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► **To cite this version:**

Naresh Magan, Natasha Sahgal. Potential for detection and discrimination between mycotoxigenic and non-toxigenic spoilage moulds using volatile production patterns. *Food Additives and Contaminants*, 2007, 24 (10), pp.1161-1168. 10.1080/02652030701519096 . hal-00577419

HAL Id: hal-00577419

<https://hal.science/hal-00577419>

Submitted on 17 Mar 2011

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Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2007-165.R1
Manuscript Type:	Special Issue
Date Submitted by the Author:	14-Jun-2007
Complete List of Authors:	Magan, Naresh Sahgal, Natasha; Cranfield University, Applied Mycology
Methods/Techniques:	Mycology
Additives/Contaminants:	Mycotoxins
Food Types:	Cereals and grain

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Manuscripts

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8 3 **Potential for detection and discrimination between mycotoxigenic and non-**
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10 4 **toxigenic spoilage moulds using volatile production patterns – a review**
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16 6 NATASHA SAHGAL, RACHEL NEEDHAM AND F. JAVIER CABAÑES* AND
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45 **Key words:** volatile fingerprints, electronic nose technology, mycotoxins, spoilage moulds,
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1

2 **Abstract**

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4 There has been interest in the development of techniques for the rapid early detection of
5 mycotoxigenic moulds in the food production chain. The development of sensor arrays which
6 respond to the presence of different volatiles produced by such moulds have been examined
7 as a potential method for the development of such detection systems. Commercial devices
8 based on such sensor arrays, so called electronic noses have been examined extensively for
9 the potential application for determining the presence of mycotoxigenic moulds in food raw
10 materials. There is also interest in using the qualitative volatile production patterns to
11 discriminate between non-mycotoxigenic and mycotoxigenic strains of specific
12 mycotoxigenic species, e.g. *Fusarium* section *Liseola*, *Penicillium verrucosum* and
13 *Aspergillus* section *Nigri*. This paper reviews the technology and the available evidence that
14 the non-destructive analysis of the headspace of samples of food raw materials or the
15 discrimination between strains (mycotoxigenic and non-mycotoxigenic) can be determined
16 using volatile fingerprints.

17

1 Introduction

2 Food raw materials are colonised by a wide range of mycotoxigenic moulds including
3 *Aspergillus flavus* (aflatoxins), *Penicillium verrucosum* (ochratoxins, citrinin), *Aspergillus*
4 *ochraceus* (ochratoxins), *Fusarium* section *Liseola* (fumonisins), and *Aspergillus* section
5 *Nigri* species (ochratoxins) (Magan & Olsen, 2004). There has been particular interest in the
6 development of prevention strategies to minimise the exposure of consumers to mycotoxins
7 (Aldred *et al.*, 2004). This strategy requires the early and rapid detection of mycotoxigenic
8 moulds and their toxins. There is also significant interest in being able to discriminate
9 between strains of a species with regard to the ability to produce specific mycotoxins.

10 Volatiles can be used to detect early mould contamination of cereals. Studies by
11 Abramson *et al.* (1983) and Tuma *et al.* (1989) suggested that in stored barley and wheat 3-
12 octanone, 1-octanol, and 3-methyl-1-butanol were key volatiles. These studies depended on
13 the use of GC-MS to obtain the information. These volatile fingerprints also vary with
14 individual microorganisms. For example, using GC-MS it has been shown that the volatile
15 profiles produced by *Pseudomonas fragi*, *Saccharomyces cerevisiae* and *Penicillium*
16 *verrucosum* are very different (Needham *et al.*, 2005). However, this requires significant
17 technical and analytical expertise. The development of sensor arrays, so called electronic nose
18 systems, which could simulate the odour detection in the human olfactory system were
19 developed to facilitate the detection of volatile fingerprints in headspaces of samples to enable
20 rapid detection systems to be developed for applications in a wide range of areas, including
21 food spoilage. It is well known that microbial contaminants produce a range of volatiles.

22

23 Sensor arrays for detection of volatile production patterns

24 Gardner and Bartlett (1994) defined sensor arrays used for detection of volatile fingerprints in
25 the following way: “an electronic nose is an instrument which comprises an array of
26 electronic chemical sensors with partial specificity and an appropriate pattern recognition

1 system, capable of recognising simple or complex odours". Table 1 summarises the
2 development of such sensor arrays from a historical perspective. Table 2 shows the range of
3 food matrices in which this technology has been examined.

4 The system consists of three basic building blocks: the vapour phase flows over a
5 sensor array, the interaction with the sensor surfaces occurs, and this response is analysed
6 using software for an output and interpretation. There are a number of different types of
7 sensor arrays which are used. Sensor technology has developed rapidly over the past decade
8 and this has resulted in a range of different sensor formats and the development of complex
9 microarray sensor devices. In the specific area of electronic nose systems, several different
10 physicochemical techniques have been used to produce sensor arrays for odour
11 characterisation. The type of sensor arrays used include: conducting polymers, metal oxides,
12 metal oxide silicon field effect sensors, piezoelectric crystals, surface acoustic wave devices,
13 optical sensors and electrochemical sensors (Turner & Magan, 2004).

