines, MIBP and MIPP, in the fungal samples were identified by the comparison of their retention times, as well as their mass spectra to the retention times, and also mass spectra obtained by analysing certified reference materials (CRMs). Where no CRMs were available the non-target pyrazines were tentatively identified by the comparison of their recorded mass spectra with that of the spectral data from the Wiley and NIST libraries.

3.4.3. Confirmation of the target pyrazines

Methoxypyrazines detected in ***Penicillium purpurogenum*** (MRC 181) and ***Penicillium rubrum*** (MRC 1723) were confirmed as reflected in figures 4 to 10. After completion of the mass spectral analyses the end of the GC column was

removed from the TOFMS and inserted through the detector port of the GC. The carrier gas thus exhausted into the atmosphere. Duplicate samples were analysed exactly as for the TDS-CIS-GC-TOFMS analyses, with the only difference being that the TOFMS was now replaced by the human nose as detection method. The pyrazines were sniffed as they eluted from the end of the GC column. The retention times and the aromas were noted (Table 4) to confirm that the target pyrazines were indeed responsible for emitting the desired aroma. Other aromas were also noted as indicated in table 5.

3.5. DISCUSSION AND CONCLUSION

3.5.1. Preliminary study: Identification of aroma producing fungi by sensory evaluation prior to chemical analyses

The trial study was done to establish when flavours are produced over a fourteen day period. Results indicated that the fungi were able to produce flavours on a certain day during the preliminary study and for a certain period of time, after which the aroma either changed, disappeared or stabilised (Table 2 and figure 3). It was found that ***Ceratocystis fimbriata*** (CMW 2473), ***Geotrichum*** sp. (MRC 1998), ***Gloeophyllum trabeum*** (WR 172), ***Leptographium reconditum*** (CMW 15), ***Mucor*** sp. (MRC 2430), ***Gondwanamyces capense*** (CMW 1000), ***Gondwanamyces capense*** (CMW 978), ***Penicillium griseofulvum*** (MRC 312), ***Rhizopus stolonifer*** (MRC 4050) and ***Thallographium*** sp. A (CMW 601) all produced the caramel aroma. In most cases this aroma was observed during the first four days and at low intensities. However, ***Leptographium reconditum*** (CMW 15) and ***Gondwanamyces capense*** (CMW 978) are exceptions. The caramel aroma was noted during all fourteen days in ***Leptographium reconditum*** (CMW 15). In ***Gondwanamyces capense*** (CMW 978), the carmel aroma was noted at a higher intensity compared to the other organisms producing this flavor..

It was only with ***Fusarium scirpi*** (MRC 3774) that a chocolate aroma was observed. ***Mucor*** sp. (MRC 2430) was the only fungus that produced a coffee aroma over a long period, whereas ***Leptographium procerum*** (CMW 522) was the most prominent in the production of the nutty aroma. ***Penicillium***

purpurogenum (MRC 181) and ***P***. ***rubrum*** (MRC 1723) were the main producers of the green flavour, where the green pepper flavour was detected throughout the fourteen day trial in ***P***. ***purpurogenum*** (MRC 181). A nutty odour was noted in ***P***. ***rubrum*** (MRC 1723) after the first day of incubation, and thereafter a strong green pea odour was observed.

All three isolates of ***Ceratocystis albifundus*** produced a banana aroma. It was found that production started at different days amongst the 3 isolates although the intensity in each of them seemed to be similar.

Eleven isolates (Table 6) were selected based on the intensity of the aroma and their possible market demand, and chemically analysed for the presence of pyrazines. The volatiles were extracted on the day indicated in table 6 and also the odour noted on the day of extraction.

3.5.2. Chemical analyses: TDS-CIS-GC-TOFMS screening

Pyrazines were tentatively identified using the deconvolution (Chromalynx) software. This software automatically searches for peaks in a chromatogram and compares the mass spectra of each to that of the NIST library. Manual searches were also performed and these spectra were compared to that of the Wiley library. From the results (Table 3), it was found that four of the eleven fungi did not produce any pyrazines. These fungi produced the caramel and coffee aromas on the day of the extraction (Table 6). It is thus possible that there are other compounds responsible for these aromas, or that the pyrazines responsible were present at levels below the limit of detection of the GC-TOFMS.

It is also evident that a considerable number of pyrazines can be produced by a single isolate. For example, ***Ceratocystis albifundus*** (CMW 4069) produced a total of fifteen different pyrazines and ***Gondwanamyces capense*** (CMW 978) produced a total of fourteen pyrazines. However, this could be problematic when it comes to the development of a fermentation process and specifically during purification. Normally the flavour markets demand pure flavour substances that can be mixed according to their own specifications.

Separating these substances could make the use of such isolates expensive and economically non-viable.

It was decided to focus on the methoxypyrazines detected in the samples because of their commercial value. The presence of the target methoxypyrazines were confirmed by comparing the retention times and mass spectra of the CRMs and spectra of the Wiley library to that of the pyrazines produced by the fungi. A 79% match was found between MIPP produced by ***P. rubrum*** (MRC 1723) and that of the Wiley library. For MIBP there was an 87% and an 89% match for ***P. purpurogenum*** (MRC 181) and ***P. rubrum*** (MRC 1723) respectively. A better match can be obtained by taking the mass spectrum at the leading or descending edges of the peak and not at the apex of the peak as was done here. The reason for this is that the mass spectrum at the apex is overloaded due to the high concentration of the pyrazine present.

The sensory evaluation of the pyrazines eluting from a GC column confirmed that the pyrazines produced by the fungi were responsible for the green/green pepper aromas. The retention times of the GC-TOFMS were compared to those obtained by sniffing the aroma's eluting at the end of the GC column and a 1-1.5 min difference was noted between the two sets of results. For the GC-TOFMS analyses the GC outlet was at vacuum whilst for the sniffing at the end of a GC column exercise the GC outlet was at atmospheric pressure. Therefore, there was a difference in the retention times between the two sets of results. For example, under atmospheric conditions, MIBP, produced by ***P. rubrum*** (MRC 1723), eluted at a retention time of between 21 and 25 min whereas for the GC-TOFMS, the retention time of this pyrazine was 21.47 min.

Pyrazine compounds were not the only flavour compounds detected by GC-MS. Other flavour compounds of interest were the banana flavour (isoamyl acetate) produced by ***Ceratocystis albifundus*** (CMW 4069) and a number of sesquiterpenes produced by ***Fusarium scirpi*** (MRC 3774).

In conclusion, results from the TDS-CIS-GC-TOFMS analyses and the sensory evaluation of the pyrazines, eluting from the end of the GC column, showed that pyrazines can be produced by a variety of mycelial fungi from South African environments. Methoxypyrazines in particular, exhibiting a green pepper odour, are in demand in the flavour industry and ***Penicillium*** species seem to be good candidates for the production of methoxypyrazines. Previous studies demonstrated that methoxypyrazines can be produced by ***Penicillium caseicolum*** (Rizzi, **1988**) and ***Penicillium vulpunim*** (Larsen & Frisvad, **1994**). However, this study shows that other ***Penicillium*** species can also produce methoxypyrazines, including ***P. purpurogenum*** (MRC 181) that produces MIBP and ***P. rubrum*** (MRC 1723) that produces both MIBP and MIPP.

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Table 1: Fungi screened and culture media used in the trial study.

Name of fungi	*CC No.*	*Maintenance*	*Culture media*
Ceratocystis albifundus	CMW[1] 4069	PDA[3]	Tryptic Soy Broth
Ceratocystis albifundus	CMW 4092	PDA	Tryptic Soy Broth
Ceratocystis albifundus	CMW 4095	PDA	Tryptic Soy Broth
Ceratocystis fimbriata	CMW 2473	PDA	Modified Czapeck Dox
Fusarium scirpi	MRC[2] 3774	PDA	Tryptic Soy Broth
Geotrichum sp.	MRC 1998	PDA	Modified Czapeck Dox
Gloeophyllum trabeum	WR 172	MEA[4]	Modified Czapeck Dox
Leptographium procerum	CMW 522	MEA	Tryptic Soy Broth
Leptographium reconditum	CMW 15	PDA	Modified Czapeck Dox
Mucor sp.	MRC 2430	MEA	Modified Czapeck Dox
Gondwanamyces capense	CMW 1000	PDA	Modified Czapeck Dox
Gondwanamyces capense	CMW 978	MEA	Tryptic Soy Broth
Penicillium griseofulvum	MRC 312	PDA	Modified Czapeck Dox
Penicillium purpurogenum	MRC 181	PDA	Tryptic Soy Broth
Penicillium rubrum	MRC 1723	MEA	Tryptic Soy Broth
Phoma exigua var.foveata	MRC 590	PDA	Tryptic Soy Broth
Rhizopus stolonifer	MRC 4050	MEA	Modified Czapeck Dox
Sporothrix sp.	CMW 400	PDA	Tryptic Soy Broth
Thallographium sp. A	CMW 601	MEA	Modified Czapeck Dox
Thallographium sp. B	CMW 601	MEA	Tryptic Soy Broth

[1]CMW = FABI culture collection, University of Pretoria, [2]MRC = CSIR culture collection, Pretoria, [3]PDA = potato dextrose agar, [4]MEA = Malt Extract Agar

Table 2: Odour intensities of flavours produced by 20 selected fungi over a period of 14 days.

Name of fungi	*CC No*	*Days and Intensity*									
		1		**2**		**3**		**4**		**5**	
Ceratocystis albifundus	CMW 4069	No odour		No odour		Banana	+	Banana	++	Banana	+++
Ceratocystis albifundus	CMW 4092	Coffee	+	Banana/passion fruit	++	Banana	+++	Banana	+++	Banana	++
Ceratocystis albifundus	CMW 4095	No odour		No odour		No odour		Blank		No odour	
Ceratocystis fimbriata	CMW 2473	Caramel	+	Caramel	+	Caramel	++	Caramel	++	Caramel	+
Fusarium scirpi	MRC 3774	Ammonia	+	Chocolate/nutty	++	Chocolate	++	Chocolate	+	Ammonia	++
Geotrichum sp.	MRC 1998	Caramel	+	Boiled green veg	++	Boiled green veg	+	Caramel	+	Sweet	+
Gloeophyllum trabeum	WR 172	Caramel	+	Caramel	+	Caramel	++	Green/sweet	+	Green/sweet	++
Leptographium procerum	CMW 522	Nutty	+	Nutty	++	Nutty	++	Nutty	+	Nutty	+
Leptographium reconditum	CMW 15	Coffee/ caramel	+	Caramel	++	Caramel	++	Caramel/ roasted	++	Caramel/ roasted	++
Mucor sp.	MRC 2430	Potato/caramel	+	Potato/caramel	+	Potato	+	Potato	+	No odour	
Gondwanamyces capense	CMW 1000	Caramel	+	Caramel	+	Caramel	+	Caramel	+	Green	+
Gondwanamyces capense	CMW 978	No odour		Caramel	+++	Caramel/Chococlate	++++	Coffee/nutty	+	Nutty	+
Penicillium griseofulvum	MRC 312	Caramel/nutty	+	Caramel	+	Boiled green veg	+	No odour		No odour	
Penicillium purpurogenum	MRC 181	No odour		Green/boiled peanuts	++	Green pepper	+++	Green pepper	++++	Green pepper	+++++
Penicillium rubrum	MRC 1723	No odour		Nutty/meaty	++	Raw green peas	++	Raw green peas	+++	Raw green peas	+++
Phoma exigua var.foveata	MRC 590	No odour		No odour		No odour		No odour		No odour	
Rhizopus stolonifer	MRC 4050	Caramel	+	Caramel	++	Potato	+	Potato	+	Potato	+
Sporothrix sp.	CMW 400	No odour		Nutty	+	Nutty	+	Nutty	+	Nutty	+
Thallographium sp. A	CMW 601	No odour		No odour		Caramel	+	Caramel	+	Caramel	+
Thallographium sp. B	CMW 601	No odour		Ammonia	+	Cabbage	+++	Cabbage	++++	Cabbage	+++

(+): Odour intensities ranked from 1+ to 5+'s, where 1+ indicates a very slight odour and 5+'s indicate a very strong odour

No odour = no additional odour observed besides the medium

Table 2 (continued): Odour intensities of flavours produced by 20 selected fungi over a period of 14 days.

Name of fungi	CC No	Days and intensity									
		6		7		8		9		10	
Ceratocystis albifundus	CMW 4069	Banana	++++	Banana	++++	Banana	++++	Banana	++++	Banana	++++
Ceratocystis albifundus	CMW 4092	Banana	+	Banana/green	+	Green	++	Green	++	Green	+
Ceratocystis albifundus	CMW 4095	No odour		No odour		Banana	+	Banana	++	Banana	++
Ceratocystis fimbriata	CMW 2473	Caramel	+	Caramel	+	Green/sour	+	Green/sour	+	Green/sour	+
Fusarium scirpi	MRC 3774	Unpleasant	+++	Unpleasant	+++	Ammonia	+++	Ammonia	++	Ammonia/stinky	++
Geotrichum sp.	MRC 1998	Sweet	+	Sweet	+	Sweet	+	Sweet	+	Sweet	+
Gloeophyllum trabeum	WR 172	Milk sweets	++	Green/sour	++	Green/sour	++	Green/sour	++	Green/sour	+
Leptographium procerum	CMW 522	Sweet	+	Sweet	+	Ammonia	+	No odour		No odour	
Leptographium reconditum	CMW 15	Caramel/ roasted	++	Caramel/ roasted	++	Caramel/ roasted	++	Caramel/ roasted	++	Caramel/ roasted	++
Mucor sp.	MRC 2430	No odour		Coffee	+	Coffee	++	Coffee	+	Coffee	+
Gondwanamyces capense	CMW 1000	Green/cauliflower	+	Green/sweet	+	Green/sweet	+	Green/sweet	+	Green/sweet	+
Gondwanamyces capense	CMW 978	Nutty	+	Nutty	+	Nutty	+	Unpleasant	+	Unpleasant	++
Penicillium griseofulvum	MRC 312	No odour		No odour	+	No odour		No odour		No odour	
Penicillium purpurogenum	MRC 181	Green pepper	++++	Green pepper	+++	Green pepper	+++	Green/ earthy	++	Green/earthy	+
Penicillium rubrum	MRC 1723	Raw green peas	+	Raw green peas	+	Raw green peas	+	Unpleasant	+++	Unpleasant	+++
Phoma exigua var.foveata	MRC 590	No odour		No odour		No odour		No odour		No odour	
Rhizopus stolonifer	MRC 4050	Potato	+	Potato	+	Sweet	+	Roasted	+	Sweet	+
Sporothrix sp.	CMW 400	Ammonia	+	Ammonia	+	Ammonia	+	Ammonia	+	No odour	
Thallographium sp. A	CMW 601	Caramel	+	Caramel	+	Caramel	+	Sweet	+	No odour	
Thallographium sp. B	CMW 601	Cabbage	+++	Cabbage	++	Cabbage	+	Cabbage	++	Cabbage	+

(+): Odour intensities ranked from 1+ to 5+'s, where 1+ indicates a very slight odour and 5+'s indicate a very strong odour

No odour = no additional odour observed besides the medium

Table 2 (continued): Odour intensities of flavours produced by 20 selected fungi over a period of 14 days.

Name of fungi	CC No	Days and Intensity							
		11		12		13		14	
Ceratocystis albifundus	CMW 4069	Banana	+++	Banana	++	Banana	+	Banana	+
Ceratocystis albifundus	CMW 4092	Green	+	Green/cauliflower	+	Green	+	Green/cauliflower	+
Ceratocystis albifundus	CMW 4095	Banana	+++	Banana	+++	Banana	+++	Banana	+++
Ceratocystis fimbriata	CMW 2473	Green/sour	+	Green/sour	+	Green/sour	+	Green/sour	+
Fusarium scirpi	MRC 3774	Ammonia/stinky	++	Ammonia/stinky	+++	Ammonia/stinky	++	Ammonia/stinky	+++
Geotrichum sp.	MRC 1998	Sweet	+	Sweet	+	Sweet	+	Sweet	+
Gloeophyllum trabeum	WR 172	Green/sour	+	Green/sour	+	Green/sour	+	Green/sour	+
Leptographium procerum	CMW 522	Sweet	+	Ammonia	+	Ammonia	+	Meaty	+
Leptographium reconditum	CMW 15	Caramel/ roasted	++	Caramel/ roasted	++	Caramel/ roasted	+	Caramel/ roasted	+
Mucor sp.	MRC 2430	Coffee	+	Coffee	+	Coffee	+	Coffee	+
Gondwanamyces capense	CMW 1000	Green/sweet	+	Green/sweet	+	Green/sweet	+	Green/sweet	+
Gondwanamyces capense	CMW 978	Unpleasant	++	Unpleasant	++	Unpleasant	++	Unpleasant	+
Penicillium griseofulvum	MRC 312	No odour		No odour		No odour		No odour	
Penicillium purpurogenum	MRC 181	Green	+	Green	*	Green	+	Green	+
Penicillium rubrum	MRC 1723	Unpleasant	+++	Unpleasant	+++	Unpleasant	+++	Unpleasant	+++
Phoma exigua var.foveata	MRC 590	No odour		No odour		No odour		No odour	
Rhizopus stolonifer	MRC 4050	No odour		No odour		No odour		No odour	
Sporothrix sp.	CMW 400	No odour		No odour		Cauliflower	+	Meaty	+
Thallographium sp. A	CMW 601	No odour		No odour		No odour		No odour	
Thallographium sp. B	CMW 601	Cabbage	+	Cabbage	+	No odour		Ammonia	+

(+): Odour intensities ranked from 1+ to 5+'s, where 1+ indicates a very slight odour and 5+'s indicate a very strong odour

Blank = no additional odour observed besides the medium

Table 3: Presence of pyrazines in eleven selected fungi based on GC-MS.

