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U.S. ARMY CHEMICAL AND BIOLOGICAL DEFENSE COMMAND

ERDEC-TR-443

**RAPID IDENTIFICATION OF AIRBORNE BIOLOGICAL PARTICLES
BY FLOW CYTOMETRY, GAS CHROMATOGRAPHY, AND
GENETIC PROBES**

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September 1997

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Aberdeen Proving Ground, MD 21010-5423

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U.S. Army Edgewood Research, Development and Engineering Center
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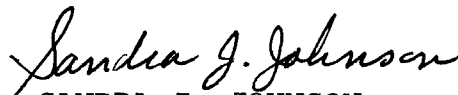
TITLE RAPID IDENTIFICATION OF AIRBORNE BIOLOGICAL
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13. ABSTRACT (Maximum 200 words) Detection of airborne biological particulates is a primary mission of the U.S. Army Edgewood Research, Development and Engineering Center biological defense program. If biological particles could be characterized according to their unique physical and biochemical profiles, detection and perhaps even identification of the particles might be possible. This study focused upon microbial particles, more specifically upon fungal spores, yeast cells, and bacterial cells. Physical characteristics of the particles, it was proposed, could be detected by flow cytometry, while their biochemical profiles could be determined by gas chromatography, and their genetic identity could be obtained by either a suitable genetic probe or by matching its genetic fingerprint. Genetic techniques were not attempted in the work reported here, but the approach was investigated further. Trial results were encouraging.				
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PREFACE

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CONTENTS

1.	INTRODUCTION	9
2.	METHODS AND MATERIALS	9
2.1	The Cultures	9
2.2	Flow Cytometry	10
2.3	Gas Chromatography	11
2.4	Genetic Probes	11
3.	RESULTS AND DISCUSSION OF FLOW CYTOMETER ANALYSIS	12
4.	RESULTS AND DISCUSSION OF GAS CHROMATOGRAPH ANALYSIS	26
5.	RESULTS AND DISCUSSION OF GENETIC PROBE ANALYSIS	34
6.	CONCLUSIONS	36
	REFERENCES	39

FIGURES

1a.	<u>Cladosporium</u> and <u>Mucor</u>	15
1b.	<u>Cladosporium</u> and <u>Heterobasidion</u>	16
1c.	<u>Cladosporium</u> and <u>Penicillium</u>	17
1d.	<u>Mucor</u> and <u>Heterobasidion</u>	18
1e.	<u>Heterobasidion</u> and "Yeast 1"	19
1f.	<u>Heterobasidion</u> and "Yeast 2"	20
1g.	<u>Heterobasidion</u> and <u>Aspergillus</u>	21
1h.	"Yeast 1" and "Yeast 2"	22
1i.	"Yeast 2" and <u>Aspergillus</u>	23
1j.	<u>Penicillium</u> and "Bacteria"	24
2a.	"Blank 1"	27
2b.	"Blank 2"	28
2c.	<u>Mucor</u> spores	29
2d.	<u>Aspergillus</u> spores	30
2e.	<u>Cladosporium</u> spores	31
2f.	<u>Penicillium</u> spores	32

TABLES

1. Average Shapes and Sizes of Fungal Spores and
Yeast and Bacterial Cells by Direct Observation 14

2. Summary of Retention Times of Detected Peaks
During Gas Chromatography Analysis Using 95% Ethanol
Extractant; the Retention Times in Parentheses are
Regarded as Errors 33

3. Comparison of the Times of the Detected Peaks
as Occurring After the Time of the Solvent Peak
(Retention Time of Detected Peaks Minus Retention Time
of Solvent Peak); the Superscripts Estimate Which
Peaks May Be the Same Component in Each of the Samples 33

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RAPID IDENTIFICATION OF AIRBORNE BIOLOGICAL PARTICLES
BY FLOW CYTOMETRY, GAS CHROMATOGRAPHY AND GENETIC PROBES

1. INTRODUCTION

The purpose of this study is to characterize airborne biological particles based on their unique physical and biochemical profiles to enable the detection, and perhaps, the identification of the particles. The biological particles of interest are microbial, more specifically, fungal spores, yeast cells, and bacterial cells, and they are studied because they can be important to the health of humans, animals and plants.

It was proposed that the detection of the particles could be based on their physical characteristics as detected by flow cytometer analysis. The identification of their biochemical profiles could be achieved by gas chromatography. The genetic identity of any biological particle could be obtained by the application of a suitable genetic probe or by matching its genetic fingerprint.