15 **Detection of mould spoilage in food raw materials**

16 It has been shown that raw materials such as grain which is poorly stored have off-odours due
17 to alcohols, esters, ketones and mono and sesquiterpenes and aldehydes. The dominant
18 volatiles in grain were found to be 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone (Magan
19 & Evans, 2000). Studies with grain having different levels of moulding and natural samples
20 showed that by using a standardised grain amount and a conducting polymer array of sensors
21 and a radial based-neural network it was possible to carry out real time analyses of grain
22 samples in 10 mins (Evans *et al.*, 2000). This enabled a decision to be made on whether grain
23 was acceptable or not. Interestingly, results were very comparable with those produced by an
24 odour panel classification. This approach was based on acceptable or unacceptable and
25 showed a small group of samples in a so-called zone of uncertainty which suggested that they
26 were in the process of going mouldy and thus needed further investigation.

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2
3 1 Other studies using metal oxide sensors have suggested that the presence of 1%
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5 2 contaminated grain could be discriminated (Costello *et al.*, 2003). However, in trials with
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7 3 harvested naturally contaminated grain some false positives were obtained when compared to
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9 4 other criteria used at intake facilities for processing. The results were, importantly, not found
10
11 5 to be linked to grain moisture content. These studies all suggest that quality of grain can be
12
13 6 effectively managed using this approach. Similar studies by Börjesson *et al.* (1996), Jonsson
14
15 7 *et al.* (1997) and Olsson *et al.* (2002) showed that there was some value in quality grading of
16
17 8 cereals using the electronic nose approach.
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22 9 Research has also been carried out using a mass spectrometry-based e-nose system to
23
24 10 examine mould contamination of Spanish bakery products. By using a range of data analysis
25
26 11 techniques including PCA, DFA and fuzzy ARTMAP and solid phase micro-extraction to
27
28 12 concentrate the volatiles produced by spoilage moulds successful (98%) discrimination of
29
30 13 mould growth from controls was possible within 48 hrs (Vinaixa *et al.*, 2004). This was
31
32 14 possible prior to any visible growth being observed. After 96 hrs, 88% discrimination
33
34 15 between a range of spoilage species was achieved. Further studies on spoilage fungi in bakery
35
36 16 products using this same approach has recently been reported by Marin *et al.* (2007). They
37
38 17 employed the MS-electronic nose to detect *Eurotium*, *Penicillium* and *Aspergillus* species in
39
40 18 Spanish cake products. They found that volatile production patterns were positively correlated
41
42 19 with ergosterol as a biomass marker of fungi after 2, 4 and 7 days. This measurement
43
44 20 approach using a relatively sophisticated e-nose system required 20-25 mins per sample. This
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46 21 also has potential to be included in a QA system for processing of raw materials.
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23 Use of volatiles to detect mycotoxigenic spoilage moulds

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57 24 Fungi producing secondary metabolites are known to use different biosynthetic pathways,
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59 25 depending on the final product. For example, production of aflatoxins by *A. flavus* group
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26 follows a general scheme of acetate → polyketide → anthraquinones → xanthones → → to

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2
3 1 aflatoxins. Zeringue *et al.* (1993) studied aflatoxigenic and non-aflatoxigenic *A. flavus* strains
4
5 2 in submerged culture and found that *A. flavus* produced distinct compounds such as α -
6
7 3 gurjunene, trans-carophyllene and cadinene which were only present in aflatoxigenic strains.
8
9 4 There was some correlation between production of these compounds and aflatoxin synthesis.
10
11 5 These studies used standard analytical techniques such as GC and GC-MS. Jelen *et al.* (1995,
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13 6 1997a, 1998) also attempted to find potential marker volatiles for trichothecene production by
14
15 7 *Fusarium sambucinum* and suggested that different sesquiterpenes were produced by
16
17 8 toxigenic and non-toxic strains. More recently, Jelen & Grabarkiewicz-Szczesna (2005)
18
19 9 examined a range of *Aspergillus ochraceus* and other *Aspergillus* species and tried to relate
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21 10 volatile production patterns using GC-MS and ochratoxin production. They found that,
22
23 11 regardless of toxin production capacity, similar key volatiles such as 1-octen-3-ol, 3-octanone,
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25 12 3-octanol, 3-methyl-1-butanol, 1-octene, and limonene were produced. The predominant
26
27 13 volatile was 1-octen-3-ol and was produced by all strains. While environmental conditions of
28
29 14 temperature and water content optima for quantified volatiles and ochratoxin production were
30
31 15 different, the volatiles did not facilitate discrimination of *A. ochraceus* strains. In studies of
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33 16 barley grain Börjesson *et al.* (1990) found that the highest production of volatile fungal
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35 17 metabolites precedes ochratoxin production.