Name of Pyrazine	*Retention Time*	*Abundance*	*Library*
***Ceratocystis albifundus* (CMW4069)**			
2-Methyl-5-(1-methylethyl)pyrazine	16.7406	2238	NIST ϕ2
	16.7446	2620	
	17.0306	5674	
	17.0354	6168	
2,5-Dimethyl-3-isopropylpyrazine	20.1156	1363	NIST ϕ2
2,3,5-Trimethyl-6-propylpyrazine	20.6379	2753	NIST ϕ2
2,3-Dimethyl5-n-propylpyrazine	22.1019	125	NIST ϕ2
2,5-Dimethyl-3-(2-methylpropyl)pyrazine	22.1100	202	NIST ϕ2
2,5-Dimethyl-3-(3-methylbutyl)pyrazine	22.7802	1882	NIST ϕ2
3,5-Dimethyl-2-isobutylpyrazine	22.9905	446	NIST ϕ2
2-(2-Methylpropyl)-3-(1-methylethyl)pyrazine	23.6049	7020	NIST ϕ2
	24.3096	7458	
2-(2-Methylpropyl)-3,5,6-trimethylpyrazine	23.6655	556	NIST ϕ2
	23.6768	453	
Trimethyl-1-popenyl-(Z)pyrazine	23.6913	8004	NIST ϕ2
Trimethyl-1-popenyl-(E)pyrazine	23.6954	7491	NIST ϕ2
Trimethylpyrazine	24.3790	77	NIST ϕ2
Tetraethylpyrazine	24.6558	4085	NIST ϕ2
	24.9102	15749	
	24.9176	15689	
	24.9261	17099	
2-(3-Methylbutyl)-3,5-dimethylpyrazine	25.4101	646	NIST ϕ2
	25.4188	640	
2,6-Bis(2-methylpropyl)pyrazine	26.1405	3448	NIST ϕ2
	26.1448	5980	
	26.8226	4795	
	26.8232	6326	
	26.8358	7560	
***Ceratocystis fimbriata* (CMW2473)**			
No pyrazines detected	-	-	-
***Fusarium scirpi* (MRC 3774)**			
2,5-Dimethylpyrazine	12.3776	1688	NIST ϕ2
			Wiley
***Leptographium procerum* (CMW 522)**			
2,5-Dimethylpyrazine	12.3613	1737	NIST ϕ2
	12.3670	2029	
2,3,5-Trimethylpyrazine	14.93	-	Wiley
2-(3-Methylbutyl)-3,5-dimethylpyrazine	20.2198	464	NIST ϕ2
	20.2360	543	Wiley
***Leptographium reconditum* (CMW 15)**			
No pyrazines detected	-	-	-
***Mucor sp*. (MRC 2430)**			
No pyrazines detected	-	-	-
***Penicillium rubrum* (MRC 1723)**			
2,3-Dimethylpyrazine	10.5	-	Wiley
Dimethylpyrazine	12.5	-	Wiley
2-Methoxy-3, 5/6-isopropylpyrazine	18.7596	14	NIST ϕ2
2-Methoxy-3-isobutylpyrazines	21.4762	1082	NIST ϕ2

Table 3 (continued): Presence of pyrazines in eleven selected fungi based on GC-MS.

Name of Pyrazine	Retention Time	Abundance	Library
***Penicillium purpurogenum* (MRC 181)**			
Pyrazine	6.72	-	Wiley
2,5-Dimethylpyrazine	12.5199	1562	NIST ϕ2
2-Methoxy-3-isobutylpyrazine	21.51	-	Wiley
***Gondwanamyces capense* (CMW 978)**			
2,5-Dimethylpyrazine	12.4249	164	NIST ϕ2
2-Methyl-6-propylpyrazine	15.3645	2207	NIST ϕ2
	15.3741	2227	
2-Methyl-3-isopropylpyrazine	15.572	-	Wiley
2-Isobutyl-3-methylpyrazine	17.2843	1770	NIST ϕ2
	17.2945	1739	
3,5-Dimethyl-2-(1-propylenyl)-(E)pyrazine	17.8125	15030	NIST ϕ2
2,3,5-Trimethyl-6-propylpyrazine	18.3929	40	NIST ϕ2
	19.8094	329	
5-Butyl-2,3-dimethylpyrazine	18.9700	543	NIST ϕ2
3,5-Diethyl-2-methylpyrazine	19.0951	1288	NIST ϕ2
3-(1-methylethy)(1H)pyrazolo[3,4-b]pyrazine	19.3392	7677	NIST ϕ2
2-(2-methylpropyl)-3-(1-methylethyl)pyrazine	19.8528	2476	NIST ϕ2
2,3-Dimethyl-5-isopentylypyrazine	20.2534	775	NIST ϕ2
3-Butyl-2,5-dimethylpyrazine	20.2626	774	NIST ϕ2
2,6-Bis(2-methylpropyl)pyazine	20.6520	1409	NIST ϕ2
	20.6655	3017	
	20.6707	3174	
3,6-Dipropyl-2,5-dimethylpyrazine	20.8212	163	NIST ϕ2
	20.8323	35	
***Rhizopus stolonifer* (MRC 4050)**			
No pyrazines detected	-	-	-
***Thallographium* sp. (CMW 601)**			
2,5-Dimethylpyrazine	12.502	-	Wiley
2-Ethyl-3,5-dimethylpyrazine	18.302	-	Wiley

Table 4: Odour observation by sniffing at the end of the GC column.

Anylate	***Standard***	***Penicillium purpurogenum (MRC 181)***		***Penicillium rubrum (MRC 1723)***	
	Retention Time (min)	**Retention Time (min)**	**Aroma**	**Retention Time (min)**	**Aroma**
Pyrazine	-	8.2	Slight green pepper	-	-
2,5/2,6-Dimethylpyrazine	-	10.8	Burnt caramel	9.5	Green pepper
		11.9	Nutty	10.5	Green pepper
2-methoxy-3,5/6-isopropylpyrazine	-	-	-	17.5	Green pepper
				18.7	Green pepper
2-methoxy-3-isobutylpyrazine	22.2-22.5	19.5	Strong green	21.6	Green pepper
	25	21.8	Green	22.5	Green pepper
		22.5	Strong green	25	Faint green pepper
		25.3	Faint Green pepper		

Table 5: Other aromas noted during the sniffing at the end of the GC column.

***Penicillium purpurogenum* (MRC 181)**		***Penicillium rubrum* (MRC 1723)**	
Retention Time (min)	**Aroma**	**Retention Time (min)**	**Aroma**
12.3	Pineapple	11	Sweet
13.3	Green	15.6	Green
14	Sweet fruity	22	Caramel
		23	Chocolate

Table 6: List of fungi that were chemically analysed.

Name of fungi	CC No.	Odour	Day of Extraction
Ceratocystis albifundus	CMW 4069	Banana	6
Ceratocystis fimbriata	CMW 2473	Caramel, roasty	4
Fusarium scirpi	MRC 3774	Extremely sweet	3
Leptographium procerum	CMW 522	Nutty	3
Leptographium reconditum	CMW 15	Caramel, roasty	5
Mucor sp.	MRC 2430	Coffee, chocolate	3
Gondwanamyces capense	CMW 978	Unpleasant, Nutty	3
Penicillium purpurogenum	MRC 181	Green pepper	5
Penicillium rubrum	MRC 1723	Green	4
Rhizopus stolonifer	MRC 4050	Coffee, Potato	3
Thallographium sp. B	CMW 601	Cabbage	4

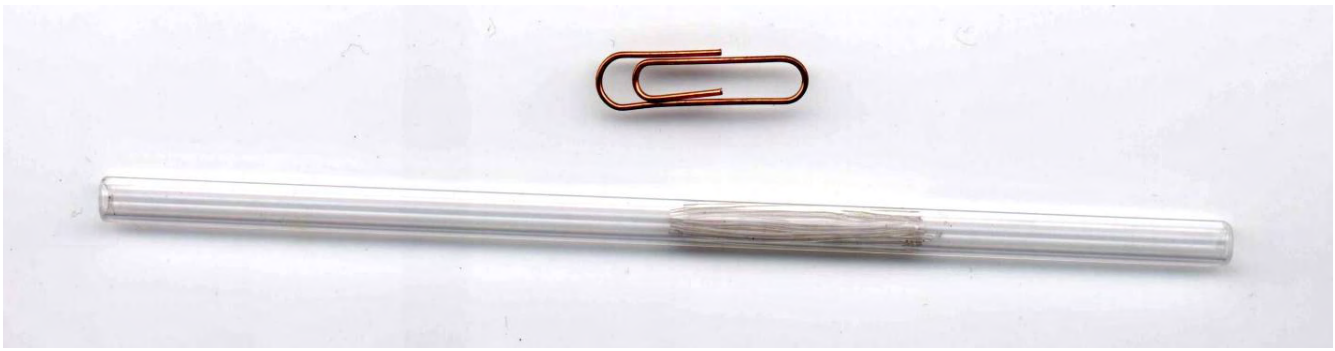
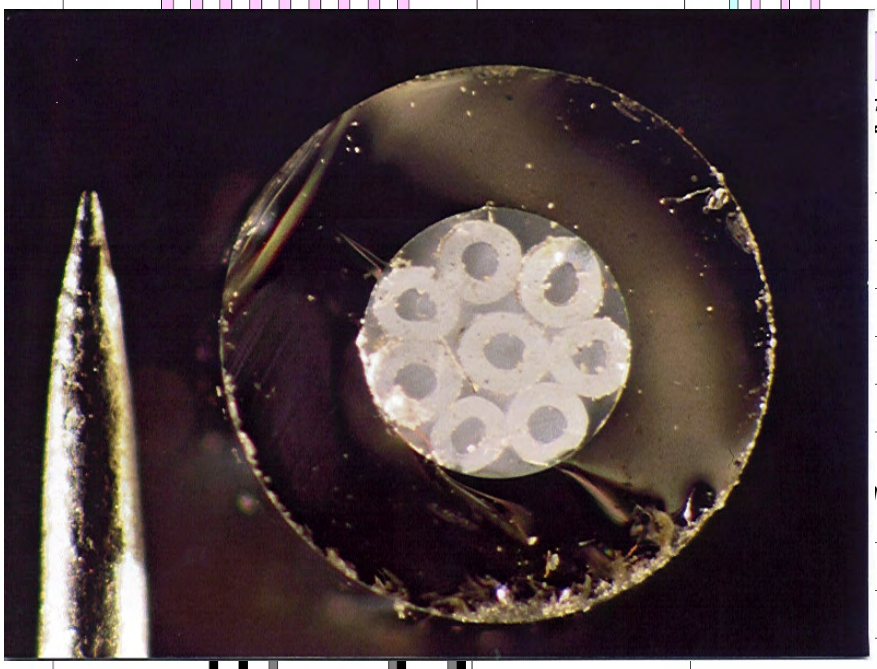


Figure 1: A multi-channel silicone rubber trap.

Figure 2: Cross-section through a multi-channel silicone rubber trap.



undus (CMW 4092)

nbriata (CMW 2473)

Fusarium scirpi (MRC 3774)

Leptographium procerum (CMW 522)

Geotrichum sp.(MRC 1998)

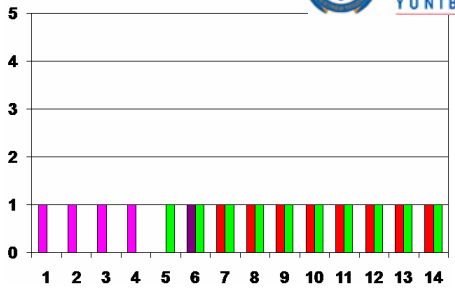
Gloeophyllum trabeum (WR 172)

Leptographium reconditum (CMW 15)

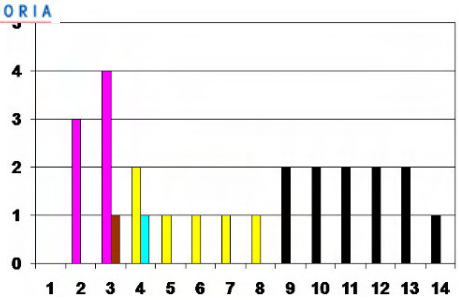
Mucor sp. (MRC 2430)

Caramel, Meaty, Nutty, Chocolate, Coffee, Green, Potato, Sweet, Banana, Ammonia, Green/sour, Blank, Unpleasant, Cabbage/ cauliflower, Passion fruit, Roasted

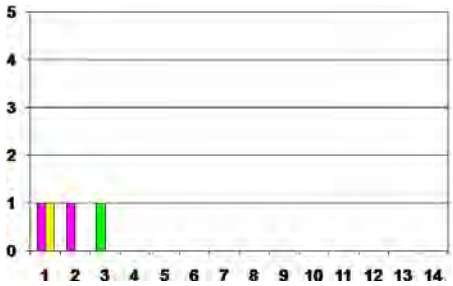
Figure 3: Aromas and intensities of flavour production by each fungus (1 = low odour; 5 = very strong odour).



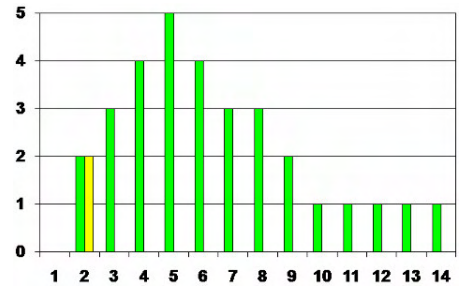
***Gondwanamyces capense* (CMW 1000)**



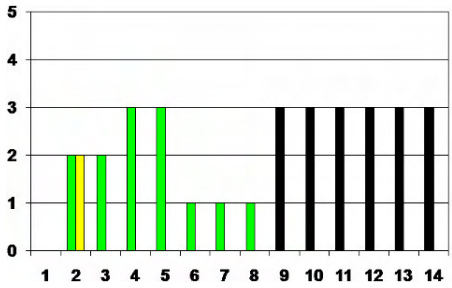
***Gondwanamyces capense* (CMW 978)**



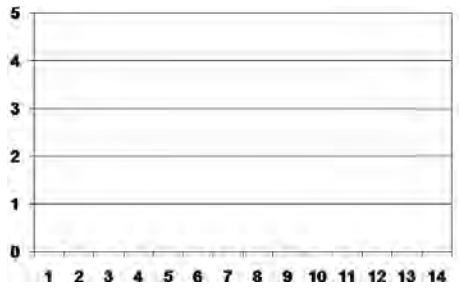
***Penicillium griseofulvum* (MRC 312)**



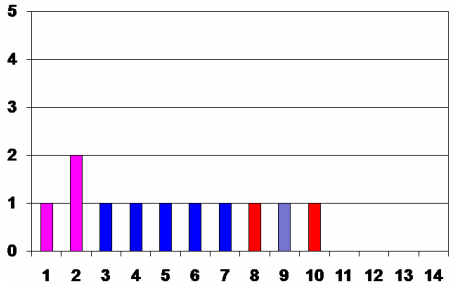
***Penicillium purpurogenum* (MRC 181)**



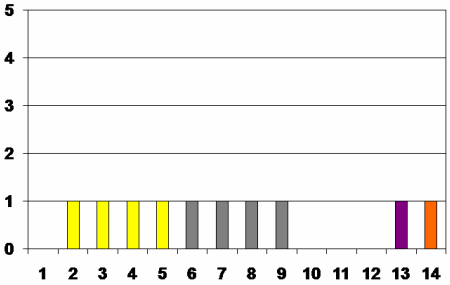
***Penicillium rubrum* (MRC 1723)**



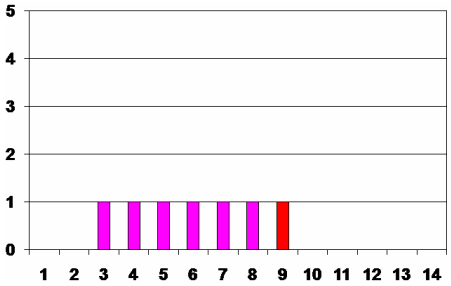
***Phoma sorghina* (MRC 590)**



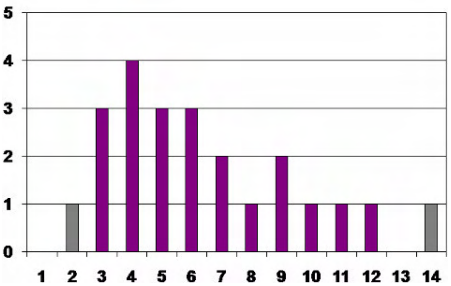
***Rhizopus stolonifer* (MRC 4050)**



***Sporothrix* sp. (CMW 400)**



***Thallographium* sp. A (CMW 601)**



***Thallographium* sp. B (CMW 601)**

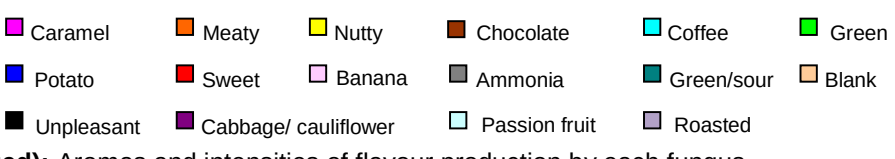
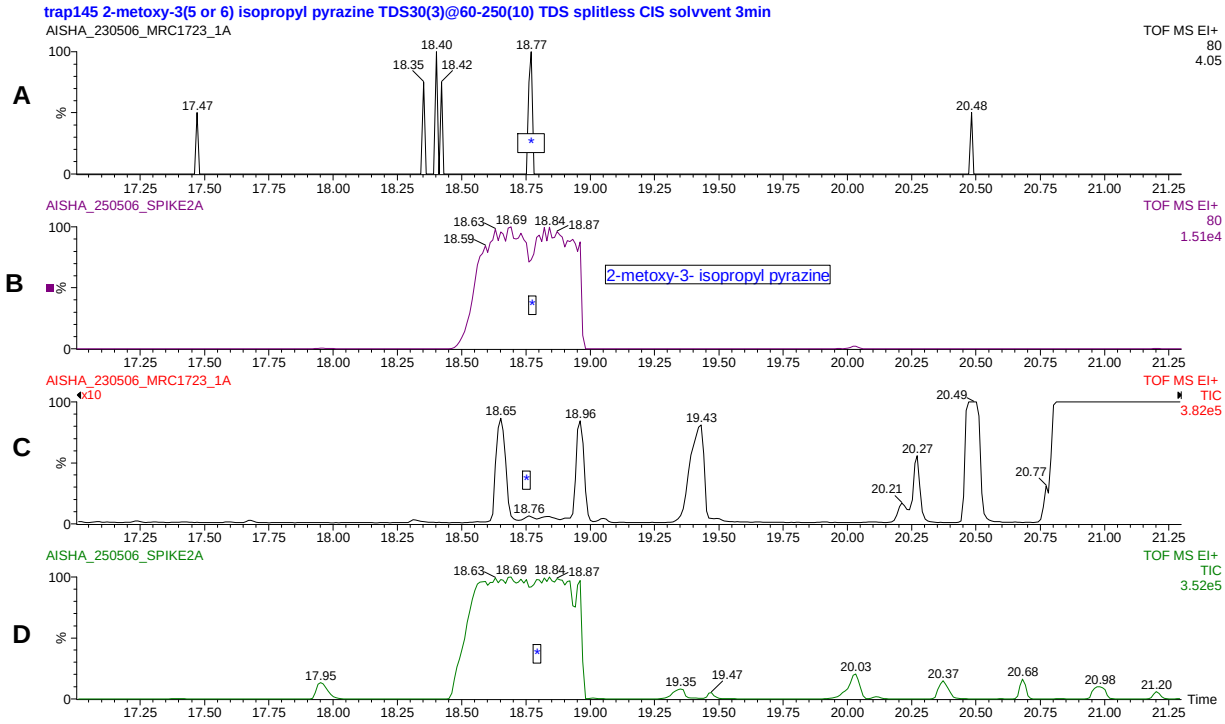


Figure 3 (Continued): Aromas and intensities of flavour production by each fungus (1 = low odour; 5 = very strong odour).