Initial trials of the techniques of flow cytometry and gas chromatography in the characterization of some airborne microbial particles are reported. Genetic techniques were not attempted, but the approach was further explored.

2. METHODS AND MATERIALS

2.1 The Cultures

To obtain microbial cultures for the study, ten 3-1/2"-diameter petri dishes of malt extract agar were exposed for one hour on the campus of the University of Washington. Colonies were isolated from these dishes to obtain specimens from which to set up stock cultures.

The fungal specimens were identified by direct observation to genus level. The most commonly occurring fungal spores encountered during the time of collection (October 1993) were of Penicillium species. Mucor, Cladosporium, and Aspergillus were also collected and included in the study, as was a previously isolated culture of Heterobasidion annosum. The yeast and bacterial specimens have not been identified, since their identifications require biochemical testing.

From the collected cultures, two yeast and one bacteria cultures were chosen arbitrarily to be included for comparisons to the fungal spores. These stock cultures were grown on malt extract agar (20 g malt extract, 20 g Bacto-agar, 1000 mL distilled water). To ensure the harvest of numerous spores for analyses, the fungal cultures were then grown on V-8 agar (200 mL V-8 juice, 3 g calcium carbonate, 20 g Bacto-agar, approximately 800 mL distilled water) which caused heavy sporulation.

The yeast and bacteria grew adequately on malt extract agar. Incubation was at temperatures ranging from 15 C in an incubator to 25 C in the laboratory. The age of harvest of the spores varied among samples and between dates of analysis.

2.2 Flow Cytometry

For flow cytometry analysis, the spores and cells of each sample specimen were collected by washing the culture with 10 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris/HCl) buffer with 0.1% Nonidet P-40 (Sigma Chemical, St. Louis MO), a surfactant. After centrifugation, the buffer solution was decanted, and the spores and cells were fixed in 70% ethanol for at least 24 hours. Before analysis, each sample was washed twice with the Tris/HCl buffer solution and stained with 10 mg/mL 4'-6-diamino-2-phenylindole (DAPI) in phosphate buffer solution. Staining took place at 0°-4°C, in darkness, for a minimum of 20 minutes to a maximum of about 60 minutes. This method of sample preparation was adapted from the studies by Allman (1992), Miller and Quarles (1990), and Van Dilla et al. (1983).

The samples were analyzed on a Coulter Elite flow cytometer equipped with two argon lasers. The first laser obtained information about the forward angle light scattering and right-angle light scattering properties of the biological particles at the visible wavelength of 488 nm. The second laser obtained information about the fluorescence levels of the stained DNA by exciting the fluorophore at a wavelength of 350 nm to 360 nm. Sorting of particles was not performed, as the particles apparently were too small to be sorted.

Pure cultures of the specimens underwent some preliminary analyses to test the capabilities of the technique. When necessary, the rate of injection of the particles was adjusted for efficiency of analysis, as was the laser voltage adjusted for stronger fluorescence of a sample. However, to make comparisons between the samples, the settings of the laser voltages must be the same to equalize the magnitudes of the output signals. In the final analysis, ten mixed samples of two specimens each were examined by the flow cytometer at fixed settings to attempt discrimination between the two different microbial particles.

2.3 Gas Chromatography

For gas chromatograph analysis, the spores from each fungal sample were collected by suction onto a Teflon PTFE membrane (0.2 mm pore size) from the surface of the culture. As many spores as possible were collected, which filled a volume estimated at 5 mL. The cells of each yeast and bacterial sample were collected by lifting with a spatula approximately 10 mL of the cells off colonies on each plated culture. Samples were placed in 1.0 mL vials capped with PTFE-lined silicone liners.

The chromatograph used was an HP5792A gas chromatograph equipped with a flame ionization detector containing a Durabond DB-5 column (30 m by 0.318 mm I.D.) with a film thickness of 1.0 mm (J&W Scientific, Folsom CA).

A simple extraction of any volatile compounds from the biological particles was preferred. A preliminary exploration involved headspace analysis after heating at 80°C for 20 minutes without an extractant. Ten microliters of the gas sample was injected. Secondly, 0.5 mL 95% ethanol was used as an extractant. One microliter of the ethanol was injected in splitless mode. The injector port temperature was 140°C and the detector was at 250°C. The column oven temperature was programmed from an initial temperature of 120°C to 195°C at 25°C/min, followed by a temperature ramp from 195° to 230°C at 30°C/min. The temperature was held at 230°C for 5 minutes. The carrier gas was helium flowing at 1 mL/min. This temperature program was adapted from the study by Cundy et al. (1991).