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37 18 Quantitative studies of volatile organic compounds with a trichothecene-producing
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39 19 strain of *Fusarium sporotrichioides*, and ochratoxigenic and non-ochratoxigenic strains of *P.*
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41 20 *verrucosum* suggested that volatile terpenes were linked to the formation of trichothecenes by
42
43 21 *Fusarium* spp. and probably accelerated production of volatile ketones by the ochratoxigenic
44
45 22 strain of *P. verrucosum* (Pasanen *et al.*, 1996). Jelen *et al.* (1997b) found that trichodiene, an
46
47 23 intermediate of trichothecene production was elevated in harvested grain spikes colonised by
48
49 24 *Fusarium* head blight species when harvested and incubated. They suggested that this could
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51 25 be a good marker for differentiation between infection by toxigenic and non-toxic strains.
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53 26 Almost all these studies used GC or GC-MS and different quantitative techniques.
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1 2 3 1 4 5 2 **Use of electronic nose systems for discriminating mycotoxigenic species and strains**

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8 3 The question arises as to whether the use of qualitative volatile fingerprints which can be
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10 4 detected by using e-nose systems can be used for the rapid early discrimination of toxin and
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12 5 non-toxin producers. Studies by Keshri & Magan (2000) used a conducting polymer based e-
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14 6 nose system (BH114, Scensive Technologies, Leeds, U.K.) to try and use the volatile
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16 7 production patterns to try and differentiate between strains of *F. verticillioides* and also *F.*
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18 8 *proliferatum* able to produce fumonisins and strains that did not produce any. They grew up
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20 9 these fungi on cereal-based substrates for 48-96 hrs and examined the volatile production
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22 10 patterns using PCA and Cluster analyses. This showed that it was possible to differentiate
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24 11 between the control blanks, the non-toxicogenic and three toxicogenic strains of each of these
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26 12 species (Figure 1). Interestingly, parallel studies carried out on hydrolytic enzyme activity,
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28 13 showed that both total and specific enzyme activity changes indicative of growth occurred
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30 14 only after 96 hrs, much later than that detected with the e-nose (48-72 hrs).

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36 15 Subsequently extensive studies were conducted with both toxicogenic and non-toxicogenic
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38 16 strains of *A. flavus* (three of each; aflatoxins) and *P. verrucosum* (two to three of each;
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40 17 ochratoxin, citrinin, both or none; Needham, 2005). Strains of *A. flavus* were supplied by Dr.
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42 18 A. Neschi, Rio Cuarto University, Argentina and *P. verrucosum* strains were provided by the
43
44 19 Dr. P.V. Nielsen, BioCentrum-DTU, Lyngby, Denmark. Studies for *A. flavus* were conducted
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46 20 on milled maize-based media and those with *P. verrucosum* on 2% wheat agar and on bread
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48 21 analogues for comparison at 0.95 water activity. The substrates were inoculated with 100 μ l
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50 22 of 10^6 CFU ml⁻¹ of relevant strains and then incubated for 24, 48 and 72 hrs. The methods for
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52 23 analyses of headspace were as described previously by Keshri & Magan (2000). Figure 2
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54 24 shows an example of the dendrogram for *A. flavus* strains examined at the 95% confidence
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56 25 limits. This showed that there was no clear clustering of strains based on their ability to

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3 1 produce aflatoxins. This suggested that it was difficult to separate strains of this species
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5 2 effectively.

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8 3 Figure 3 shows the PCA plot obtained for the different strains when incubated for 72
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10 4 hrs. This shows that PC1 and PC2 accounted for 92% of the variance and showed
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12 5 discrimination between some of the strains. An examination of the Cluster analyses of the
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14 6 data on bread analogues showed that it was possible to separate out the ochratoxin and
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16 7 ochratoxin + citrinin producers (Figure 4). However, there was less discrimination between a
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18 8 citrinin only producer and that which produced none.

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22 9 Recently we have also examined the potential of using alternative sensor formats to
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24 10 evaluate the potential of using volatile production patterns for discrimination between
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26 11 members of the *A. niger* Section *Nigri* species. Up to now, the taxonomy of black aspergilli is
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28 12 not very clear because it is primarily based on morphological criteria and in some cases (e.g.
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30 13 *A. niger* aggregate) the differences between species are very subtle (Abarca et al., 2004).
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32 14 There is already good information on the molecular differentiation between these strains
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34 15 detailed in Table 3 (Abarca *et al.*, 2004). We have examined the volatile production patterns
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36 16 on different nutrient media. In this case we found that grape juice-based media were best for
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38 17 optimising volatile production. These studies showed that it was possible to differentiate
39
40 18 between species based on the volatile production patterns detected with metal oxide sensor
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42 19 array using an AlphaMoss 3000 machine. A similar protocol was followed as used by
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44 20 Needham *et al.* (2005) except that agar discs (1 cm diam) were placed in 10 ml glass vials and
45
46 21 crimp sealed before randomising and placing in the autosampler. In all cases at least 5
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48 22 replicates were used per treatment. Figure 5 shows the discrimination achieved between the
49
50 23 species examined and this accounted for 96% of the data in PC1 and PC2. The Cluster
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52 24 analyses based on the Euclidian distances showed some discrimination between the species of
53
54 25 the *A. niger* section *Nigri* used is shown in Figure 6. Subsequent studies on cracked maize
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56 26 with different ochratoxin producing species such as *P. verrucosum*, *A. ochraceus* and *A.*