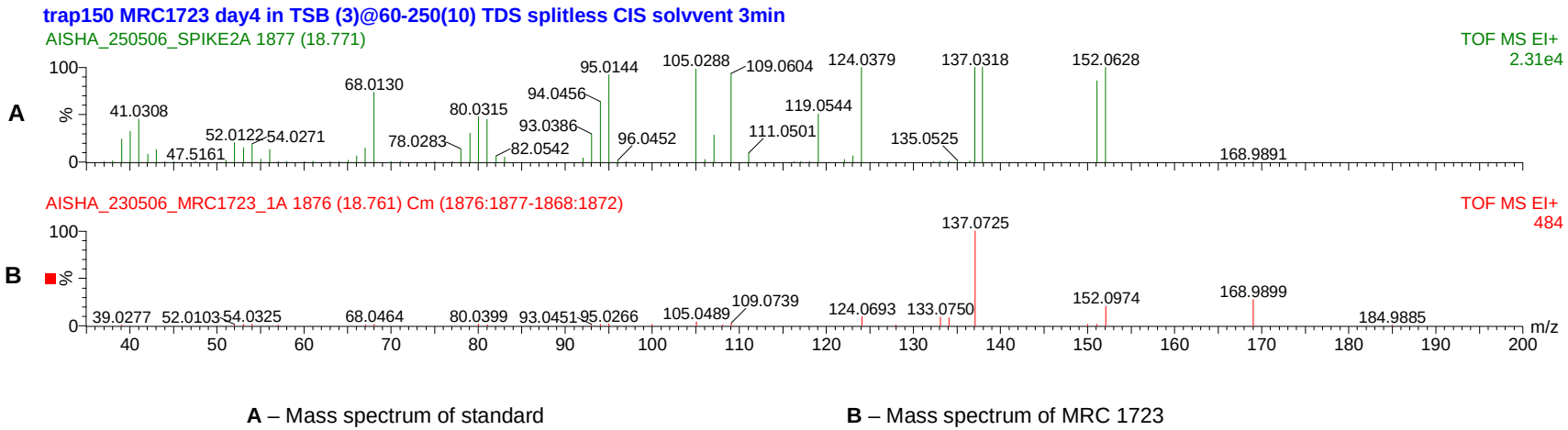
A – Chromatogram of MRC 1723 at EI+80.

B – Chromatogram of standard at EI+80

C – Total Ion Chromatogram of MRC 1723

D – Total Ion Chromatogram of standard

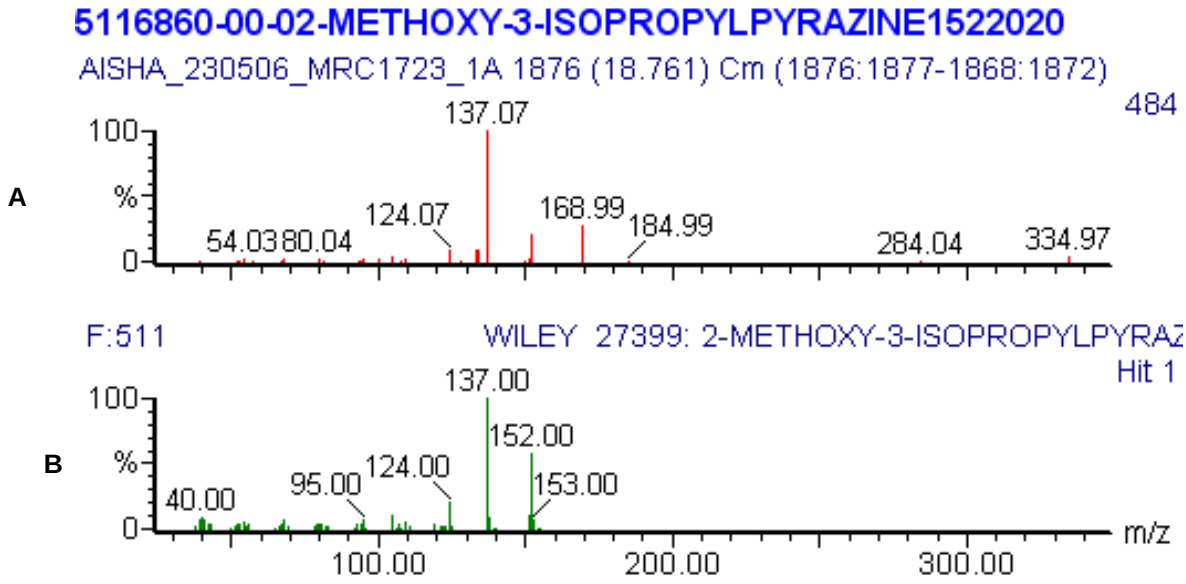
Figure 4: Comparison of retention times of ***Penicillium rubrum*** (MRC 1723) to that of the chemical standard 2-methoxy-3-isopropylpyrazine, herein referred to as spike 2A (see Materials and Methods, paragraph 2).



A – Mass spectrum of standard

B – Mass spectrum of MRC 1723

Figure 5: Comparison of mass spectra of ***Penicillium rubrum*** (MRC 1723) to that of the chemical standard 2-methoxy-3-isopropylpyrazine, herein referred to as spike 2A (see Materials and Methods, paragraph 2).



A – Mass spectrum of MRC 1723

B – Mass spectrum of pyrazine found in Wiley

Figure 6: Comparison of mass spectra of ***Penicillium rubrum*** (MRC 1723) to that of the Wiley library.

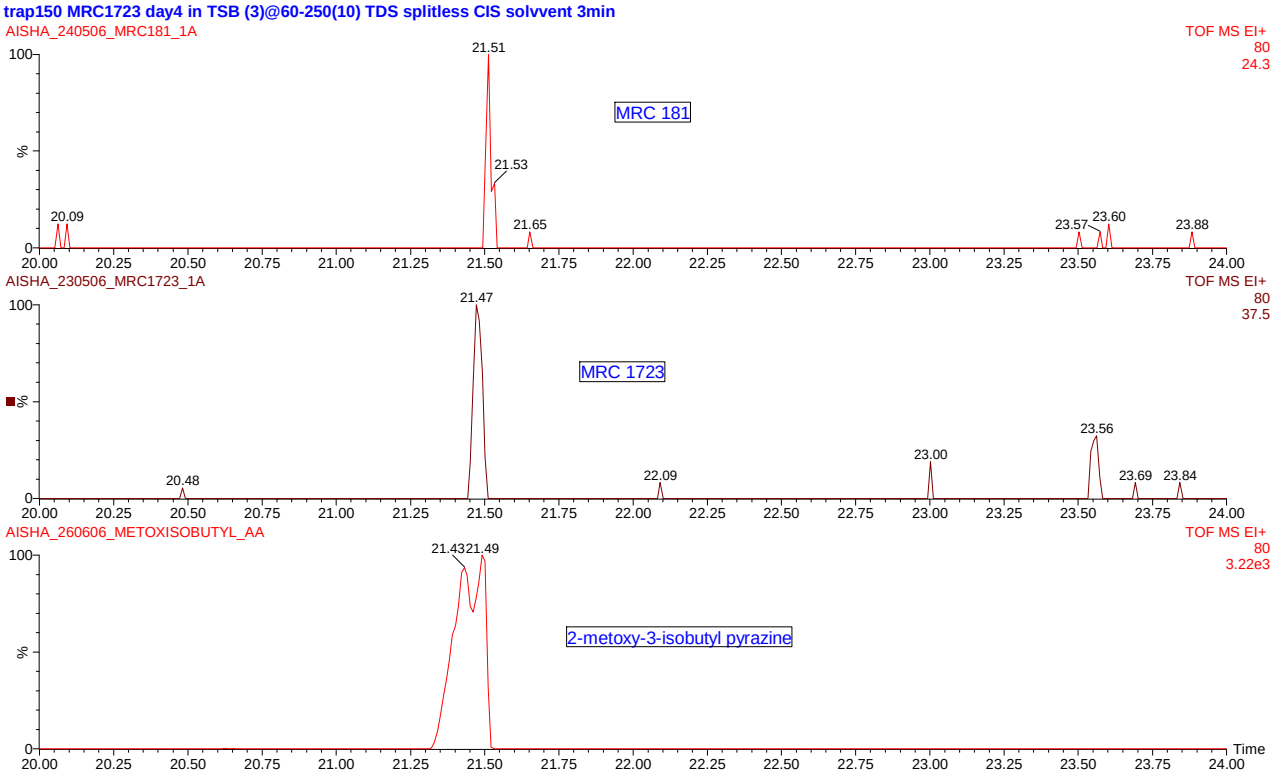


Figure 7: Comparison of the retention times of ***Penicillium rubrum*** (MRC 1723) and ***Penicillium purpurogenum*** (MRC 181) to that of the chemical standard 2-methoxy-3-isobutylpyrazine.

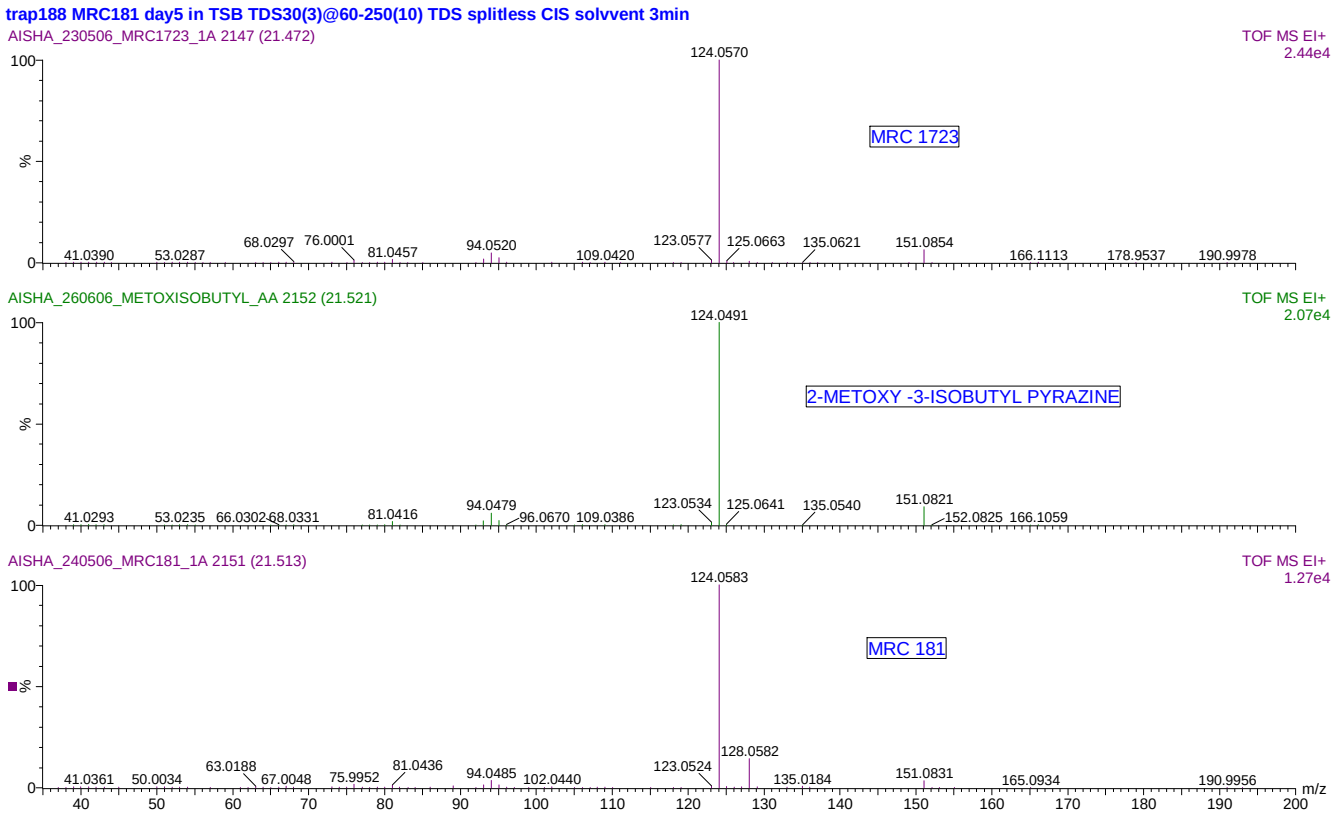
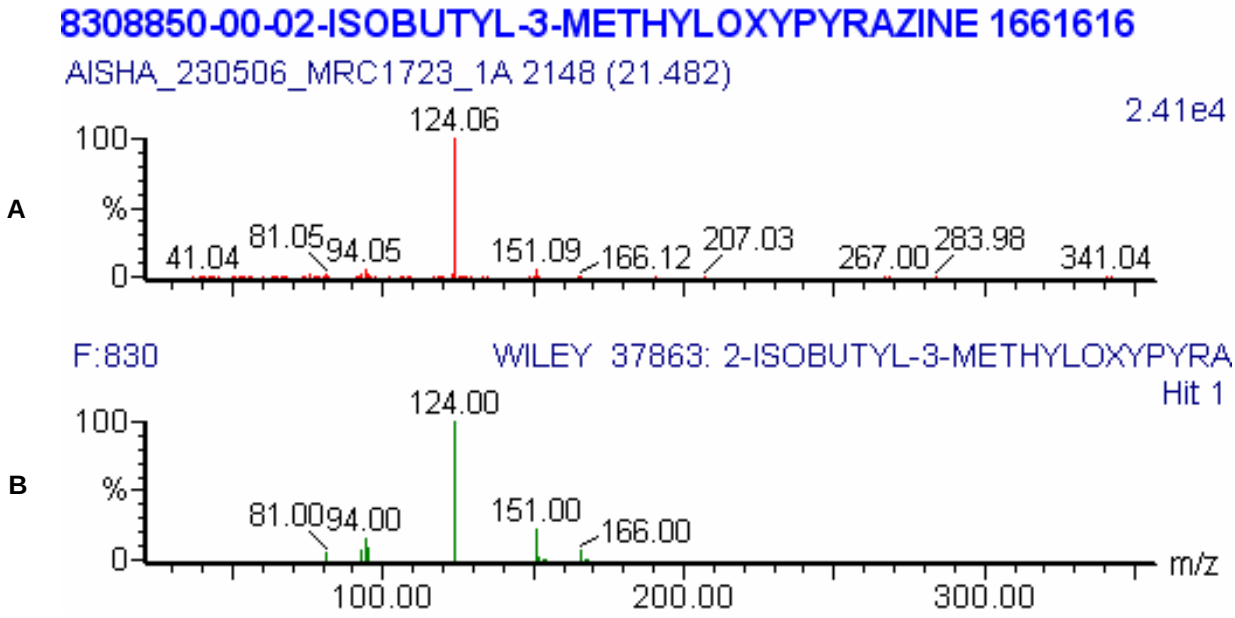


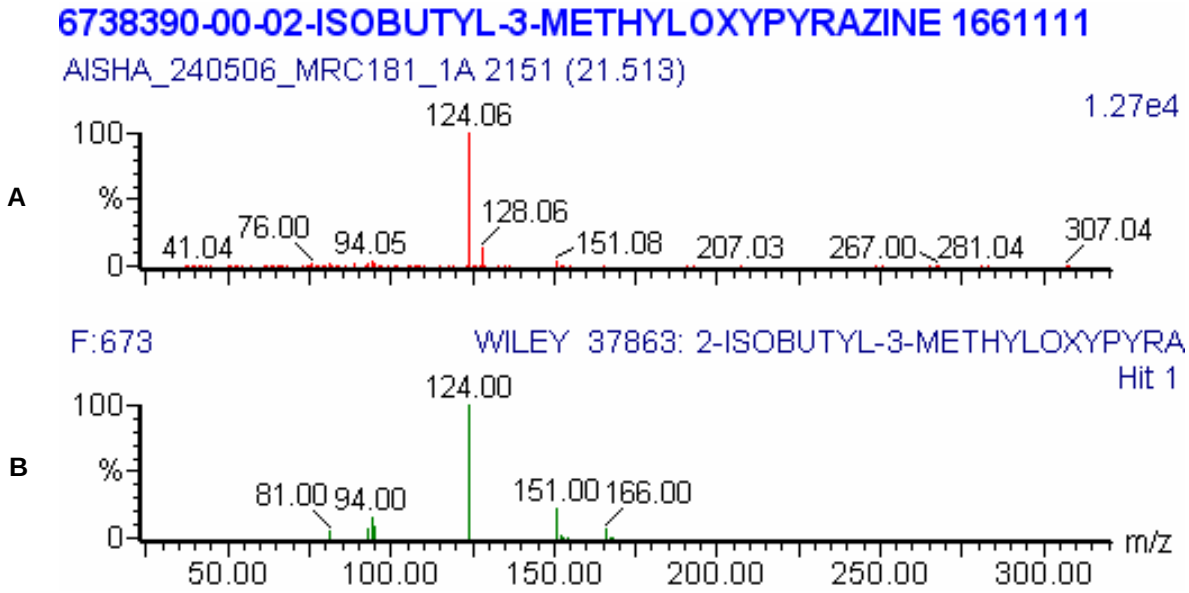
Figure 8: Comparison of the mass spectra of ***Penicillium rubrum*** (MRC 1723) and ***Penicillium purpurogenum*** (MRC 181) to that of the chemical standard 2-methoxy-3-isobutylpyrazine.



A – Mass spectrum of MRC 1723

B – Mass spectrum of pyrazine found in Wiley

Figure 9: Comparison of the mass spectra of ***Penicillium rubrum*** (MRC 1723) to that of the Wiley library.



A – Mass spectrum of MRC 181

B – Mass spectrum of pyrazine found in Wiley

Figure 10: Comparison of the mass spectra of ***Penicillium purpurogenum*** (MRC 181) to that of the Wiley library.

CHAPTER 4

Analytical method development and quantification of pyrazines produced by fungi

4.1. ABSTRACT

Methoxypyrazines have a characteristic green bell pepper odour and are of importance to the flavour and fragrance industry. It has been indicated that methoxypyrazines can be produced by ***Penicillium rubrum*** and ***Penicillium purpurogenum*** (Chapter 3). To determine whether pyrazine production by these fungi would be economically feasible the yields needed to be determined. Thus a solid-liquid and a liquid-liquid extraction method were developed in this study. A solvent extraction method using dichloromethane was used. Acidification of the sample gave the best results where 69% MIPP and 97% MIBP were recovered from the liquid-liquid extraction and 76% MIPP and 99% MIBP were recovered from the solid-liquid extraction. The pyrazines from the liquid-liquid extractions were then quantified by GC-MS-SIM. Results indicated that ***Penicillium rubrum*** produced 0.38 µg MIPP.L^{-1} and 0.88 µg MIBP.L^{-1} and ***Penicillium purpurogenum*** produced 0.88 µg MIPP.L^{-1} and 2.15 µg MIBP.L^{-1}.when grown in TSB medium for 4 and 5 days respectively. Yields obtained in this study are not seen as adequate enough for a feasible process and, therefore, further optimisation of the fermentation process is needed to increase the yields of methoxypyrazines.

4.2. INTRODUCTION

Methoxypyrazines are important aromatic compounds to the flavour and fragrance industry (Rowe, **2002**). They occur naturally in foods such as bell peppers and peas, and are known to have a characteristic green bell pepper odour (Maga, **1982**). These pyrazines occur in trace amounts but are significant due to their low odour thresholds (Rizzi, **1988**; Rowe, **2002**). The odour threshold of 2-methoxy-3-isobutylpyrazine (MIBP) is as low as 0.002 µg.L^{-1} in water and that of 2-methoxy-3-isopropylpyrazine (MIPP) is 10 µg.L^{-1} (Leffingwell & Associates, **1998**; Buttery ***et al.,*** **1969**). In addition, these pyrazines are important ingredients that contribute to the flavour of many food products (FAIA, **2002**).