Unfortunately, equipment difficulties in the middle of this procedure prevented completion of this step in the study.

The next steps planned were to analyze the samples of both headspace and liquid samples after using dichloromethane (DCM) to extract any volatile fatty acids, and acetone to extract any volatile sugars. The use of a silanizing reagent in DCM was also considered which would produce a highly volatile extract after the silanization reaction. These single-step extraction methods are based on those used in plant products analyses.

2.4 Genetic Probes

For genetic characterization of the fungal spores, yeast and bacterial cells, the availability and suitability of probes for the organisms of interest was explored. Searches of scientific literature were conducted through medical and biological databases on CD-ROM (such as Medline, Life Sciences Collection, Biosis), and genome banks (such as GenBank and European Molecular Biology Library (EMBL)) available on the Internet computer network, and through Biological Abstracts.

3. RESULTS AND DISCUSSION OF FLOW CYTOMETER ANALYSIS

Results of flow cytometer analyses for distinguishing populations are represented by histograms and scatterplots. Contour plots can also be generated and are useful for pinpointing values of FS (forward-angle light scattering) and RA (right-angle light scattering) on the plots. Electronic gating of particles may be applied, and each gate is designated by a letter. The scale at the x-axis represents channel numbers of the relative fluorescence strengths.

The plots chosen for display usually include those that:

- (1) show how doublet discrimination was accomplished in either the total population or an electronically gated population of particles. The method uses integral (area) pulse versus peak (height) pulse data, shown as the plot of "A/DAPI" versus "B/DAPI PEAK." A cell doublet appears as twice the integral pulse amplitude (the total amount of dye contained in a cell or cell doublet) of a cell singlet, but the peak pulse amplitude (the amount of dye as it passes through the focused excitation beam at any time) of a cell doublet is approximately the same as that of a cell singlet (Bauer *et al.*, 1993). In general, cell doublets represent the population in the lower right of the plot and are gated off.
- (2) illustrate the fluorescence intensity versus particle count in both the total population and in a gated population, shown as the plots of "A/DAPI" versus "Count."
- (3) show which gated populations were selected for further analysis based on the FS and RA properties of the population, represented by the plot of the logarithm of RA, "2/RA LG," versus "FS." Cell discrimination is affected by the cell's size, index of refraction, and absorptive properties. The signal from FS is approximately proportional to cell size, and it is particularly useful for discrimination between cells and debris and different cell types. RA contains information about the cell's internal structure. However, size and index of refraction also affect this signal, so that variations in the side-scatter signal may be a reflection of size variability and/or differences in internal cellular structure (Bauer *et al.*, 1993).
- (4) illustrate the relationship between fluorescence intensity and forward-angle light scatter signals in the total population, shown as the plot of the logarithm of

DAPI levels, "B/DAPI LG," versus "FS" as another means of looking at the population groupings. (This was displayed for only four of the flow cytometry samples.)

Particles were identified as debris to be gated off if they had very low levels of right-angle light scatter, fluorescence, and/or forward light scatter. The particles may be poorly stained biological particles of interest, but if they are not stained properly, they are not useful in characterizing the population of interest.

Variability in the flow cytometric data as a result of biological variability or temporal variations in flow cytometer performance is expressed as a coefficient of variation (C.V.). The C.V. was automatically calculated on designated peaks. In theory, $C.V. = s/m \times 100$, where s is the standard deviation and m is the mean for the parameter under study, for example, fluorescence intensity. In practice, the standard deviation is often approximated by determining the "full width at half maximum (FWHM)," in which case $C.V. = FWHM/m \times 0.425$. Fluorescence intensities of gated regions are listed in the "single parameter statistics" output table, and FS and RA of gated areas are listed in the "dual parameter statistics" output table.

Individual profiles of pure culture samples were studied earlier, at varying laser voltages, for general methodology and characterizations. Preparation of samples was also improved during these four initial runs. These earlier results are not included in this report, because the different laser settings used throughout those trials disallow meaningful comparisons between samples.

The estimated sizes of the particles are shown in Table 1, and they represent averages seen in the cultures used in the trials and in the flow cytometer run presented in this report. The sizes of the spores and the yeast may depend on the age of the culture and the stage of development of the individual particle at the time of observation. The samples which produced these flow cytometer results were seven days old at the time they were fixed in ethanol, and they were analyzed nine days later.