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3 1 *carbonarius* suggests that it is possible to use headspace analyses of qualitative volatiles to
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5 2 differentiate between contamination with these different mycotoxigenic spoilage fungi
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7 3 (*Cabañes et al.*, 2006).
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10 4 11 12 5 **Discussion and conclusions** 13

14
15 6 This review of recent work carried out on application of e-nose technology for the rapid early
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17 7 diagnosis of mycotoxigenic spoilage moulds suggests that there is some promise in this
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19 8 approach as part of a “prevention strategy”. Previous studies have shown that potential exists
20
21 9 for using sensor arrays to differentiate between toxigenic and non-toxigenic strains of
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23 10 *Fusarium* section *Liseola*, *Penicillium verrucosum* and *Aspergillus* section *Nigri* species. For
24
25 11 example, Keshri & Magan (2000) carried out *in vitro* studies on grain-based media using
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27 12 strains of *Fusarium verticillioides* and *Fusarium proliferatum*. Using the same e-nose system,
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29 13 based on a 14 conducting polymers sensor array, they were able to discriminate between
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31 14 uninoculated controls, a non-toxigenic strain and 3 mycotoxin producing strains of both
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33 15 organisms after 48-72 hrs with PCA and Discriminant function analyses. This was much
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35 16 earlier than hydrolytic enzyme production of β -D-glucosidase, α -D-galactosidase and N-
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37 17 acetyl- β -D-glucosaminidase. These were only demonstrated to significantly increase after 72
38
39 18 hrs incubation. Olsson *et al.* (2002) used an e-nose to detect ochratoxin A in barley grains
40
41 19 and to also predict if the level of the toxin was below the legislative limit of 5 $\mu\text{g kg}^{-1}$.
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43 20 Studies by Needham & Magan (2003) showed that it was not possible to discriminate a
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45 21 citrinin producing strain of *P. verrucosum* *in situ* although this was possible *in vitro* on
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47 22 unmodified 2% wheat agar. This discrimination was concentration dependent and when using
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49 23 a much lower initial spore concentration a reduced number of strains was observed which
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51 24 could be effectively discriminated, based on ability to produce ochratoxin or citrinin.
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53 25 However, in bakery products such as bread sometimes, even if the mycotoxigenic strain is
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3 1 inoculated on the surface, no mycotoxins are produced (Legan, 1993), which may account for
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5 2 the difficulty in sometimes being able to discriminate between such strains. However, the
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7 3 ability to detect spoilage fungi and mycotoxins prior to visible moulding is a key criteria and
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9 4 in some cases this is achievable using qualitative volatile production patterns. Also analysis
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11 5 and differentiation is prior to visible growth being observed. This is important as an approach
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13 6 which could give an early indication of potential for mycotoxin contamination in raw
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15 7 commodities. This may have particular applications as a tool for discriminating between
16
17 8 strains as an additional measurement to morphological and molecular approaches as well as in
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19 9 quality assurance systems. Potential also exists for application in the animal feed chain where
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21 10 particular mycotoxins, e.g. zearalenone, some trichothecenes and aflatoxins are particularly
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23 11 important contaminants.

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29 12 The rapid development of sensor technologies and miniaturisation of sensor arrays
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31 13 suggests that the sensitivity and robustness have been improving significantly. This will
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33 14 provide opportunities for more sensitive hand held devices to be used and the data analyses to
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35 15 be carried out remotely by using Neural Networks and other bioinformatics approaches. We
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37 16 are also now examining the potential for correlating the qualitative fingerprints with SIFT-MS
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39 17 (selective ion flow tube mass spectrometry), to monitor trace gases in real time without
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41 18 sample pre-treatment and can cope with a wide dynamic range, e.g. percentage concentrations
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43 19 in water or a few ppb of a trace compound in the same sample analyses to identify the key
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45 20 volatile compounds responsible for the discrimination achieved (Turner et al., 2006).

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3 1 Table 1. A brief historical perspective on development of sensor array systems for odour
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5 2 detection and development of electronic nose type approaches
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10 4 1962: Stereo-chemical theories of olfaction
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12 5 1978: Structure-activity relationships in human chemoreception
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15 6 1982: First development of a model nose using three sensors with broad
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17 7 sensitivity (Persaud & Dodd)
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19 8 1988: Term 'electronic nose' used for 1st time
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22 9 1990s: First commercial devices developed using conducting polymer sensor arrays
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24 10 1990s: Applications in food, environment, medicine using generic research-based devices
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27 11 >2000 onwards: More targeted approaches of e.nose systems for specific use in food,
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29 12 environment and medical applications
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8 3 Table 2. Summary of the range of food matrices and types of spoilage which have been
9 4 examined using e.nose systems
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6 Food product	Types of spoilage
7 Grain/flour	Moulds/insects/toxin
8 Bread	bacteria/yeasts/moulds
9 Milk	bacteria/yeasts
10 Cheese	moulds
11 Coffee	bacteria/yeasts/moulds
12 Fish	microbial taints/freshness
13 Tea	cultivars/speciality coffees
14 Beers	tainting
15 Wines	tainting/yeasts
16 Nuts	quality/toxins
17 Fruit	quality/disease
18 Meat	microbial taints/freshness

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Table 3. Strains of *A.niger* section *Nigri* used in this study which have been discriminated previously using molecular methods (Abarca et al., 2004). Key: N, two fragments 519 and 76 bp; and T, one fragment of 595 bp.