Microorganisms such as ***Pseudomonas*** species have shown to produce methoxypyrazines, where a mutant strain of ***Pseudomonas perolens*** was found to produce as much as 12.5 mg.L^{-1} of 2-methoxy-3-isopropylpyrazine

(Cheng ***et al.,*** **1991**). Studies indicated that methoxypyrazines can be produced by fungi such as ***Penicillium*** and ***Aspergillus*** species (Buchanan & Houston, **1982**; Gallois & Grimont, **1985**; Larsen & Frisvad, **1994**). However, the yields obtained from these fungi have not been quantitatively determined.

It was demonstrated that ***Penicillium purpurogenum*** (MRC 181) produced MIBP and ***Penicillium rubrum*** (MRC 1723) produced MIBP and MIPP (see chapter 3). Although it has been proven that these fungi can produce methoxypyrazines, it is now needed to determine the pyrazine levels to establish whether these fungi can be used as candidates in a fermentation process for mass production.

In order to determine the concentration of the pyrazines produced by these fungi, this study aimed to develop a method for the extraction of methoxypyrazines by liquid-liquid extraction using dichloromethane (DCM). This step is needed to isolate the pyrazines present in the sample for quantification purposes. Pyrazines can be produced by either submerged or solid state fermentation processes. Soy press cake, for example, is one of the solid substrates considered for pyrazine production. Therefore, a solid-liquid extraction method using DCM was also developed in this study.

4.3. MATERIALS AND METHODS

4.3.1. Preparation of standards

The pyrazines used in this study were purchased as certified reference material (neat compounds) from Sigma-Aldrich, South Africa. Chemical information regarding the pyrazines used in this study is indicated in table 1.

A 100 µg.mL^{-1} stock solution of the two target pyrazines was prepared by adding 10 µL of each pyrazine to hexane (B&J) (Anatech, SA) in a 100 mL volumetric flask. From this solution, 1 mL was diluted to 100 mL hexane to make a 1 µg.mL^{-1} solution. Calibration standards were prepared by dilution of the 1 µg.mL^{-1} solution (Table 2).

4.3.2. Liquid-liquid extraction

Tryptic soy broth (TSB) (Merck, SA) was prepared according to the manufacturers instructions. A total of 100 mL TSB was spiked with 1 µL (0.0996 µg MIPP and 0.0990 µg MIBP added) of 100 $\mu g.mL^{-1}$ pyrazine spike solution to give a final concentration of 0.996 $\mu g.L^{-1}$ MIPP and 0.99 $\mu g.L^{-1}$ MIBP respectively. The latter solution was prepared in the same way as that of the 100 $\mu g.mL^{-1}$ stock solution but water was used as a diluent instead of hexane. The pH of the spiked sample was then adjusted to pH 3 using HCl (Saarchem/Merck, SA) and saturated with 30 g NaCl (Saarchem/Merck, SA). This was then extracted with 5 x 20 mL DCM (Anatech, SA). Thereafter the pH was adjusted to pH 9 with NaOH (Saarchem/Merck, SA) and once again extracted with 5 x 20 mL DCM. The organic phase was filtered through a Whatman no 1 filter paper in a funnel filled with anhydrous Na_2SO_4 (ACE, SA). Two hundred microlitres of toluene (Saarchem, SA) was added to the DCM extracts to act as a "keeper" before concentration of the extracts. The DCM extractions were concentrated by using a rotary evaporator (Laboroto 4001-efficient, Heidolph) with the water bath at ambient temperature (25^{o}C). The flasks were rinsed with 500 µL hexane and transferred to 1.5 mL amber vials. A volume of 2 µL of 2-methoxy-3-(1-methylpropylpyrazine) was added to the vials as a qualitative monitoring compound during the Gas chromatography (GC) analysis. The extracts were analysed for methoxypyrazines by Gas Chromatography – Mass Spectrometry – Selected Ion Monitoring (GC-MS-SIM). The extraction was repeated with a higher volume of the spike solution (100 $\mu g.mL^{-1}$) at 10 µL (0.996 µg MIPP and 0.990 µg MIBP) to give final concentrations of 9.96 $\mu g.L^{-1}$ MIPP and 9.9 $\mu g.L^{-1}$ MIBP respectively.

The effect of pH on extraction efficiency was evaluated by extracting at different pH levels. In extraction method 1, an acid-base extraction was done by first extracting the sample at pH 3, followed by an additional extraction at pH 9 of the same sample. In addition, extractions were also done separately under acidic conditions at pH 3 only (extraction method 2), and lastly with no pH adjustment to the sample (extraction method 3).

An extraction was also done directly from the samples where the fungi were growing in the broth. The fungus (***Penicillium purpurogenum***) was grown as described previously in chapter 3, section 3.3.1., page 124. Thereafter 10 µL of the 100 µg.mL^{-1} spike solution was added to 250 ml of the fungal broth, (0.996 µg MIPP and 0.990 µg MIBP added). An aliquot of 100 mL (0.4 µg), to give a final concentration of 4 µg.L^{-1}, was taken and then filtered through a Whatman no 1 filter paper and extracted as explained above.

4.3.3. Solid-liquid extraction

Twenty five grams of pasteurised soy cake were weighed into a 250 mL Erlenmeyer flask and 15 mL water was added. This was spiked with 10 µL (0.996 µg MIPP and 0.990 µg MIBP) of the 100 ppm spike solution. It was then acidified to pH 3 using HCl and 100 mL DCM was added to the flask. This was left on a shaker (Shake-O-Mat, Labotec) at 160 rpm for 2 hrs and the extract was filtered through a Whatman 1 filter paper in a funnel filled with anhydrous Na_2SO_4 (ACE, SA). Thereafter, 200 µL of toluene was added to the flask as a "keeper" and the DCM was evaporated under the same conditions as for the liquid-liquid extraction. The flasks were rinsed with 1 mL hexane and transferred to 1.5 mL amber vials. The samples were then analyzed by GC-MS-SIM for the presence of methoxypyrazines.

4.3.4. Analyses of pyrazines: GC-MS-SIM

Analytes were separated using an HPGC G1530A gas chromatograph coupled to a Hewlett Packard 5973 mass selective detector. An RTX 1 MS 30 m x 0.25 mm x 0.25 µm column obtained from Restek (Chromspec CC) was used. Manual injections were performed and a septumless sample head was used at the inlet. The inlet temperature was 250^{o}C. An injection volume of 1 µL was used and the injection mode was splitless. The purge flow was 60 mL.min^{-1} starting at 1.5 min after injection. The inlet pressure was 65 kPa in the constant pressure mode resulting in a carrier gas (Helium Ultra High Purity, Afrox) velocity of 39 cm.s^{-1} (1.2 mL.min^{-1}) at 60^{o}C. The GC oven was temperature programmed from 60^{o}C (5 min) at 2^{o}C.min^{-1} to 105^{o}C, then at 50^{o}C.min^{-1} to 250^{o}C (1 min). The run time was 31.4 min. A column post run was done at 280^{o}C (2 min) at an elevated 100 kPa inlet pressure.

The GC-MS transfer line was at 280^{o}C. The MS electron impact ionisation (EI) source temperature was 230^{o}C and the quadrupole analyser was at 150^{o}C. The ionizing electron energy was 70 eV and the electron multiplier voltage was 1706 V. There was no MS solvent delay and MS SIM parameters used are in appendix A, table 1.

For the quantification of the methoxypyrazines an external standard multi-level (n=5) calibration was used. The main ions used for the quantification, as well as the qualifier ions, are indicated in appendix A, table 1. To quantify the pyrazines present in ***P. purpurogenum*** (MRC 181) and ***P. rubrum*** (MRC 1723) the fungi were grown as described in chapter 3, section 3.3.1., page 123. The fungal broth was filtered and the filtrate extracted at pH 3.

4.4. RESULTS

4.4.1. Liquid-liquid extraction

Different extraction conditions were used and table 2 (Appendix A), which shows the results obtained during the development of the method for the solvent extraction of the soy broth. A 0% recovery was obtained for both methoxypyrazines when the broth was spiked with 1 µg.L^{-1} (0.996 µg.L^{-1} MIPP and 0.99 µg.L^{-1} MIBP) spike solution and with no solvent added prior to evaporation to act as a "keeper". No pyrazines were detected in the reagent blank. To determine the loss of pyrazines during the evaporation of the solvent, 1 µg.L^{-1} (0.996 µg.L^{-1} MIPP and 0.99 µg.L^{-1} MIBP) of the spike solution was added to 100 mL of DCM. The solvent was evaporated using a rotary evaporator and results indicated that there was 100% loss of MIPP and a 90% loss of MIBP when only 25 µL toluene was added as a "keeper" prior to evaporation (Appendix, B, tables 1-2 and figure 1). A reagent blank was also analysed and results are indicated in Appendix B, figure 2.

The extraction method recovery determination was repeated at a higher spike concentration of 10 ug.L^{-1} (9.96 µg.L^{-1} MIPP and 9.9 µg.L^{-1} MIBP) spike level in broth and 200 µL toluene added as a "keeper". When doing an acid-base extraction 30% of MIPP and 39% of MIBP were recovered in the acidic fraction (Appendix B: table 3 and figure 3), whereas only 0.5% MIPP and 3%

MIBP were recovered in the basic fraction (Appendix B: table 4 and figure 4). When repeating the extraction at pH 3 only, it was found that 69% of MIPP and 97% of MIBP were recovered (Appendix B: table 5 and figure 5). The increased efficiency noted when the extraction was repeated at pH 3 is likely due to experimental variation. With no pH adjustment of the sample, 24% of MIPP and 40% of MIBP were recovered (Appendix B: table 6 and figure 6).

4.4.2. Solid-liquid extraction

Results obtained for the solid-liquid extractions of the soy press cake under different pH conditions are indicated in appendix A, table 3. It was found that only 16% MIPP and 38% MIBP were recovered when no pH adjustment was made (Appendix B: table 7 and figure 7), whereas 76% MIPP and 99% MIBP were recovered when the soy press cake was adjusted to pH 3 (Appendix B: table 8 and figure 8). A reagent blank for the solid-liquid extraction was also done (Appendix B, figure 9).

4.4.3. Quantification of methoxypyrazines

The calibration curves used in the quantification of pyrazines produced by ***Penicillium purpurogenum*** (MRC 181) and ***Penicillium rubrum*** (MRC 1723) are illustrated in appendix C (Figures 1 and 2). The curves were set up by plotting the area of the peak of the quantification ion against the concentration of the standard. Chromatograms of each standard used in setting up the calibration curve are indicated in appendix C (Table 1 and figure 3, table 2 and figure 4, table 3 and figure 5, table 4 and figure 6 and table 5 and figure 7). The correlation coefficients (R^2 values) of both curves were greater than 0.99.

The concentration of pyrazines produced by each fungus is given in appendix D (Tables 1 and 2 and figures 1 and 2). Extraction was done on a single sample. Duplicate manual injections of the sample extract were performed. Quantification results obtained for ***Penicillium purpurogenum*** (MRC 181) were 0.88 µg MIPP.L^{-1} and 2.15 µg MIBP.L^{-1}, whereas for ***Penicillium rubrum*** (MRC 1723) it was 0.38 µg MIPP.L^{-1} and 0.88 µg MIBP.L^{-1} (Appendix D, tables 3-6). The limit of detection at a confidence level of 95%, calculated from the

calibration regression line, was 0.090 µg.L^{-1} for MIPP and 0.043 µg.L^{-1} for MIBP. The repeatability of the manual injection technique (retention time and area) was determined by multiple injections of a standard (0.1 ng.µL^{-1}). The %RSD (Relative Standard Deviation) was then calculated for the retention times of the analytes and the peak areas of the quantification ions. Retention time precision of the manual injection technique expressed as %RSD (n=10) was 0.028 and 0.022 for MIPP and MIBP respectively (Appendix D, table 7). The repeatability of the peak area of the quantification ion showed a %RSD of 1.68 and 1.55 (n=5) (Appendix D, table 8).

4.5. DISCUSSION

Solvent extraction is the most common method used for the extraction of volatiles (Cai and Ho, **2001**). Akochi ***et al***. (**1994**) used a method for the extraction of pyrazines with diethyl ether. Both an acid and a base pH step were included, whereby the basic step increased the recovery of pyrazines from the samples. Serrano-Carreon ***et al.*** (**1992**) used DCM as a solvent for the extraction of pyrazines in a model system based on crushed wheat.

During the diethyl ether extractions in the method used by Akochi ***et al***. (**1994**), pyrazines can be back-extracted from the solvent using dilute HCl, which is a procedure probably used as a clean up step. Thereafter, the free bases are transformed with NaOH and are extracted with diethyl ether (Kuo ***et al***., **1989**). However, results of this study show that the pyrazines, when extracted with DCM at an acidic pH, gave the best results and a basic pH adjustment did not improve the recovery of pyrazines from the sample (Appendix A, table 2). Addition of an acid (pH 3) was found to improve the extraction efficiency of pyrazines from the soy press cake as well (Appendix A, table 3). The acid possibly serves to free the pyrazines from the oily soy cake matrix.

Observations similar to this study were found by Serrano-Carreon ***et al.*** (**1992**). These authors tried four extraction methods, two methods having only an acid step and two methods having both an acid and a base step at

two different concentrations of acid. They found that the method with the higher acid concentration and no base step gave the best results. It was also shown that basic conditions did not improve pyrazine extraction recovery. The extraction of the sample at pH3 (HCl) with DCM is, therefore, regarded as a suitable extraction method for pyrazines.

A higher percentage extraction recovery to that obtained in this study would be desirable. However, for a sample spiked at a level of 10 $\mu g.L^{-1}$, a 60-115% recovery is acceptable. In comparison to the method used by Serrano-Carreon ***et al.*** (**1992**) the extraction recovery in this study of MIBP is higher (97% compared to 60.2%). Thus this method can be used for the extraction and detection of methoxypyrazines produced by ***P. rubrum*** and ***P. purpurogenum.***

There was a significant loss of the pyrazines during the evaporation of the solvent when only 25 µL toluene was added to the extract as a "keeper" (Appendix A, table 2). Therefore, a larger volume of toluene (200 µL) was added as a "keeper" to the extract before evaporation of the solvent. This reduced loss of the analytes during evaporation and, therefore, higher percentage recoveries of the target pyrazines were achieved. This was done by spiking with 10 µL of the 100 $\mu g.mL^{-1}$ spike solution in 100 mL sample (0.996 $\mu g.L^{-1}$ MIPP and 0.99 $\mu g.L^{-1}$ MIBP) to give a final concentration of 9.96 $\mu g.L^{-1}$ MIPP and 9.9 $\mu g.L^{-1}$ MIBP broth. The amount injected for GC-MS analysis was 2 $ng.\mu L^{-1}$ extract (500 µL final extract volume) (Appendix A, table 2).

Extraction of fungal samples was difficult when performing the solvent extraction in a separating funnel due to blockages of the funnel by fungal material. Filtering of the samples prior to extraction was thus considered. Results indicated that pyrazines were recovered from the extractions and they are thus not retained on the filter (Appendix A, table 2). Therefore, it was decided to first filter the samples before doing the solvent extraction.

To determine if the GC-MS-SIM technique that was used to quantify the pyrazines could yield repeatable results, the precision of the manual injection technique and instrument response (%RSD) was determined. Low %RSD values were obtained for both the quantification ion peak areas (%RSD of 1.68 and 1.55 for MIPP and MIBP respectively) and the analyte retention times (%RSD 0.028 and 0.022 for MIPP and MIBP respectively) indicating good precision of the GC-MS-SIM technique. The pyrazines produced by the fungi were detected at levels above the detection limits and thus the methods used in this study for the extraction and quantification of pyrazines are suitable to determine whether fungi are able to produce methoxypyrazines (Appendix D, table 1). It was also found that the correlation coefficients (R^2 values) of the calibration curves were excellent as they were greater than 0.99, indicating that the methoxypyrazines were accurately quantified in this study.

4.6. CONCLUSION

A method for the solvent extraction of methoxypyrazines produced by ***P. rubrum*** and ***P. purpurogenum*** was successfully developed in this study. This method involved the use of DCM as a solvent for the extraction of pyrazines at pH 3. The pyrazines produced by fungi were present only at trace levels. Low concentrations of pyrazines obtained can lead to difficulties in detection and quantification. However, GC-MS-SIM analysis was shown to be suitable for the quantification of pyrazines at trace levels.

To produce pyrazines on a large scale, using the methods of this study, would not be economically feasible due to the low yields of pyrazines obtained and further work to optimise and increase the recovery of pyrazines is required. The method developed, however, is satisfactory for non-commercial, screening purposes. A solid-liquid extraction method was also developed that can be used in future studies for the extraction of pyrazines from fungi growing on soy press cake.

4.7. REFERENCES

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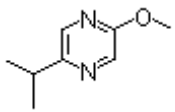
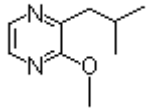
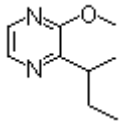
Table 1: Chemical information on pyrazines.

Name	Abbreviation	Structure	CAS	MW	MF	Density	Purity
2-methoxy-3, (5/6)-isopropyl pyrazine	MIPP		25773-40-4	152.1	$C_8H_{12}N_2O$	0.996 $g.mL^{-1}$	98%
2-methoxy-3-isobutyl pyrazine	MIBP		24683-00-9	166.11	$C_9H_{14}N_2O$	0.99 $g.mL^{-1}$	99%
2-methoxy-3-(1-methylpropyl)pyrazine	QMC		24168-70-5	166.22	$C_9H_{14}N_2O$	1.00 $g.mL^{-1}$	98%

CAS = Chemical Abstract number

MW = Molecular Weight

MF = Molecular Formula

QMC = Qualitative Monitoring Compound

Table 2: Preparation of calibration standards.

Standard	Concentration of Standard ($ng.\mu L^{-1}$)	Volume of 1 ppm stock	Volume hexane	Final Volume
1	0.025	25 µL	975 µL	1000 µL
2	0.05	50 µL	950 µL	1000 µL
3	0.1	100 µL	900 µL	1000 µL
4	0.25	250 µL	750 µL	1000 µL
5	0.5	500 µL	500 µL	1000 µL

APPENDIX A (Data produced by Y Naude, Department of Chemistry, University of Pretoria)

Table 1: MS-SIM Parameters.

Name	Rt min	Dwell	Quantification ion	Qualifier ions
2-methoxy-3,(5/6)-isopropylpyrazine	13.5	75	137	152, 124,138
2-methoxy-3-isobutylpyrazine	19.85	75	124	151, 94, 81
2-methoxy-3-(1-methylpropyl)pyrazine			-	138 (target ion), 124, 151, 137

Rt = Retention time

Table 2: Percentage recovery of MIPP and MIBP from TSB under different extraction conditions using DCM solvent (liquid-liquid) extraction.