The analysis of the mixed samples of two types of either spores and/or cells from the Mucor, Cladosporium, Penicillium and Aspergillus species, Heterobasidion annosum, two yeast samples, and one bacteria sample, are shown in Figures 1a to 1j. The flow cytometer laser settings were established and not adjusted throughout the run to allow comparisons of the results between the samples. The laser excitation voltage was set by analyzing samples of the smallest particles first to ensure their detection.

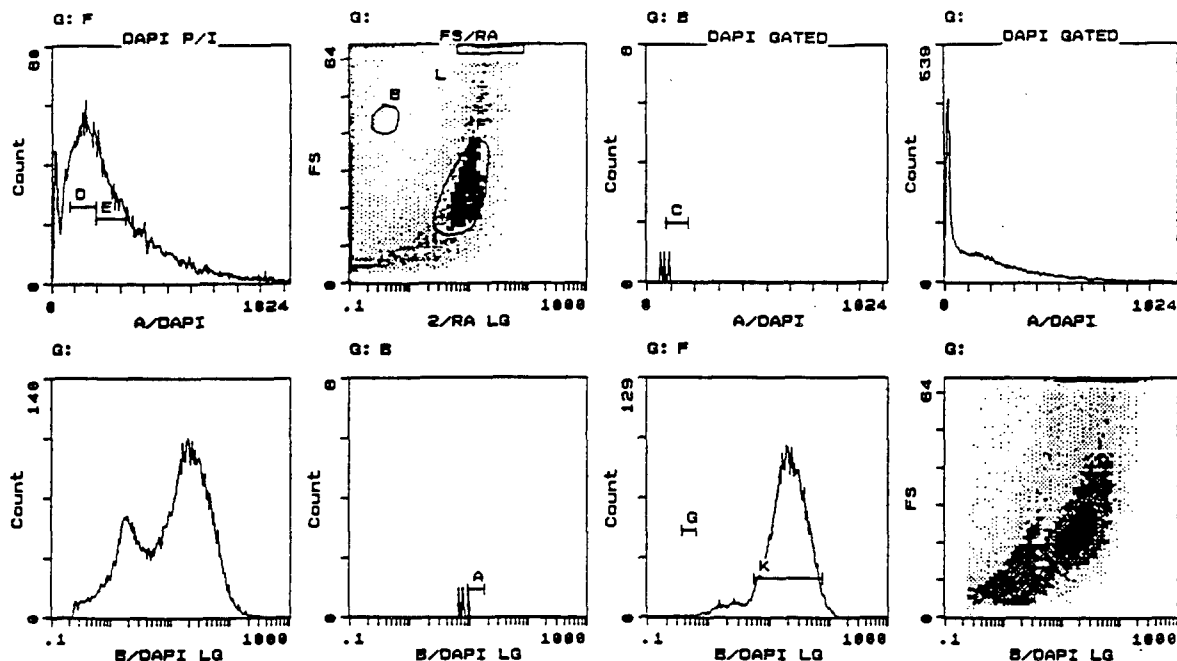
Table 1. Average Shapes and Sizes of Fungal Spores and Yeast and Bacterial Cells by Direct Observation.

Sample	Shape of Spore or Cell	Size of Spore or Cell
<u>Cladosporium</u>	Ovate; subfusiform	7.5 x 5 μm to 12.5 x 5 μm
<u>Mucor</u>	Ovate/spherical	7.5 to 12.5 μm long/diam.
<u>Heterobasidium</u> annosum	Dacryoid/lacrymoid	7.5 to 10 μm diam.
"Yeast 1"	Ovate/elliptical	2.5 x 5 to 5 x 10 μm
"Yeast 2"	Ovate/spherical	5 to 7.5 μm long/diam.
<u>Aspergillus</u>	Ovate/spherical	3.75 to 5 μm long/diameter
<u>Penicillium</u>	Spherical	2.5 to 3.75 μm diam.
"Bacteria"	Cocci	Approx. 0.5 μm diam.

Two populations of particles appeared in most of the flow cytometer runs. Plotting the DAPI levels on logarithmic scale enabled better resolution of peaks in all cases, except in Figure 1j where the linear plot of "Count vs. DAPI" obtained a clearer display of two populations. In that sample of Penicillium spores and bacterial cells (Figure 1j), difficulties arose in obtaining data about bacteria cells as a separate population. The particles in the plot that had produced the low levels of fluorescence, but no light scatter, were regarded as debris and were not analyzed.