Reference No.	Species	Source	Geographic Origin	OTA Production	RFLP pattern
A-941	<i>A.carbonarius</i>	grapes	Spain	+	
A-75	<i>A.niger</i> aggregate	feedstuff	Spain	+	N
A-946	<i>A.niger</i> aggregate	coffee	Portugal	-	N
A-947	<i>A.niger</i> aggregate	grapes	Spain	-	T

Figure legends

Figure 1. Cluster analyses of strains of *F. verticillioides* based on those able to produce fumonisins (48N, 25N, 150N) and one which in a non-producer (6N; adapted from Keshri & Magan, 2000).

Figure 2. Cluster Analysis dendrogram of mycotoxigenic and non-mycotoxigenic *Aspergillus flavus* strains based on their volatile profiles after 72 hrs incubation at 25°C on 0.95 a_w 2% milled maize agar. Key: toxigenic 1 - T20; toxigenic 2 - D106; toxigenic 3 - D69; non-toxigenic 1 – T47; non-toxigenic 2 - T48; non-toxigenic 3 - D64.

Figure 3. Principal Component Analyses (PCA) of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 48 hrs incubation at 25°C on 0.95 a_w bread analogue with an initial population of 1x10⁶ spores ml⁻¹. Key: citrinin + OTA producer- IBT22123 (MM) OTA producers- IBT5252 (PP), IBT22025 (PN), citrinin producers- IBT22116 (NN), IBT21491 (NP).

Figure 4. Cluster analysis dendrogram of mycotoxigenic and non-mycotoxigenic *Penicillium verrucosum* strains based on their volatile profiles after 48 hrs incubation at 25° on 0.95 a_w bread analogue with an initial population of 1x10⁶ spores ml⁻¹. Key: citrinin (Cit) + OTA producer- IBT22123, OTA 1- IBT22025, OTA 2- IBT5252, citrinin 1- IBT22116, citrinin 2- IBT21491 producer.

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3 1 Figure 5. PCA of the discrimination between isolates of *A.niger* section *Nigri* on white grape
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5 2 juice medium after 48 hrs at 25°C. Key: A941, *A.carbonarius* – toxin producer; A75; *A.niger*
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7 3 aggregate-toxin producer; A.946, *A.niger* aggregate, non-toxin producer; A947, *A.niger*
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9 4 aggregate , non-toxin producer.
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15 6 Figure 6. Cluster analysis using the Euclidean distances based on the volatile fingerprints after
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17 7 48 hrs incubation. This shows the groupings and the separation of the treatments. C is the
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19 8 control medium. Key: A941, *A.carbonarius* – toxin producer; A75; *A.niger* aggregate-toxin
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21 9 producer; A.946, *A.niger* aggregate, non-toxin producer; A947, *A.niger* aggregate , non-toxin
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23 10 producer.
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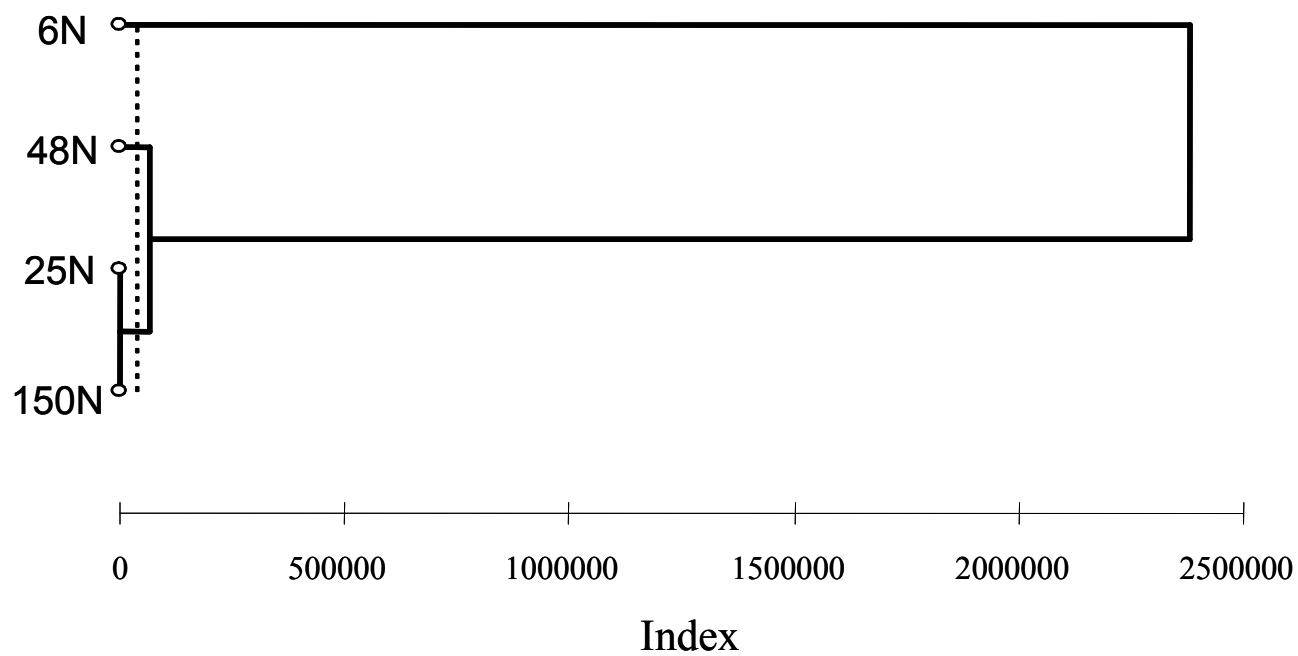