Extractions	Percentage Recovery	
	MIPP	**MIBP**
Broth spiked (1 µg.L^{-1})		
Acid fraction	ND	ND
Base fraction	ND	ND
Reagent Blank	ND	ND
Loss during evaporation of solvent (25 µL toluene added as a keeper)	ND	10
Fungus spiked at 5 ng.µL^{-1} and then filtered		
Base only fraction	24	40
Broth spiked (10 µg.L^{-1}) (200µL toluene added as a keeper)		
Extraction Method 1 (Both acid and base steps)***:***		
1.1 Acid fraction	30	39
1.2 Base fraction	0.5	3
Extraction Method 2: Acid adjustment (pH3)	69	97
Extraction Method 3: No pH adjustment	24	40

Extraction efficiencies are a rough estimate and give an indication of the approximate values of extraction efficiencies.

ND – Not detected.

Table 3: Percentage recovery of MIPP and MIBP from soy press cake under different extraction conditions using DCM in the solid-liquid extractions.

Extractions	**Percentage Recovery at 40 µg.kg^{-1}**	
	MIPP	**MIBP**
No pH adjustment	16	38
pH 3	76	99

APPENDIX B (Data produced by Y Naude Department of Chemistry, University of Pretoria)

Loss on Evaporation

Table1 : Analyte loss during rotary evaporation.

Name	Quantification Ion	Ret Time	Area	µg.L^{-1}	% Recovery (1 µg.L $^{-1}$
MIPP	137			ND	0
MIBP	124	20.223	89265	0.081625	8.163

Table 2: Table of retention time and areas of pyrazines during the determination of the loss on evaporation of the standard (0.5 ng.µL^{-1}).

Peak#	Quantification Ion	Ret Time	Area
MIPP	137	15.024	2375414
MIBP	124	20.237	2733939

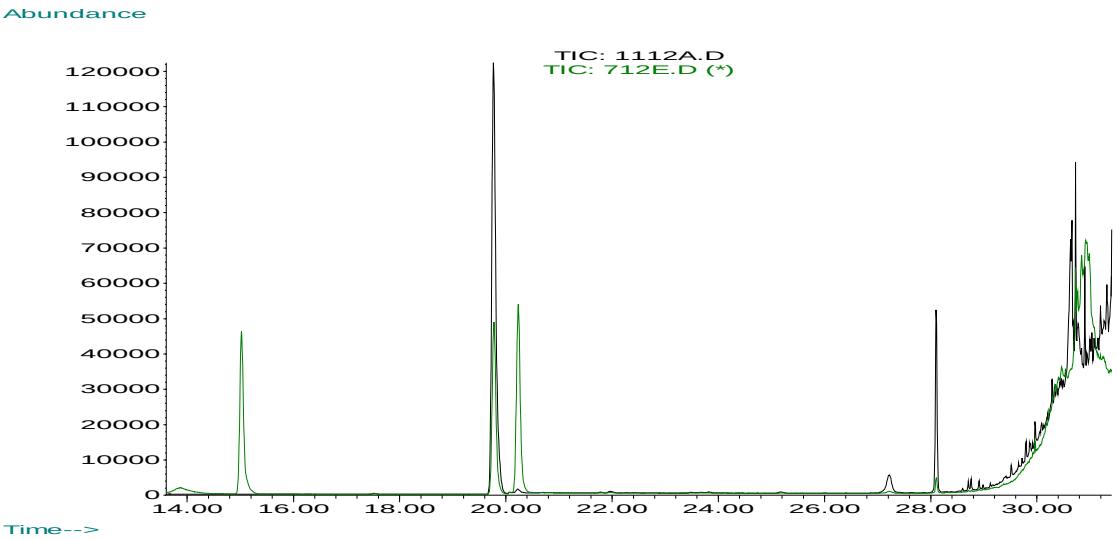


Figure 1: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines during the determination of the loss on evaporation (TIC 1112A.D) and the standard (0.5 ng.µL^{-1}) (TIC 712 E.D). The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine, originally added as a qualitative monitoring compound during GC analysis.

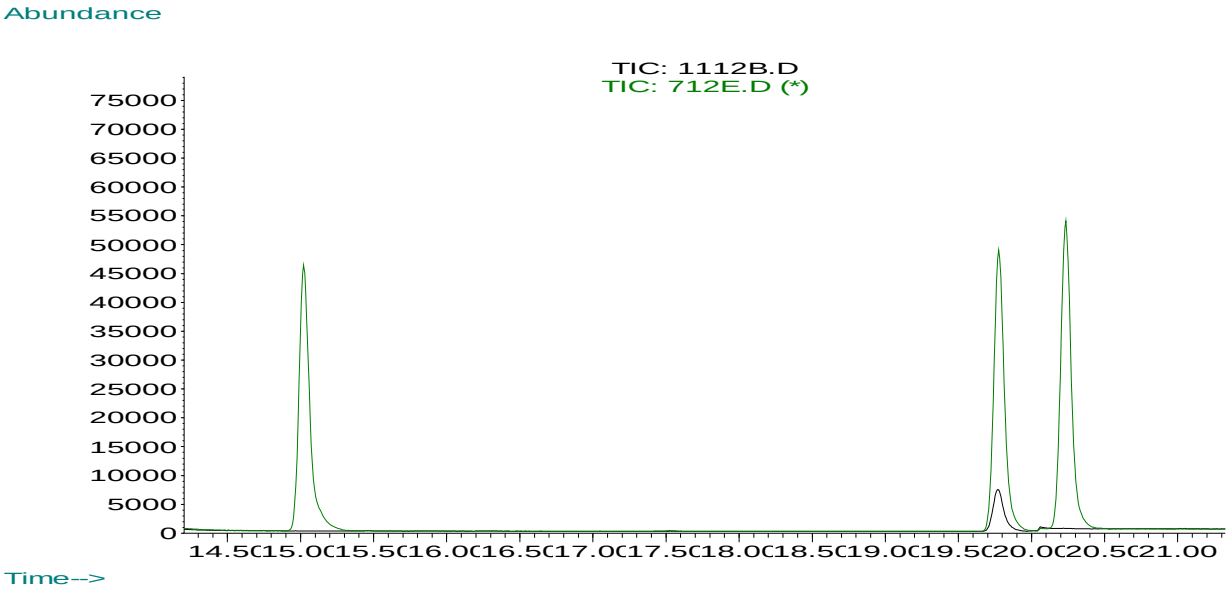


Figure 2: Overlays of the chromatograms (sum of selected ions in table 1, appendix A) of the reagent blank (TIC: 1112B.D) and a standard (0.5 ng.µL^{-1}) (TIC: 712E.D). The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

Liquid-liquid extractions

Table 3: Retention time and areas of pyrazines during the acid-base liquid-liquid extraction of the acid fraction before adjustment to pH 9.

Peak#	Quantification Ion	Ret Time	Area	µg.L^{-1}	% Recovery (10 µg.L^{-1})
MIPP	137	14.832	2431180	2.37768	23.77678
MIBP	124	20.359	3920972	3.0964	30.96399

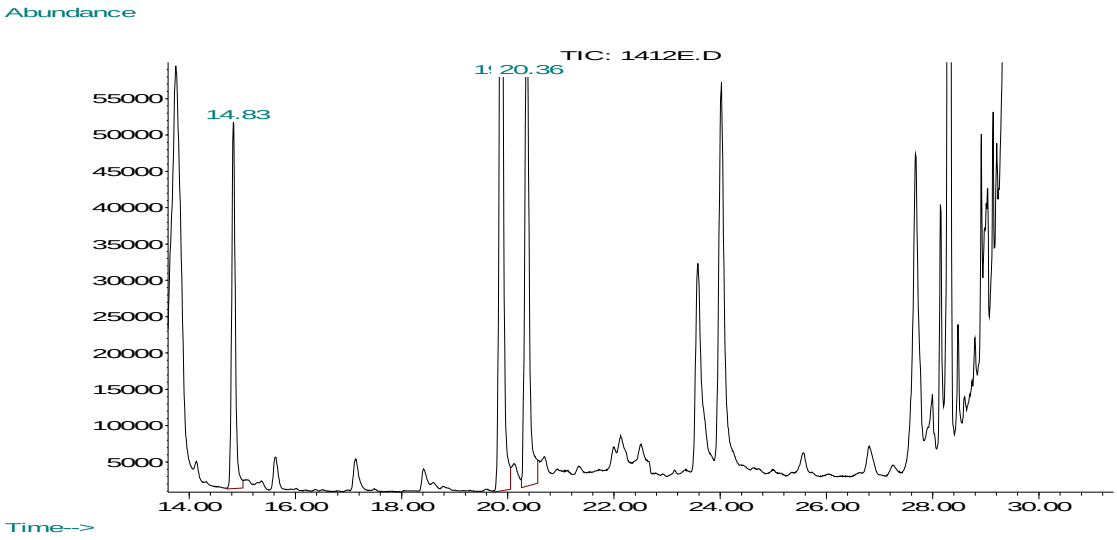


Figure 3: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines of the acid fraction (pH 3) before adjustment to pH 9 during the acid-base liquid-liquid extraction. The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

Table 4: Retention time and areas of pyrazines during the liquid-liquid extraction of the base fraction after extraction at pH 3.

Peak#	Quantification Ion	Ret Time	Area	µg.L^{-1}	% Recovery (10 µg.L^{-1})
MIPP	137	14.822	37466	0.03664	0.366415
MIBP	124	20.357	256879	0.20286	2.028578

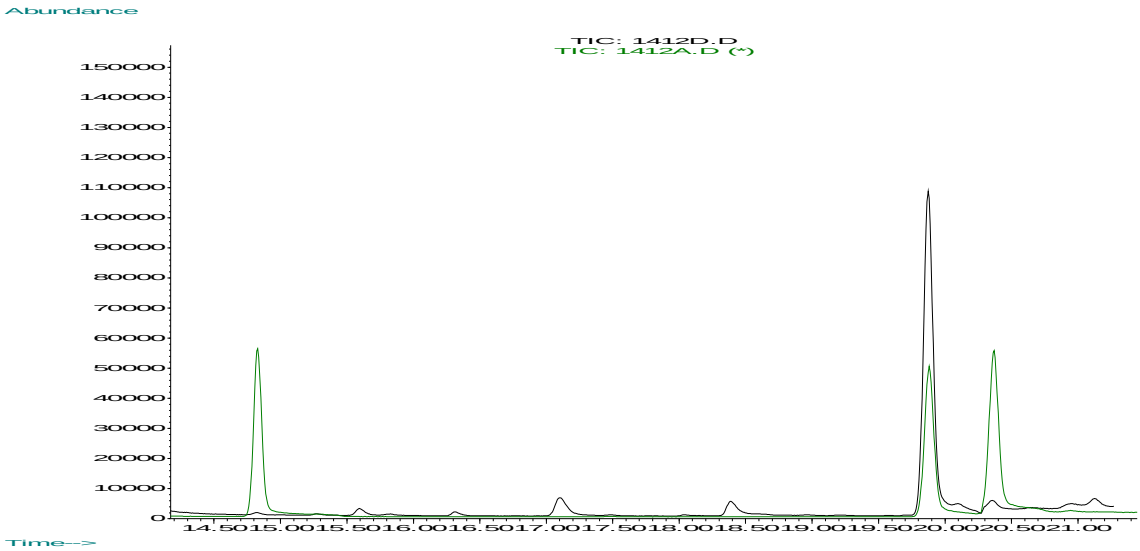


Figure 4: Chromatogram (TIC: 1412D.D) (sum of selected ions in table 1, appendix A) of pyrazines during the liquid-liquid extraction of the base fraction (pH 9) after extraction at pH 3. The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

Table 5: Retention time and areas of pyrazines during the liquid-liquid extraction at pH 3 only.

Peak#	Quantification Ion	Ret Time	Area	µg.L^{-1}	% Recovery (10 µg.L^{-1})
MIPP	137	14.945	6312668	5.47673	54.8
MIBP	124	20.468	10055943	7.701095	77.0

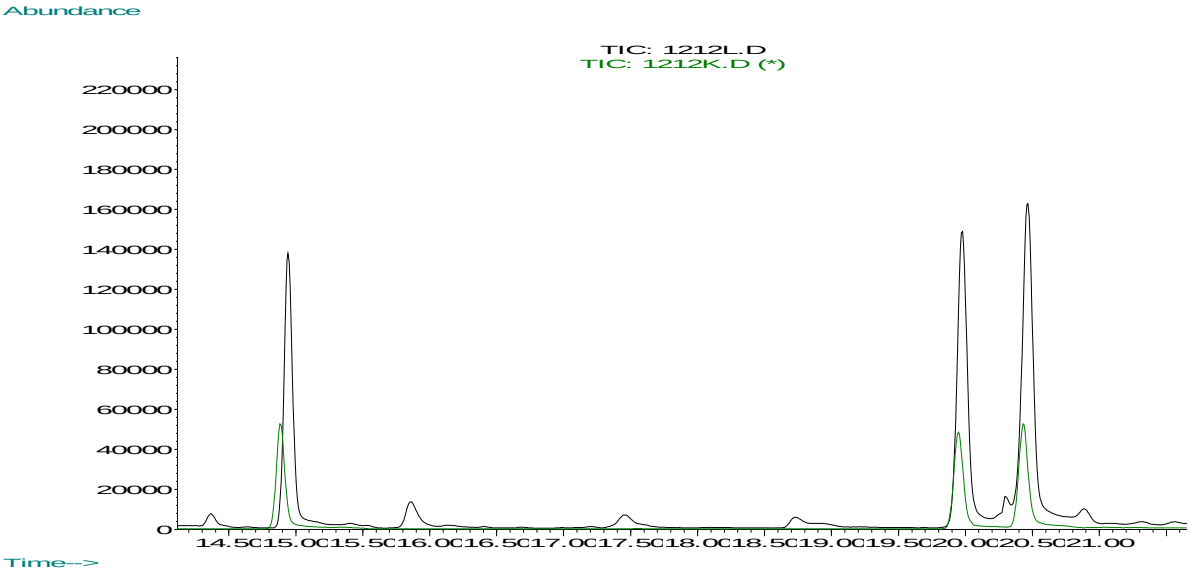


Figure 5: Overlays of the chromatograms (sum of selected ions in table 1, appendix A) (TIC: 1212L.D) of pyrazines during the liquid-liquid extraction at pH 3 only and the standard only. The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

Table 6: Retention time and areas of pyrazines during the liquid-liquid extraction at no pH adjustment.

Peak#	Quantification Ion	Ret Time	Area	µg.L^{-1}	% Recovery (10 µg.L^{-1})
MIPP	137	14.835	1941806	1.899075	18.99074
MIBP	124	20.367	4016906	3.17216	31.72158

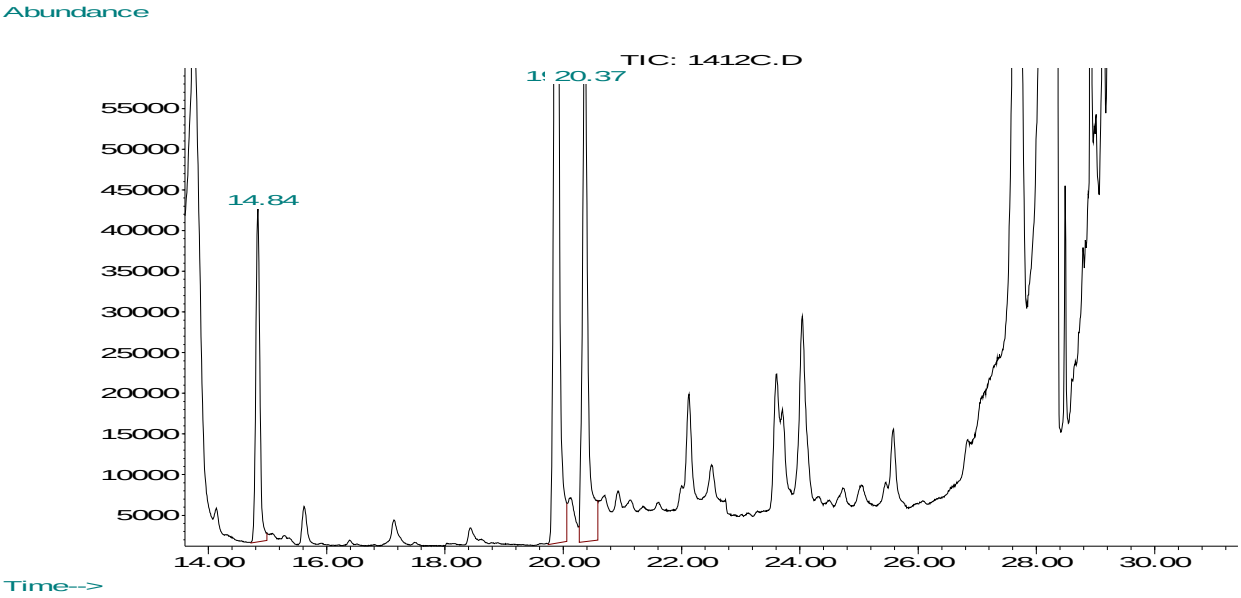


Figure 6: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines during the liquid-liquid extraction at no pH adjustment. The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

Solid-liquid Extractions

Table 7: Retention time and areas of pyrazines during the solid-liquid extraction at no pH adjustment.

Peak#	Quantification Ion	Ret Time	Area	µg.kg^{-1}	%Recovery (40 µg.kg $^{-1}$)
MIPP	137	14.853	1519505	5.27316	10.54632
MIBP	124	20.390	3943914	12.0814	24.1628

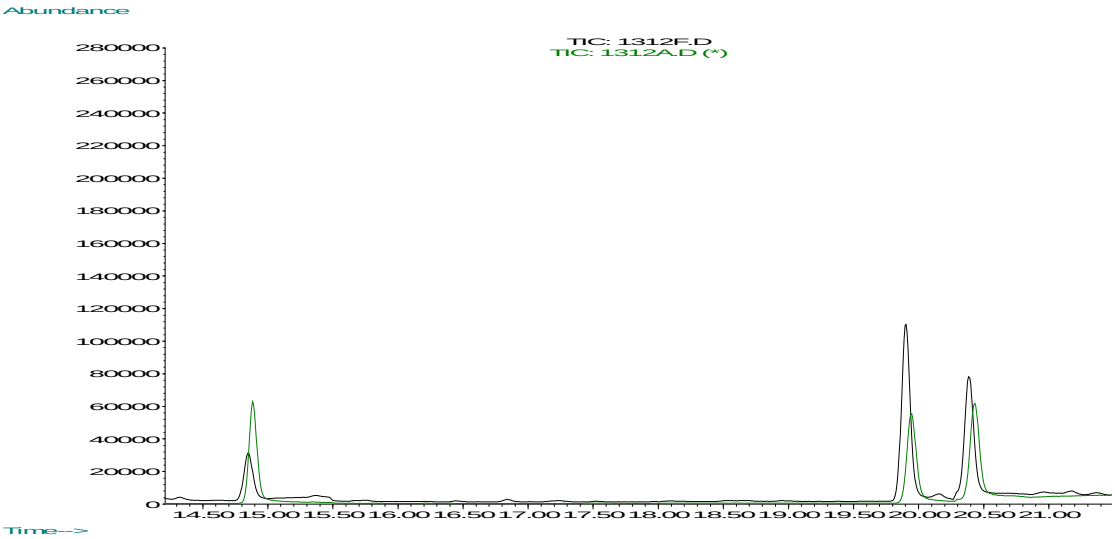


Figure 7: Chromatogram (sum of selected ions in table 1, appendix A) [soy no pH adjustment (TIC: 1312F.D) overlayed with the standard (0.5 ng.µL^{-1}) (TIC: 1312A.D)] of pyrazines during the solid-liquid extraction at no pH adjustment. The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

Table 8: Retention time and areas of pyrazines during the solid-liquid extraction at the acidic pH.