Figure 1. Sahgal et al.

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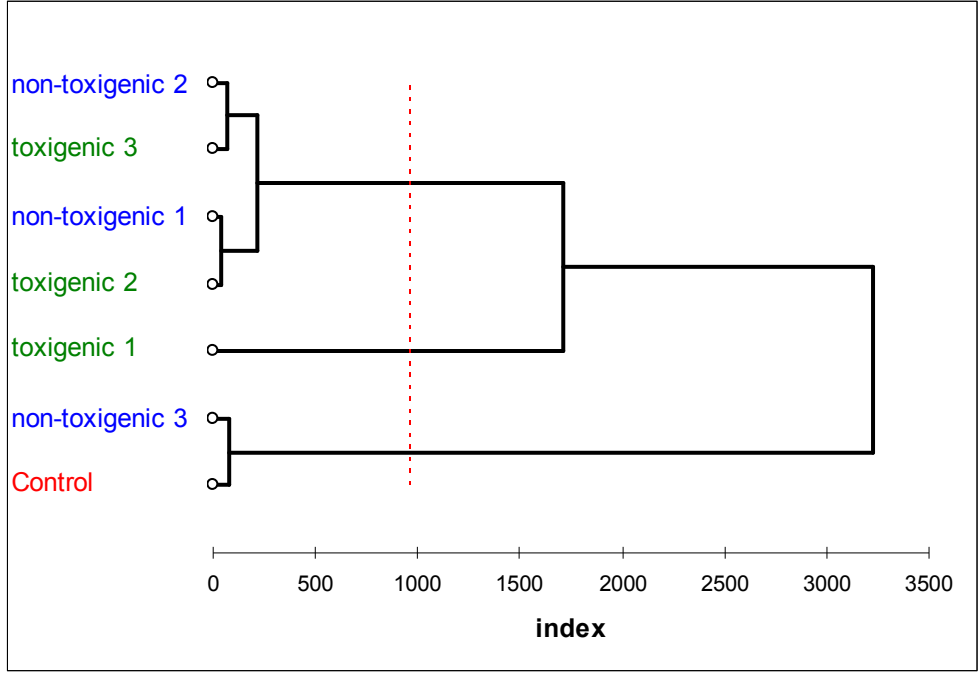


Figure 2. Sahgal et al.

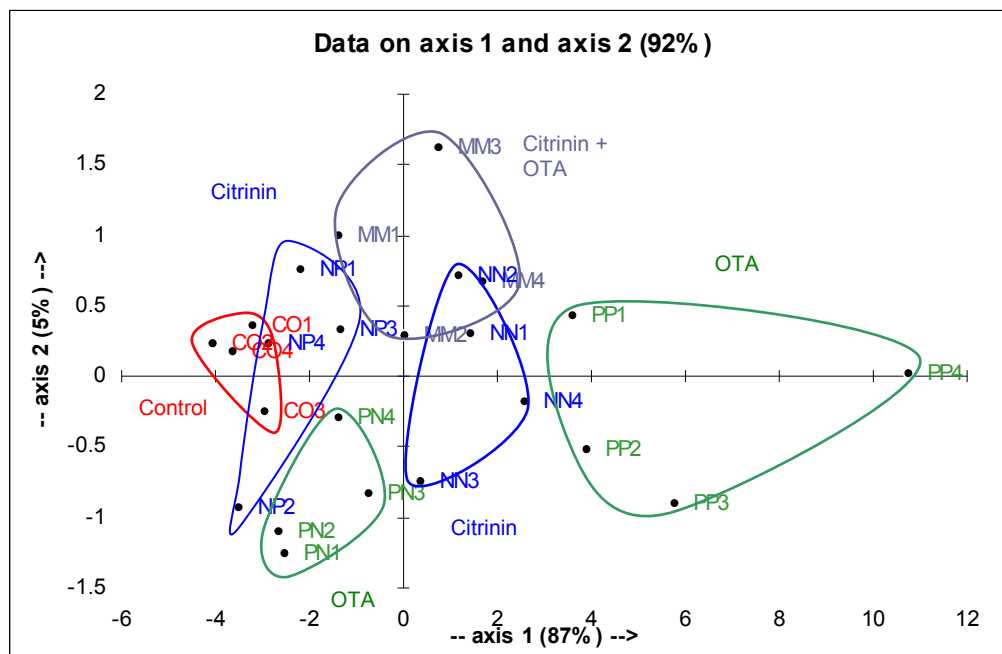


Figure 3. Sahgal et al.

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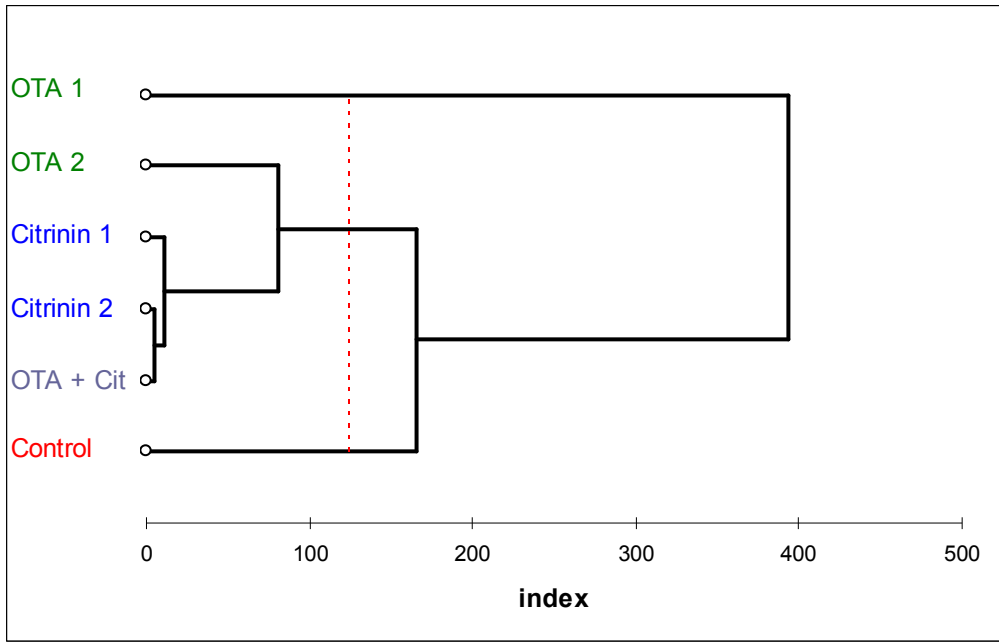


Figure 4. Sahgal et al.

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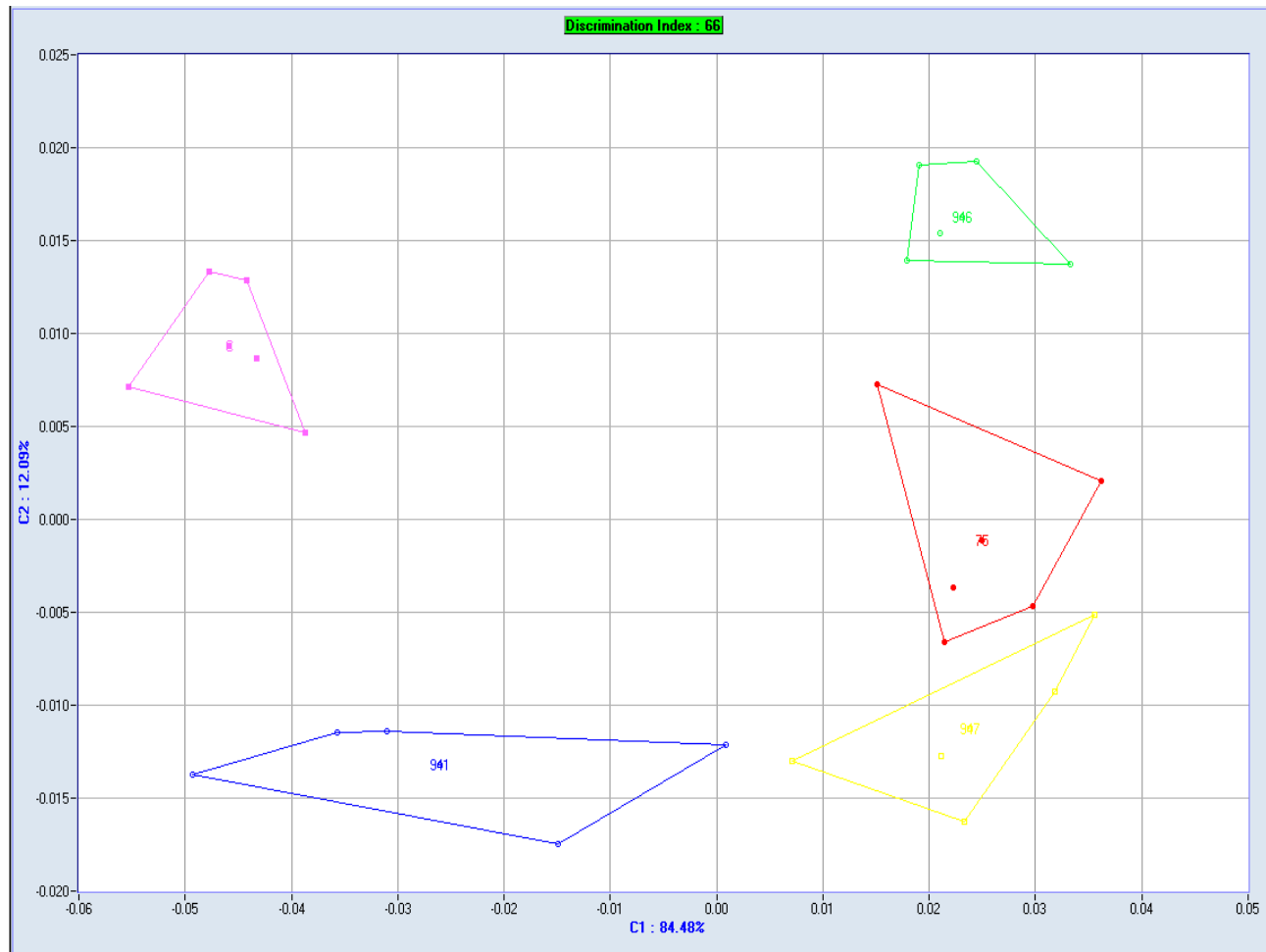