Peak#	Quantification Ion	Ret Time	Area	µg.kg^{-1}	% Recovery (40 µg.kg^{-1})
MIPP	137	14.863	6222344	24.34164	48.68328
MIBP	124	20.444	9926520	31.35596	62.71192

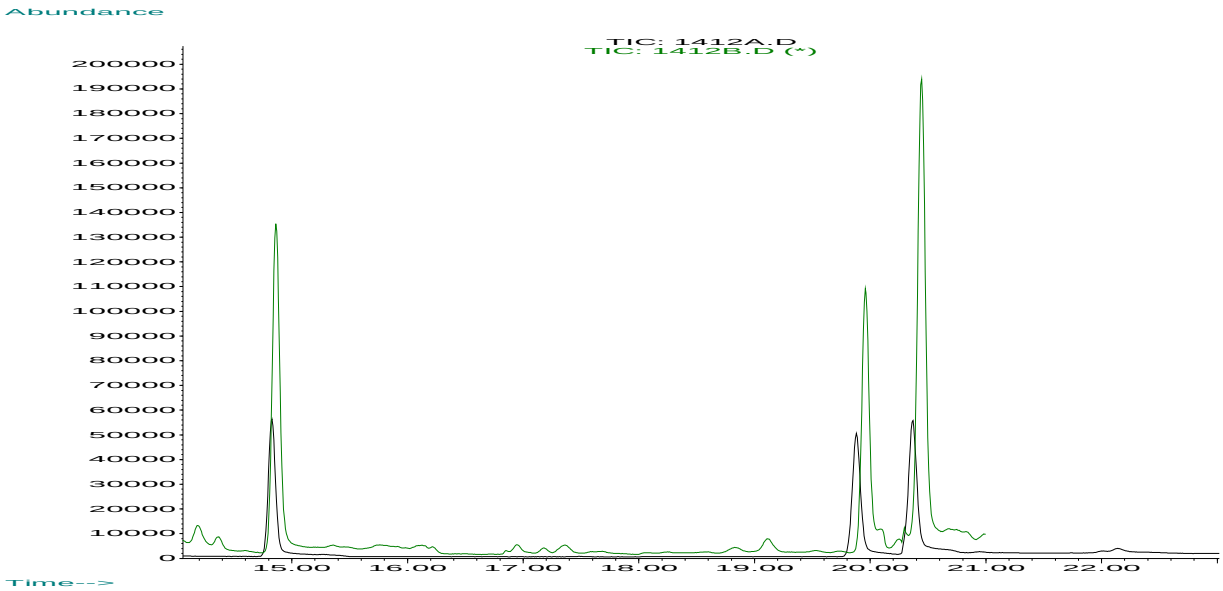


Figure 8: Chromatogram (sum of selected ions in table 1, appendix A) (TIC: 1412B.D) of pyrazines during the solid-liquid extraction at the acidic pH. The peak at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

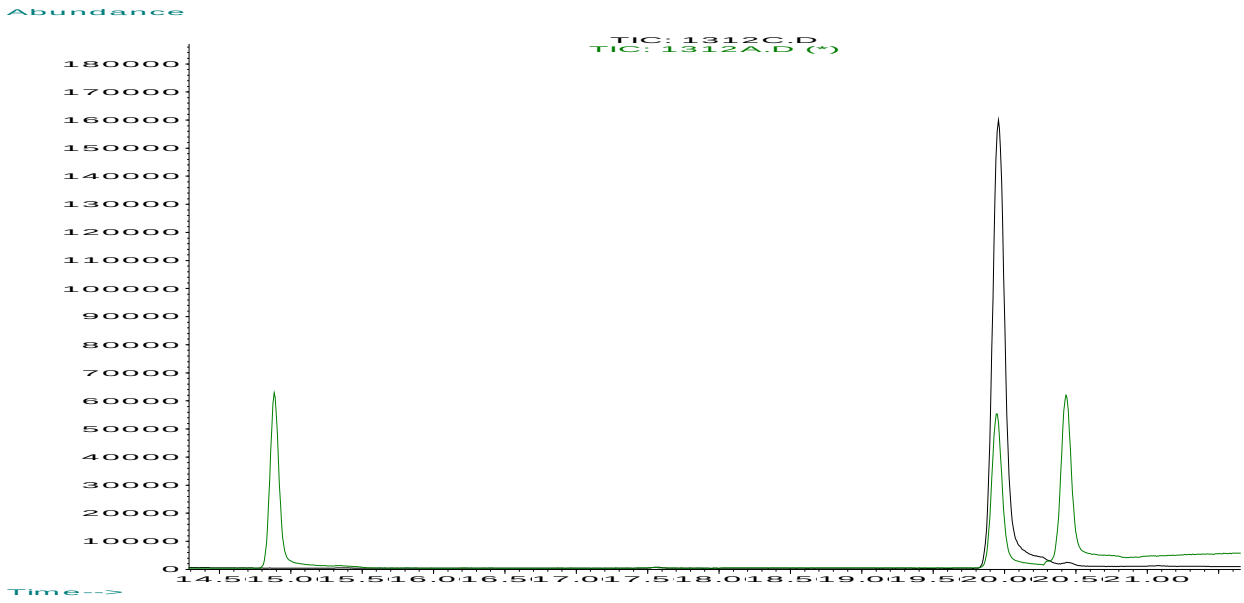


Figure 9: Overlays of the Chromatograms (sum of selected ions in table 1, appendix A) of the reagent blank and standard (0.5 ng.µL^{-1}) for the solid-liquid extractions. The peaks at retention time 19.8 min can be ignored and is due to an additional compound, 2-methoxy-3-(1-methylpropyl)pyrazine originally added as a qualitative monitoring compound during GC analysis.

APPENDIX C (Data produced by Y Naude Department of Chemistry, University of Pretoria)

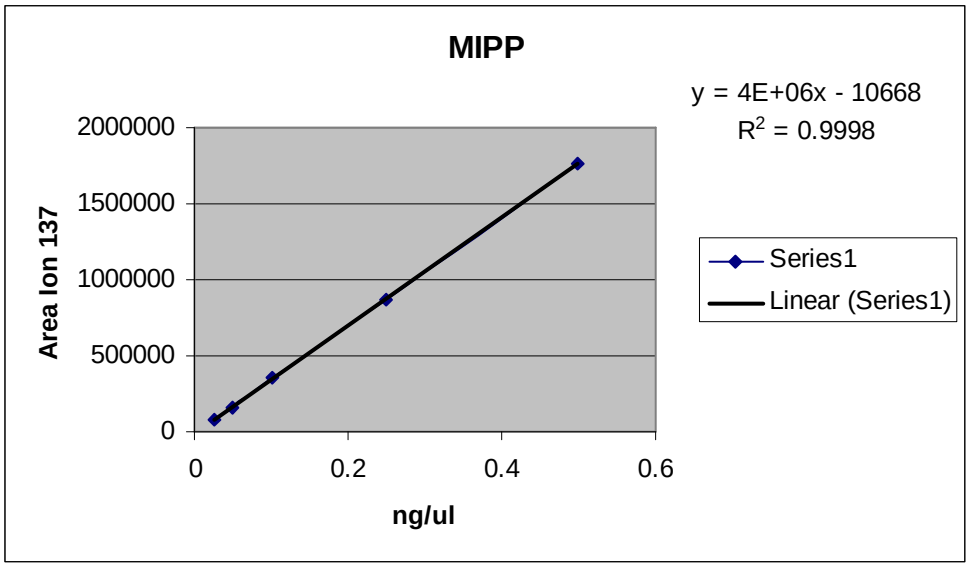


Figure 1: Calibration curve for MIPP.

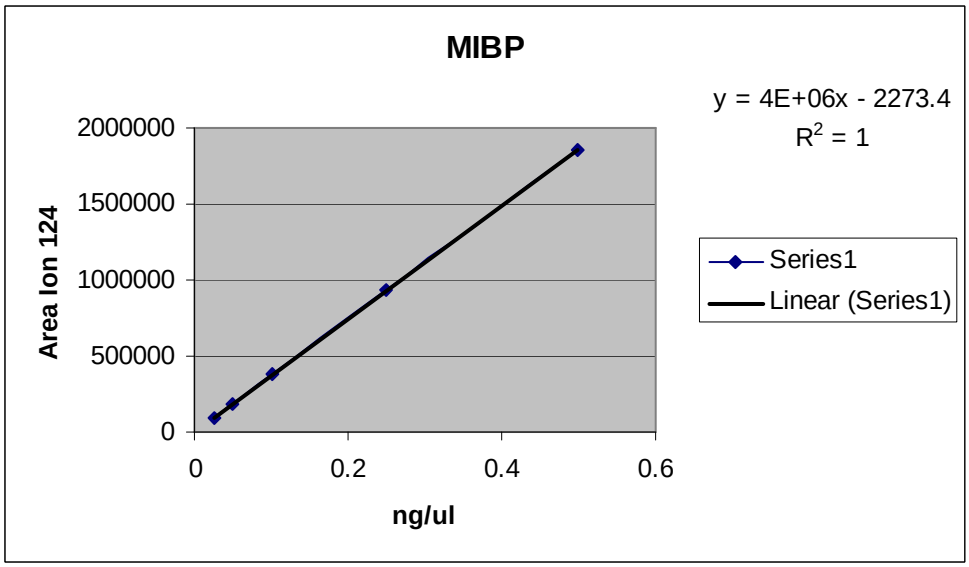


Figure 2: Calibration curve for MIBP.

Setting up the calibration curve

Table 1: Retention time and areas of pyrazines in the 0.5 ng.µL^{-1} standard.

Peak#	Quantification Ion	Ret Time	Area
MIPP	137	14.603	310754
QMC	-	19.612	2175783
MIBP	124	20.084	3137280

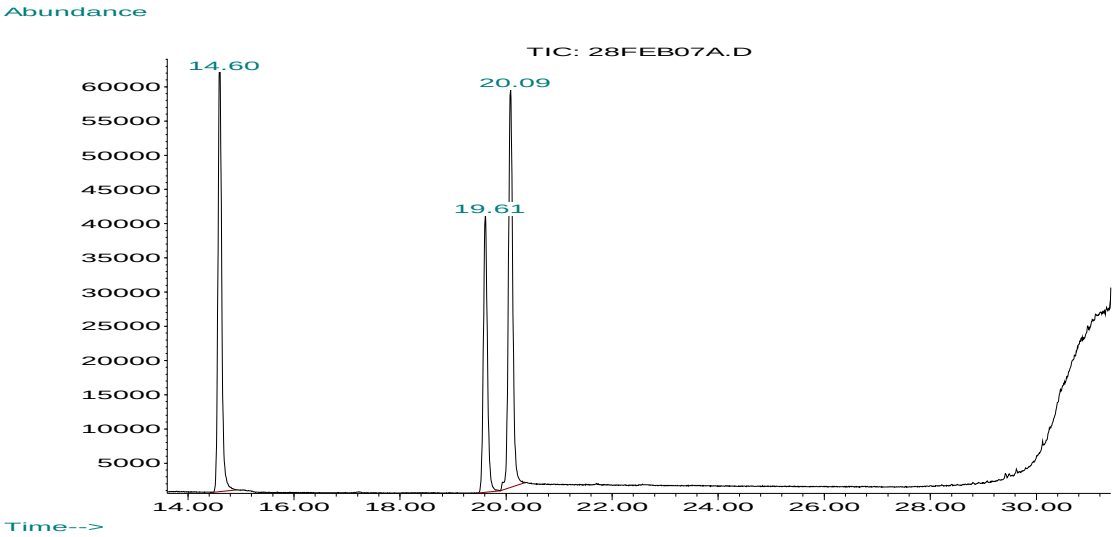


Figure 3: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines in the 0.5 ng.µL^{-1} standard.

Table 2: Retention time and areas of pyrazines in the 0.25 ng.µL^{-1} standard.

Peak#	*Quantification Ion*	*Ret Time*	*Area*
MIPP	137	14.610	1501710
QMC	-	19.621	1164356
MIBP	124	20.093	1587228

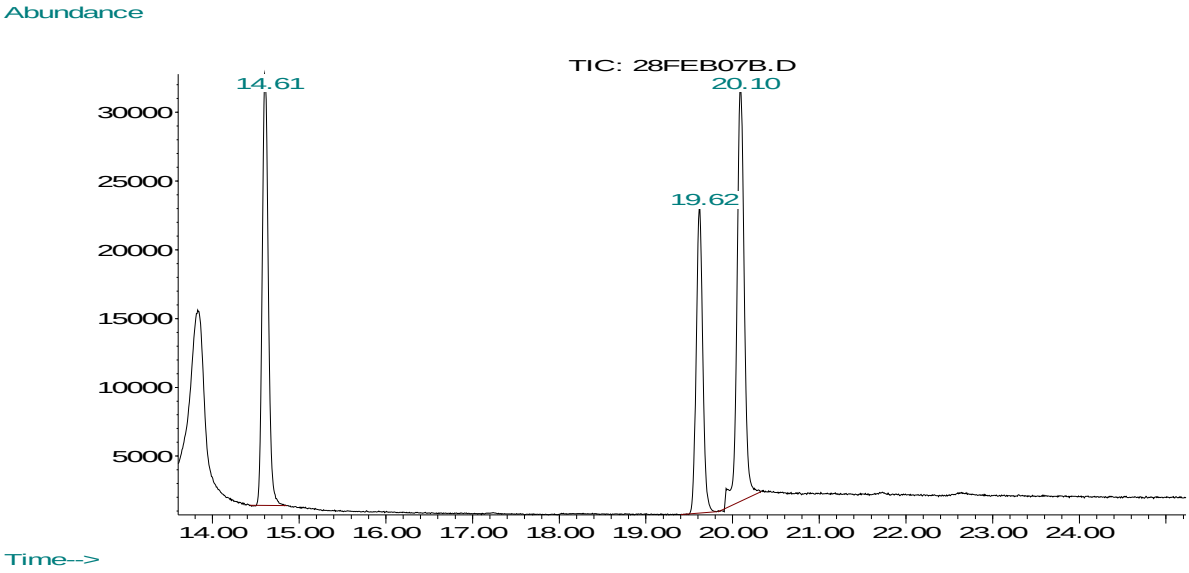


Figure 4: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines in the 0.25 ng.µL^{-1} standard.

Table 3: Retention time and areas of pyrazines in the 0.1 ng.µL^{-1} standard.

Peak#	Quantification Ion	Ret Time	Area
MIPP	137	14.613	624222
QMC	-	19.620	1800740
MIBP	124	20.093	634671

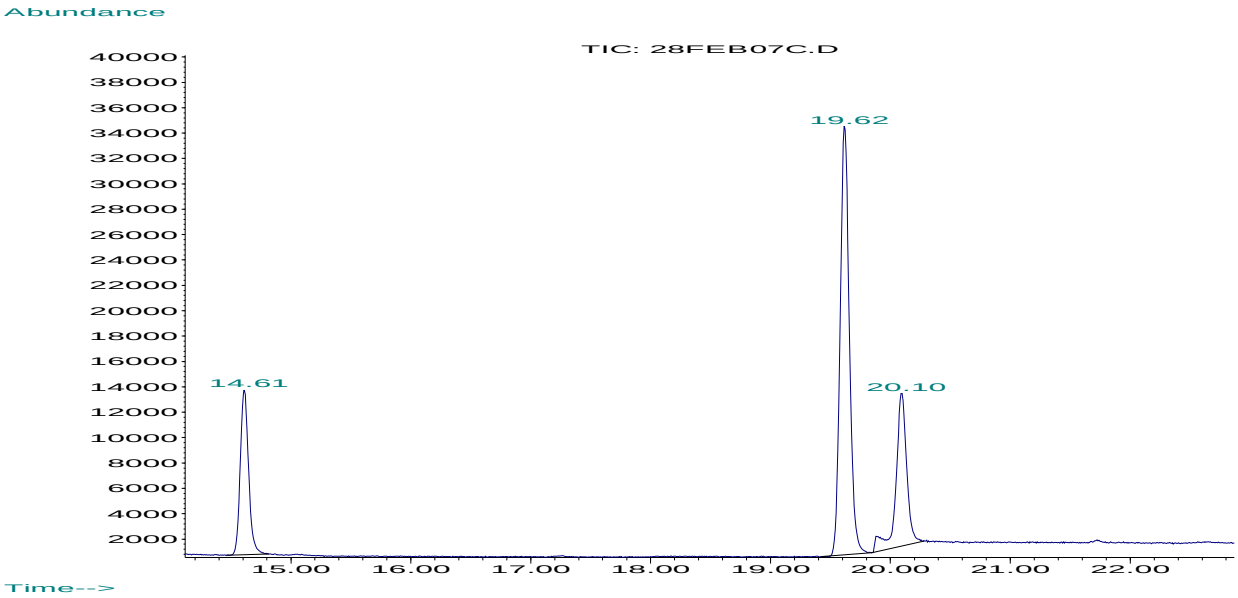


Figure 5: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines in the 0.1 ng.µL^{-1} standard.

Table 4: Retention time and areas of pyrazines in the 0.05 ng.µL^{-1} standard.

Peak#	Quantification Ion	Ret Time	Area
MIPP	137	14.607	274598
QMC	-	19.617	1552109
MIBP	124	20.087	312878

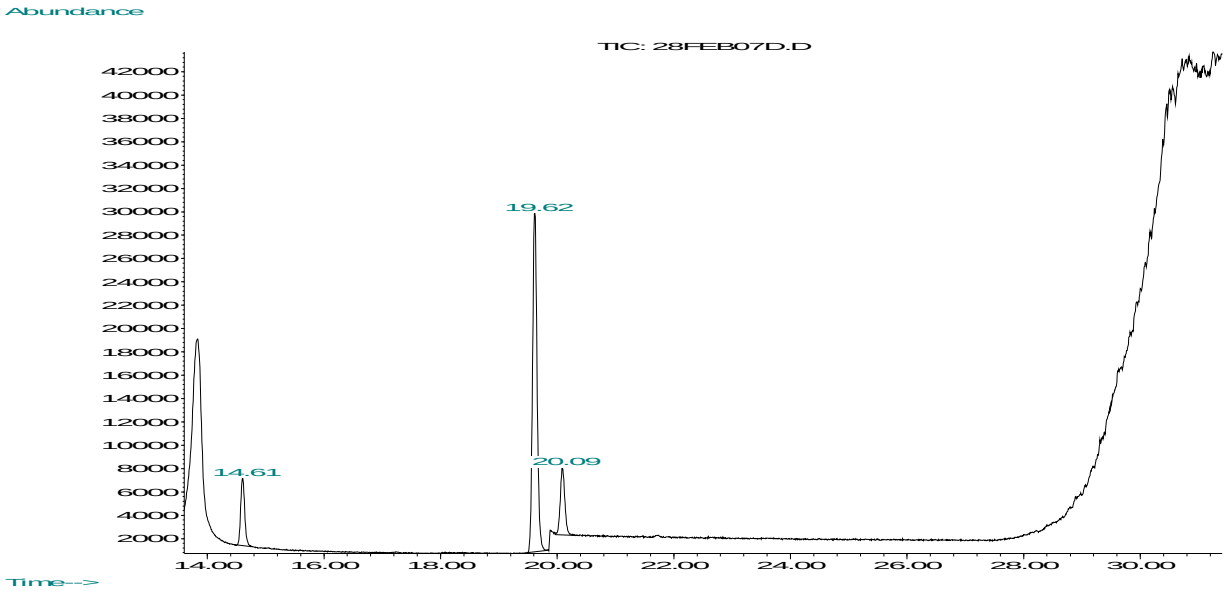


Figure 6: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines in the 0.05 ng.µL^{-1} standard.