Figure 5. Sahgal et al.

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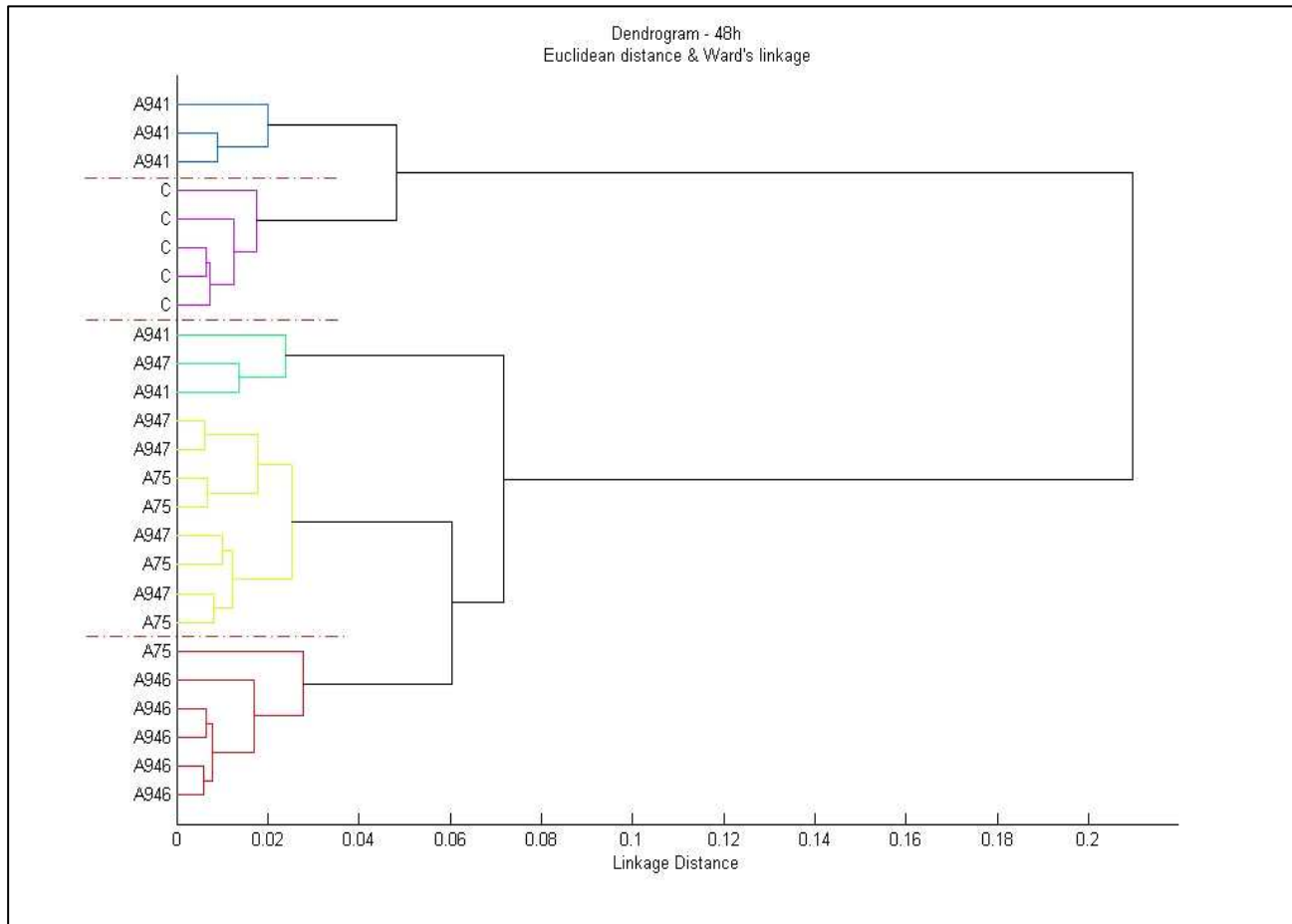


Figure 6. Sahgal et al.