Table 5: Retention time and areas of pyrazines in the 0.025 ng.µL^{-1} standard.

Peak#	Quantification Ion	Ret Time	Area
MIPP	137	14.609	136419
QMC	-	19.621	1406894
MIBP	124	20.093	157330

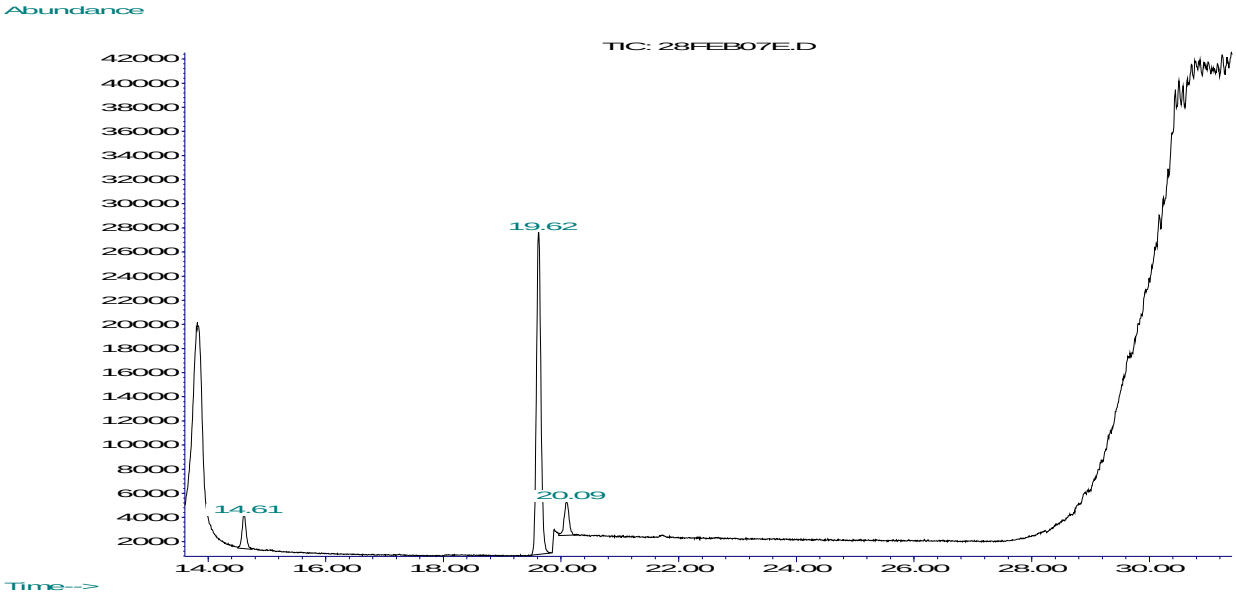


Figure 7: Chromatogram (sum of selected ions in table 1, appendix A) of pyrazines in the 0.025 ng.µL^{-1} standard.

APPENDIX D (Data produced by Y Naude, Department of Chemistry, University of Pretoria)

Quantification of MRC 181 and MRC 1723

Table 1: Quantification of methoxypyrazines produced by the fungi.

	MIPP	MIBP
Concentration in MRC 181 (n=1, m=2)	0.88 µg.L^{-1}	2.15 µg.L^{-1}
Concentration in MRC 1723 (n=1, m=2)	0.38 µg.L^{-1}	0.88 µg.L^{-1}
L.O.D (L.O.C = 95%)	0.090 µg.L^{-1}	0.043 µg.L^{-1}
% RSD Rt (min), m=10	0.028%	0.022%
% RSD Target Ion Response, m=5 (0.1 ng.L^{-1})	1.68%	1.55%
% Recovery at 10 µg.L^{-1}, n=1 (Broth)	55%	77%

Rt = Retention time

LOD = Limit of Detection

LOC = Level of Confidence

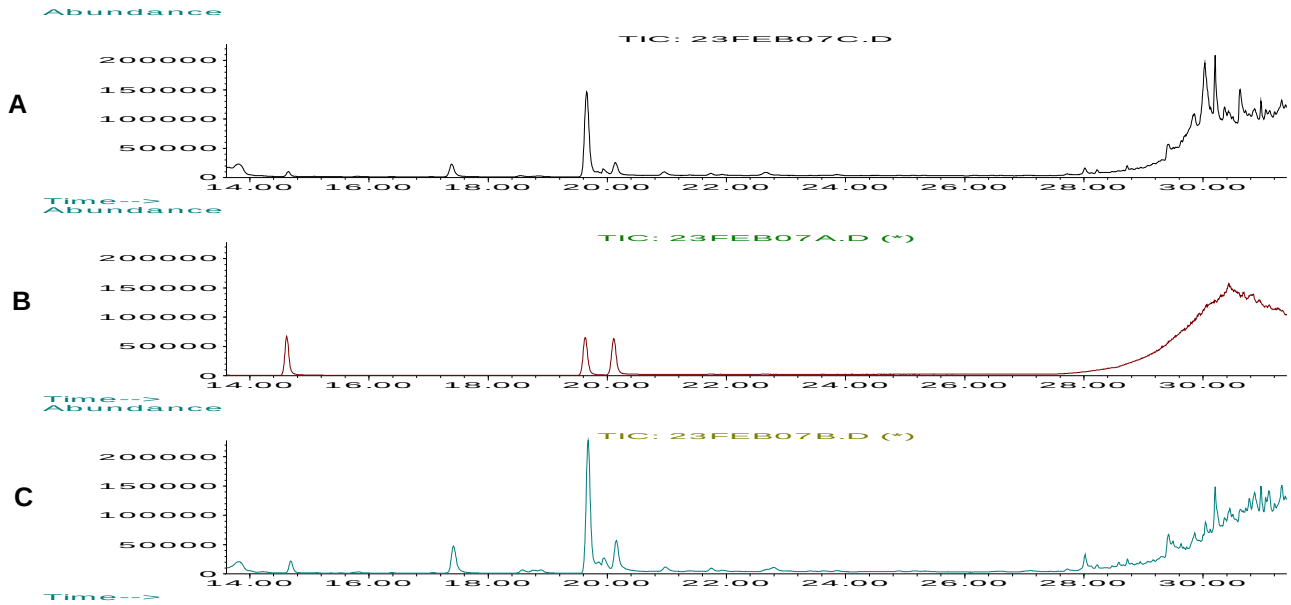
RSD = Relative Standard Deviation

n = Number of extractions

m = Number of injections

Table 2: Retention time and areas of pyrazines in MRC 1723 and MRC 181.

Peak#	Quantification Ion	Ret Time	Area
MRC 1723			
MIPP	137	14.647	458040
QMC	-	19.658	8432438
MIBP	124	20.139	1302547
MRC 181			
MIPP	137	14.690	957848
QMC	-	19.681	11988421
MIBP	124	20.155	3476605



A – Chromatogram of MRC 1723 **B** – Chromatogram of standard

C – Chromatogram of MRC 181

Figure 1: Chromatogram (sum of selected ions in Table 1 Appendix A) of pyrazines in MRC 1723 and MRC 181.

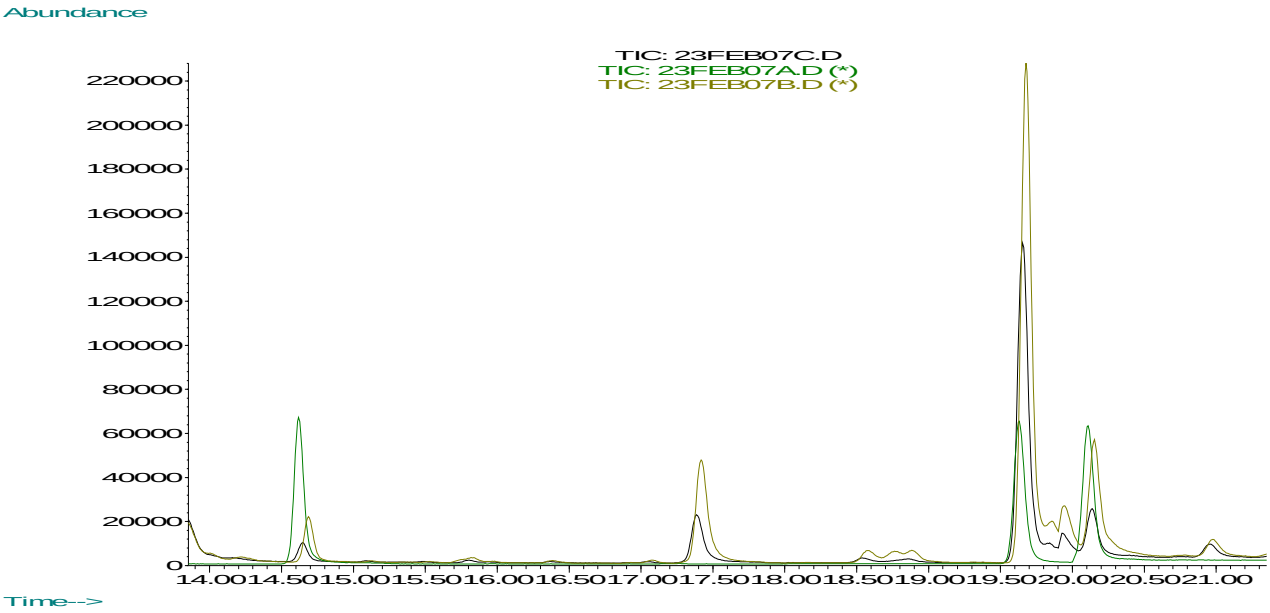


Figure 2: Overlay of the chromatograms of pyrazines produced by MRC 1723 (TIC: 23FEB07C.D) and MRC 181 (TIC: 23FEB07B.D compared to the analytical standard 0.5 ng.µL^{-1} standard (TIC: 23FEB07A.D).

Table 3: Average quantification results of MIPP produced by ***Penicillium purpurogenum*** (MRC 181).

Injection	Concentration ($\mu g.L^{-1}$)
28 Feb	0.95
23 Feb	0.8
X(m=2)	**0.875**
S	**0.11**
%RSD	**12.12**

X = Average; S = Standard Deviation; RSD =Relative Standard Deviation; m = Number of injections

Table 4: Average quantification results of MIBP produced by ***Penicillium purpurogenum*** (MRC 181).

Injection	Concentration ($\mu g.L^{-1}$)
28 Feb	2.35
23 Feb	1.95
X(m=2)	**2.15**
S	**0.28**
%RSD	**13.16**

X = Average; S = Standard Deviation; RSD =Relative Standard Deviation; m = Number of injections

Table 5: Average quantification results of MIPP produced by ***Penicillium rubrum*** (MRC 1723).

Injection	Concentration ($\mu g.L^{-1}$)
28 Feb	0.4
23 Feb	0.35
X(m=2)	**0.375**
S	**0.035**
%RSD	**9.428**

X = Average; S = Standard Deviation; RSD =Relative Standard Deviation; m = Number of injections

Table 6: Average quantification results of MIBP produced by ***Penicillium rubrum*** (MRC 1723).

Injection	Concentration ($\mu g.L^{-1}$)
28 Feb	0.95
23 Feb	0.8
X(m=2)	**0.875**
S	**0.106**
%RSD	**12.122**

X = Average; S = Standard Deviation; RSD =Relative Standard Deviation; m = Number of injections

A %RSD of 30 is considered acceptable.

Table 7: Repeatability of manual injection technique (n=10, between run).

	Rt MIPP	Rt QMC	Rt MIBP
	14.600	19.61	20.083
	14.603	19.612	20.084
	14.61	19.621	20.093
	14.613	19.62	20.093
	14.607	19.617	20.087
	14.609	19.621	20.093
	14.607	19.616	20.089
	14.603	19.61	20.084
	14.611	19.62	20.094
	14.61	19.618	20.092
X	14.6073	19.6165	20.0892
S	0.004138	0.004378	0.004367
***%RSD**	0.028328	0.022318	0.021736

X = Average

S = Standard Deviation

RSD =Relative Standard Deviation

* %RSD=S/X x 100

Table 8: Repeatability of the area of the quantification Ion (Response): manual injection of 0.1 ng.ul^{-1} pyrazine standard (n=5, between run).

	MIPP	MIBP
0.1 ng.uL^{-1}	341365	385473
	341998	378633
	346686	381137
	331971	370645
	345214	373789
X	341446.8	377935.4
S	5738.953	5871.432
***%RSD**	1.680775	1.553554

X = Average

S = Standard Deviation

RSD =Relative Standard Deviation

* %RSD=S/X x 100

RESEARCH NOTE

Production of pyrazines by *Penicillium purpurogenum* and *Penicillium rubrum* on soy press cake as substrate

5.1. INTRODUCTION

Examples of using Solid Substrate Fermentation (SSF) processes date back to more than two and a half thousand years where bread and cheese-making were established practices (Krishna, **2005**). Today SSF technologies are also used in the production of enzymes, antibiotics, single cell proteins, biofuels, organic acids and aromatic compounds (Pérez-Guerra ***et al***., **2003,** Krishna, **2005**). In the food industry, fungi are used for the production of flavours, colouring agents and protein supplements in order to enhance the taste and nutritional properties of non-meat products (Blanc, **2001**).

The use of fungi in an SSF process has several advantages. The hyphal mode of growth allows the fungus to easily penetrate and colonise the substrate and its ability to grow at a low water activity (aw) reduces the risk of bacterial contamination (Pérez-Guerra ***et al***., **2003**). In addition, solid substrates allow the products produced from the fungi to be more concentrated and therefore could contribute to more cost effective SSF processes.

Substrates that are generally ideal in SSF processes include agricultural crops and residues as well as forestry and food processing by-products. (Pandey, Soccol & Mitchell, **2000**; Pérez-Guerra ***et al***., **2003**). One potential substrate is soy press cake, a by-product from the extraction of oils from soybeans. South Africa is the second highest producer of soybeans in Africa with a 6-year (2000-05) average production at approximately 205,270 tonnes (Anonymous, **2008**). Seventy nine percent of soybean produced is converted to soy press cake (Schmidt, **2009**) of which most is converted into soybean meal and used in the animal feed industry (SAinfo, **2008**). However, soybeans contain a wide variety of amino acids such as glycine, leucine, valine and threonine (Berk, **1992**) that could serve as precursors for the production of pyrazines (Beck**,** Hansen & Lauritsen, **2003**). Therefore, a study was undertaken to evaluate soy press cake as a substrate for pyrazine production by ***Penicillium rubrum*** and ***P. purpurogenum***.

5.2. MATERIALS AND METHODS

5.2.1. Determination of fungal content in Soy press-cake

Prior to fermentation, the fungal content of the soy press-cake was determined using 2 methods. In the first method, fungal enumeration was done according to a method described by Rabie ***et al***. (**1997**). A sample was taken from the batch of the high fibre and the low fibre soy press-cake and thereafter 100 sub-samples (the size of a knife point) of each were randomly plated onto 20 potato dextrose agar (PDA) plates. Sub samples were plated onto the PDA plates by evenly placing 5 sub samples onto each petri plate. The plates were incubated for 7 days at 25^{o}C. The second method involved a serial dilution of pasteurised and unpasteurised soy press cake. One gram of soy press-cake was diluted in 10 mL water and a series of 5 dilutions (100 µL in 900 µL) was made. Thereafter, 100 µL was plated onto PDA using the spread plate technique and incubated at 25 ^{o}C until visible growth was noted. This experiment was done in triplicate. Fungi growing from the soy press-cake were morphologically investigated and identified to species level using stereo and light microscopic equipment. In certain cases, fungi were only identified up to genus level because of a lack of fruiting structures. The results of fungi identified in method 1 were expressed as a percentage of the amount of sub samples infested with a specific fungus.

5.2.2. Inoculum preparation

Penicillium purpurogenum (MRC 181) and ***Penicillium rubrum*** (MRC 1723) were separately tested for their ability to colonise soy press cake. The fungi were grown in petri dishes on PDA and malt extract agar (MEA) respectively at 25^{o}C until spores covered the surface of the plates. Spores were harvested by adding 7 mL of a 0.05% tween 80 solution to each plate. The spores were scraped off using a spatula. To maintain a constant inoculum size in all fermentations, the spore suspension, 5 mL from each plate, was transferred to a sterile container. A standard spore count was done using a haemocytometer. The soy press cake (3 kg) was inoculated with a volume of

25 mL of spore suspension, containing respectively in total about 5.75 x 10^8 spores of ***P. purpurogenum*** or 5.6 x 10^8 spores of ***P. rubrum***.

5.2.3. Fermentation

Milled pasteurised soy press cake (heated to 70-80^{o}C for a period of 10 min and done for 9 cycles) was received from the CSIR and used for SSF production of pyrazines. The fermentations were carried out in trays (36 cm x 28 cm x 8 cm depth) sterilised by wiping the surface with 76% ethanol. The fermentation medium consisted of a combination of 3 kg of pasteurised soy press cake mixed with a liquid medium. The liquid medium (total volume 1200 mL) containing $FeSO_4.7H_2O$ (0.09 g.L^{-1}), $MgSO_4.7H_2O$ (4.5 g.L^{-1}), $ZnSO_4.7H_2O$ (0.045 g.L^{-1}), KCl (4.5 g.L^{-1}), $CuSO_4.5H_2O$ (0.0225 g.L^{-1}) and glucose (45 g.L^{-1}) was autoclaved at 121^{o}C for 20 min. The effect of supplementation with glycine on pyrazine production was investigated by adding 45 g.L^{-1} (500 mL) of filter sterilised glycine to the medium. To prepare the latter medium, glycine was added to the ingredients stated above and was then made up to a volume of 1200 mL. The moisture content of the soy press cake was predetermined by weighing 10 g of each of the media and drying it in an oven (70^{o}C) over a period of 10 days and the weight of the samples monitored daily until stabilisation. The moisture content was found to be 67% for the medium containing glycine and 70% for the medium containing no glycine. The difference in moisture content is most likely because of the less water used to make the total volume of the medium to 1200 mL in the case where glycine was added.

In order to take adequate sample sizes from the trays during the fermentation 3 kg of the soy press cake was required. However, the trays were too small to carry out a fermentation of this volume. Therefore, the medium was divided into 2 trays where each tray contained 1.5 kg of the soy press-cake. Sampling was done by taking random sub-samples from +/- 5 different spots in each tray and combining into one sample to form a representative sample. The fermentations were done in triplicate for each fungus. After inoculation the trays were covered with aluminium foil and incubated for 9 days at 25^{o}C.

A total sample size of 61 g was taken daily for the analysis of pyrazines, glucose and pH. At the end of the fermentation 84% of the soy press cake was left in the trays. Glucose tests were done using Accu-Chek Active (Roche) and pH was tested by means of pH strips (pH-Fix 0-14, Machery-Nagel). Sample extraction and pyrazine analysis were done as described previously (see chapter 4, Section 4.3.3., page 157). The extracts were then sent to the CSIR, where pyrazine analyses were performed at the (National Metrology Institute of South Africa) NMISA. The method for separation of the compounds by gas chromatography is indicated in chapter 4, section 4.3.4 page 157.

5.3. RESULTS AND DISCUSSION

Results indicated that a high fungal content was present in the unpasteurised soy, where both high fibre and low fibre soy was evaluated (Table 1). These included ***Aspergillus flavus*** (100%), ***Mucor*** sp. (30%) and ***Aspergillus candidus*** (26%) found in the low fibre soy as well as ***Aspergillus versicolor***, ***Penicillium brevicompactum*** and ***Penicillium chrysogenum*** found at levels below 15%. ***Aspergillus flavus*** (28%) and ***Aspergillus fumigatus*** (24%) were found in the high fibre soy. Other fungi such as ***Penicillium brevicompactum***, ***Penicillium chrysogenum***, ***Mucor*** sp., ***Cladosporium cladosporioides***, ***Chaetomium*** sp. and ***Syncephalastrum racemosum*** were also found in the high fibre soy at a percentage below 10%. It was also found that most of the fungi identified were associated with poor storage conditions and some could have the ability of producing mycotoxins (Table 1). For example, ***Aspergillus flavus,*** present at high levels in soy is known to produce aflatoxins (Hensarling ***et al.***, **1983**). Although this fungus was present at much lower levels in the high fibre soy, the presence of other fungi not found in the low fibre soy also occurred. Therefore, the use of low fibre soy was used for the fermentation.

Fungal contaminants in the substrate could also have an effect on the production of pyrazines due to the utilisation of important precursors by competitive organisms. The presence of mycotoxigenic fungi in the soy press cake is also of concern as mycotoxins could end up in the final product, which is destined for use in the flavour and food industries. However, most

mycotoxins are not highly volatile (Cole & Cox, **1981**) and could be eliminated from the final product by optimally using the boiling point of pyrazines compared to mycotoxins during extraction by steam distillation for example.

The use of sterilisation would reduce or eliminate fungal contaminants, but is not seen as an option as this process may denature the amino acids such as glycine, leucine, valine and threonine present that are essential for pyrazine production (Berk, **1992**). Pasteurisation of the soy press cake was used in an effort to lower the fungal contaminants. Determination of the fungal content in the pasteurised soy indicated a reduction in fungi in some samples. ***Aspergillus fumigatus***, ***Aspergillus niger***, ***Cladosporium sphaerospermum***, ***Eurotium chevalieri*** and ***Mucor circinelloides*** were eliminated by pasteurisation and the average levels of ***Aspergillus flavus*** (3.2×10^3) in unpasteurised soy decreased to 1×10^3 in pasteurised soy (Table 2).

It also indicated that the pasteurisation process needs to be optimised and it should be homogenously done throughout the batch as large standard deviations were noted amongst the replicates (Table 2). Although the pasteurisation was not totally effective in the elimination of all the fungi present, high inoculums (5.75×10^8 spores of ***P. purpurogenum*** or 5.6×10^8 spores of ***P. rubrum***) were used in an effort to out-compete those organisms initially present in the soy press cake.

The changes in pH and glucose were used as an indication of the growth and metabolic activities of the fungi during the fermentation process. A change in pH reflects a change in metabolic activities. In this study, no difference was noted in the pH changes either between the two fungi or between the enriched soy press cake and the soy press cake containing no added glycine (Figure 1). The pH value in the soy press cake and the enriched soy press cake inoculated with ***Penicillium purpurogenum*** (MRC 181) and ***Penicillium rubrum*** (MRC 1723) remained at pH7 in all trays until day 5. At days 6-8 the pH decreased to pH6 and then increased again to pH7 at day 9. It is normally expected that the pH decreases initially and that indicates a secretion of

organic acids, followed by a pH increase that indicates the assimilation of organic acids (Krishna, **2005**).

The glucose concentrations in all trays were found to be very similar. The average concentration at day 0 was between 4.1 mmol.L^{-1} and 6 mmol.L^{-1}. The concentration in all trays increased to a peak value ranging from 16-21 mmol.L^{-1} on day 4 and then decreased again to a value of 7-7.9 mmol.L^{-1} on day 9. The decrease on day 9 was noted in all trays except in the tray fermented with ***Penicillium purpurogenum***, as the final reading in this tray was taken on day 8 (10.55 mmol.L^{-1}). No reading was taken on day 9 as the tray became contaminated (Figure 2). The increase in glucose concentration can be attributed to the break down of the complex carbohydrates such as raffinose and stachyose in the soy when converted to glucose (Berk, **1992**). Enzymes such as α-galactosidases are produced by many ***Penicillium*** species, including ***Penicillium purpurogenum,*** and are known to break down these complex carbohydrates (Lounteri Tekanen & Viikari, **1998**; Shibuya ***et al***., **1998**; Varbanets ***et al***., **2001**). It is thus possible that observations noted in figure 2 regarding the increase in glucose concentration is due to the production of α-galactosidases by the fungi which breaks down raffinose and stachyose, resulting in free glucose. The decrease in concentration is observed when glucose is utilised during metabolic activities.

The particle size of the substrate can also have an effect on the growth of the fungi. The soy press cake received from the CSIR was milled and, therefore, led to agglomeration and large pellets were formed. This influences the aeration negatively and can result in poor development and growth of fungi (Pandey ***et al***., **2000**). Thus, the visible growth of the fungi occurred mainly on the sides of the trays. This is most probably due to parameters such as temperature, oxygen transfer and moisture content that differs between the sides and centre of the trays. At the beginning of the fermentation all these parameters are the same in all areas of the tray but, as the fermentation progresses, a large amount of heat is generated by metabolic activities. Due to the low thermal conductivity of the materials used in SSF, heat builds up resulting in a difference in temperature at different areas in the tray, where

temperatures in the centre of the tray can be much higher than the sides (Pandey, **2003**). Due to the sides being cooler, condensate will form at the sides of the container and increase the moisture content there. Since aeration is also better on the sides, there is a better O_2 and CO_2 exchange with the environment. Thus, conditions on the sides of the container are more suitable for growth than in the centre. Larger particle size could result in better respiration and thus better growth (Pandey ***et al***., **2000**).

The high lipid content in the soy extracts interfered with the pyrazine analysis and, therefore, it could not be determined if the fungi could utilise the soy for pyrazine production. Florisil/mesh 130/140 (Analabs Incorporated, USA) is a magnesium silicate and is used to remove interfering compounds prior to analyses. This was used on a spiked sample but it was found that the florisil also removed the pyrazines in the spike and thus could not be used to clean up the extracts. Therefore, further work is needed to develop a method for the analysis of pyrazines in soy press cake with high lipid contents. However, this fell outside the scope of this study and was not further investigated.

Although pyrazines were not detected via chemical analysis, a sniffing test done on day 7 of the fermentation indicated that a chocolate odour was produced in most of the samples (Table 3). It was also noted that when the incubator door was opened a chocolate/caramel odour was observed. Possible pyrazines that could be produced are 2,3,5,6-tetramethylpyrazine, 2,3,5-trimethylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethlypyrazine, 2-acetyl-3,5(or 6)-dimethylpyrazine, 2-ethyl-3,5(or 6)-dimethylpyrazine, 2-methoxy-3-methylpyrazine and 2-methylpyrazine.

Although literature states that SSF is a promising technology for the production of value added products (Krishna, **2005**), many factors need to be taken into account when designing a fermentation process. The nature of the substrate is important and in this study the lipid content, fungal content and particle size of the soy press cake influenced fungal growth and pyrazine analyses.

In conclusion, the soy press cake in its current composition and texture does not support fermentation by ***P***. ***rubrum*** (MRC 1723) and ***P***. ***purpurogenum*** (MRC 181). Although the use of fungi in solid substrate fermentation is desirable, the downstream processing could become very expensive, especially when it comes to the recovery and purification steps. During the extraction, many additional compounds other than pyrazines will also be extracted. However, the demand from flavour companies are for high purity compounds so that they can use it in their own formulations according to their clients specifications. Thus, further purification steps may be required, which will lead to an increase in recovery costs (Manpreet ***et al***., **2005**; Robinson ***et al***., **2001**). Another factor that may also contribute to additional downstream processing expenses is the treatment of fermentation wastes where large volumes of fermentation material may be required to produce the desired volume of product. However, the product can be regarded as natural and a carefully considered process and techno-economic strategy could show otherwise.

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Table 1: Percentage of low and high fibre soy press cake samples containing fungal content.

Name of fungi	Low fiber soy	High fiber soy
Aspergillus candidus	26%	-
Aspergillus flavus	100%	28%
Aspergillus fumigatus	-	24%
Aspergillus ochraceus	6%	-
Aspergillus versicolor	12%	-
Chaetomium sp.	-	2%
Cladosporium cladosporoides	-	4%
Mucor sp.	30%	6%
Penicillium brevicompactum	10%	9%
Penicillium chrysogenum	10%	9%
Rhizopus oryzae	-	4%
Syncephalastrum racemosum	-	2%

Table 2: Differences in fungal content between pasteurized and unpasteurized soy press cake.

Fungi	Unpasteurised Soy		Pasteurised Soy	
	Average	Std Dev	Average	Std Dev
Aspergillus flavus	3.2×10^3	2.6×10^3	1×10^3	1.73×10^3
Aspergillus fumigatus	1.1×10^3	1.65×10^3	0	0
Aspergillus versicolor	1.1×10^5	1.86×10^5	4.43×10^5	7.68×10^5
Penicillium chrysogenum	8.58×10^3	6.25×10^3	1.37×10^4	2.37×10^4
Aspergillus niger	1×10^2	1×10^2	0	0
Bacteria	1×10^7	1.73×10^7	1×10^7	1.73×10^7
Trichoderma reesei	0	0	1×10^5	1.73×10^5
Cladosporium sphaerospermum	3.33×10^2	5.77×10^2	0	0
Eurotium chevalieri	1.67×10^3	2.89×10^3	0	0
Mucor circinelloides	6.67×10^1	1.15×10^2	0	0

Std Dev = standard deviation

Number of replicates = 3

Table 3: Odour noted in the different trays on day 7 of the fermentation.

Fermentations	Odour noted		
	Replicate 1	**Replicate 2**	**Replicate 3**
MRC 181	Strong caramel, medicine	Chocolate pronutro	Chocolate pronutro
MRC 181 + Glycine	Chocolate pronutro	Chocolate	Yeast-like
MRC 1723	Sweet, yeast-like	Yeast-like	Chocolate pronutro
MRC 1723 + Glycine	Yeast-like	Chocolate	Chocolate

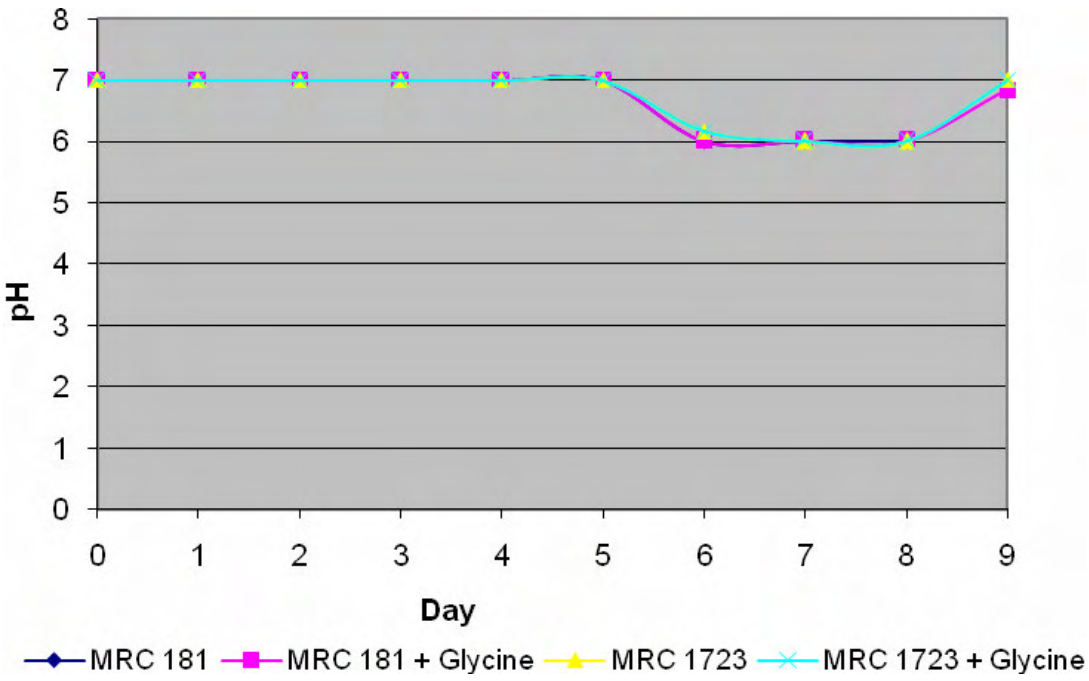

Figure 1: Comparison of pH changes amongst the fungi in the different media.

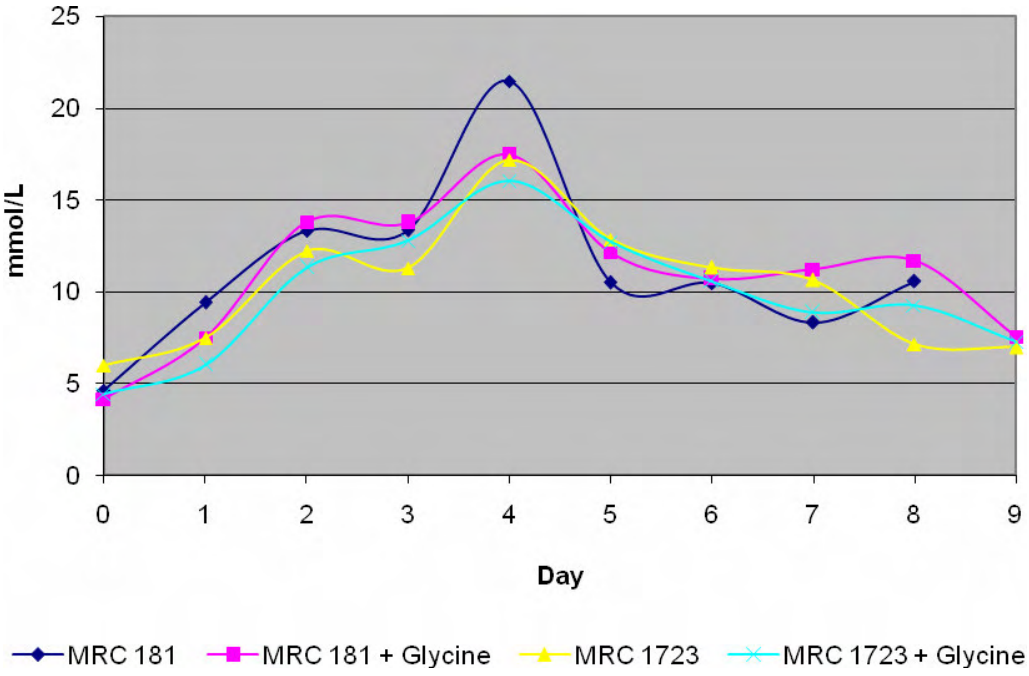

Figure 2: Comparison of glucose concentration amongst the fungi in the different media.

SUMMARY

An overview of the flavour and fragrance industry has indicated that there is a great demand for flavours to have the natural, halaal and kosher status. This has opened the door for the increase in production of flavours by microbial means. Particularly with regards to pyrazine production, bacterial species such as ***Bacillus*** are commonly used. Although previous literature indicated that fungi are not as prominent pyrazine producers compared to bacteria, this study has indicated that mycelial fungi can be considered for the production of natural pyrazine flavours. Out of the 280 fungi screened, 45% showed pyrazine flavour production as indicated in chapter 2. This chapter also showed that the content of growth media can have a substantial influence on flavour production. For example, fungi grown in Cz-medium produced more of the caramel and chocolate flavours, compared to fungi that were grown in TSB, which produced more of the nutty, meaty and potato flavours. The green flavour was, however, prominent in both media, of which mostly members of the ***Aspergillus*** and ***Penicillium*** groups produced this flavour.

Selected ***Penicillium*** species, including ***Penicillium rubrum*** and ***P. purpurogenum*** produced a green pepper odour that is indicative of the presence of methoxypyrazine. Chemical analytical methods as described in chapter 3 confirmed that ***Penicillium purpurogenum*** produced 2-methoxy-3-isobutylpyrazine (MIBP) and ***Penicillium rubrum*** produced both 2-methoxy-3-isobutylpyrazine (MIBP) and 2-methoxy-3,5/6-isopropylpyrazine (MIPP). Methoxypyrazines are high impact aroma chemicals that have a typical green pepper odour and a high market value. Due to the favourable characteristics of the ***Penicillium*** species in industrial fermentation processes (such as mass spore production and rapid colonization of substrates) and their ability to produce high value compounds, these fungi were selected for further studies.

In order to explore the potential use of these fungi in an industrial application, the methoxypyrazines produced were quantified. Prior to quantitation, a solvent extraction

method, using dichloromethane, was developed. Amongst the different pH parameters analysed acidified conditions showed the best results, where 69% MIPP and 97% MIBP were recovered from the liquid-liquid extraction and 76% MIPP and 99% MIBP were recovered from the solid-liquid extraction. Pyrazines quantified from the liquid-liquid extractions indicated that ***Penicillium rubrum*** produced 0.38 µg MIPP.L^{-1} and 0.88 µg MIBP.L^{-1}, and ***Penicillium purpurogenum*** produced 0.88 µg MIPP.L^{-1} and 2.15 µg MIBP.L^{-1} (Chapter 4).

Yields obtained from this study were not seen as feasible for the production of methoxypyrazines by the fungi on an industrial scale and, therefore, solid state fermentation was investigated as an option to improve the yields. Due to the availability of soy press cake as a by-product, as well as the variety of the amino acids present, it was selected as a possible substrate for pyrazine production. The results from this study, however, indicated that the nature of the substrate, such as the lipid content, fungal content and particle size of the soy press cake does not support fungal growth and makes pyrazine analyses problematic.

Alternate methods of improving methoxypyrazine yields thus need to be found. A limiting factor is the lack of understanding of the metabolic pathway involved in pyrazine production by ***Penicillium*** species. By having this knowledge the fermentation process can be adapted accordingly for the optimal production of methoxypyrazines by these microorganisms. Additionally, substrates that contain the necessary precursors that are cost effective would contribute significantly in the development of an economically viable fermentation process for the production of methoxypyrazines by ***Penicillium*** species.