In comparing the results of each mixed sample, it is not always certain which population is which type of particle. For example, Cladosporium and Mucor (Figure 1a) are similar in their ranges of spore size, although their shapes may be different, and thus positive identification is difficult at this point. The use of their internal complexities, measured by the right-angle light scatter, is limited, as there is little distinction in the RA properties of the two types of spores. In addition, the Cladosporium population is not placed in the same location in the plots when Figures 1a, 1b and 1c are examined. Allman *et al.* (1992) found that the assumption that the correlation of the intensity of forward light scatter with cell-size does not necessarily hold for mixed populations. Cells may give a forward scatter signal which is out of proportion to their size because of a high value for their refractive index (Allman *et al.*, 1992).

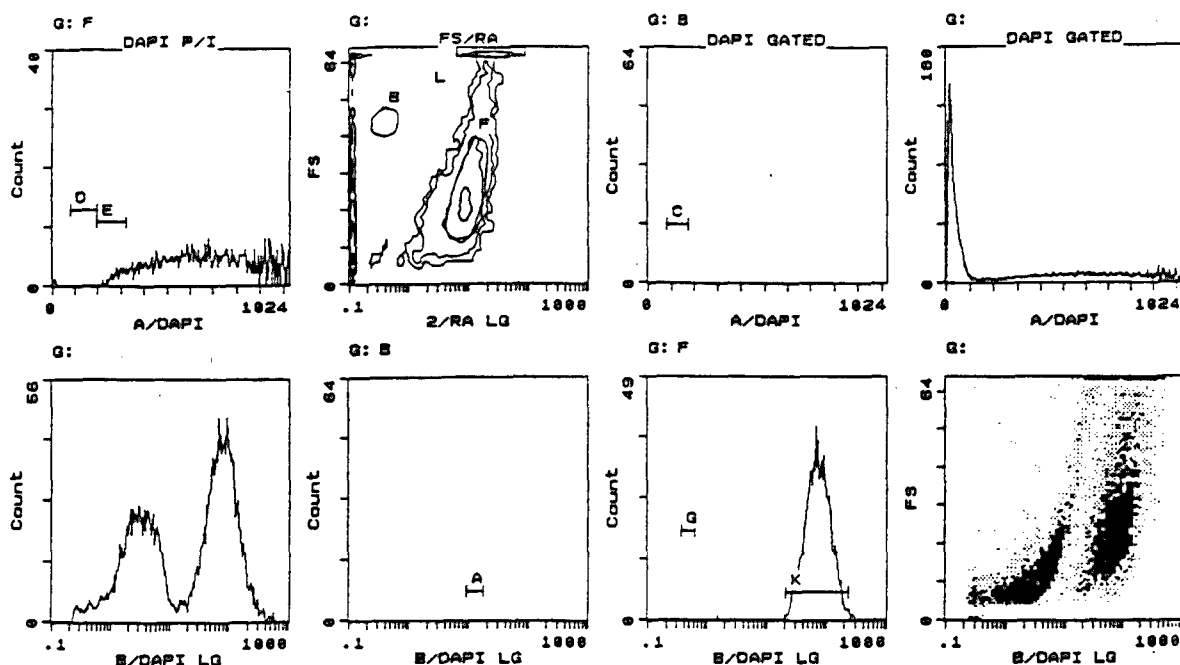
Sorting of the particles is normally possible with the flow cytometer. So that a population can be collected and taken to a fluorescent microscope for examination or identification,



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
			Peak.....	X Channel.....						
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	48.9	9	9.6	2	11.6	2.07	17.8	0.382	9.4	18
C	64.5	12	115	2	106.5	24.3	22.8	0.369	79	172
D	33.7	5817	145	80	136.3	32.3	23.7	17.2	78	192
E	25.3	4366	202	56	251.7	37.1	14.7	33.3	195	323
G	9.2	27	0.42	2	0.505	0.068	13.5	0.382	0.36	0.64
K	90.6	15650	20	129	21.2	12.7	60.0	21.3	5.5	77
DUAL PARAMETER STATISTICS										
			Peak.....	X Channel....			Y Channel.....			
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
B	0.1	22	0.24, 43	2	0.343	0.089	26.0	43.2	2.3	5.25
F	54.1	17268	6.6, 20	207	8.20	3.39	41.3	23.6	6.1	25.9
L	3.0	970	24, 63	130	25.6	12.4	48.5	62.9	0.5	0.766

Figure 1a. Cladosporium and Mucor.

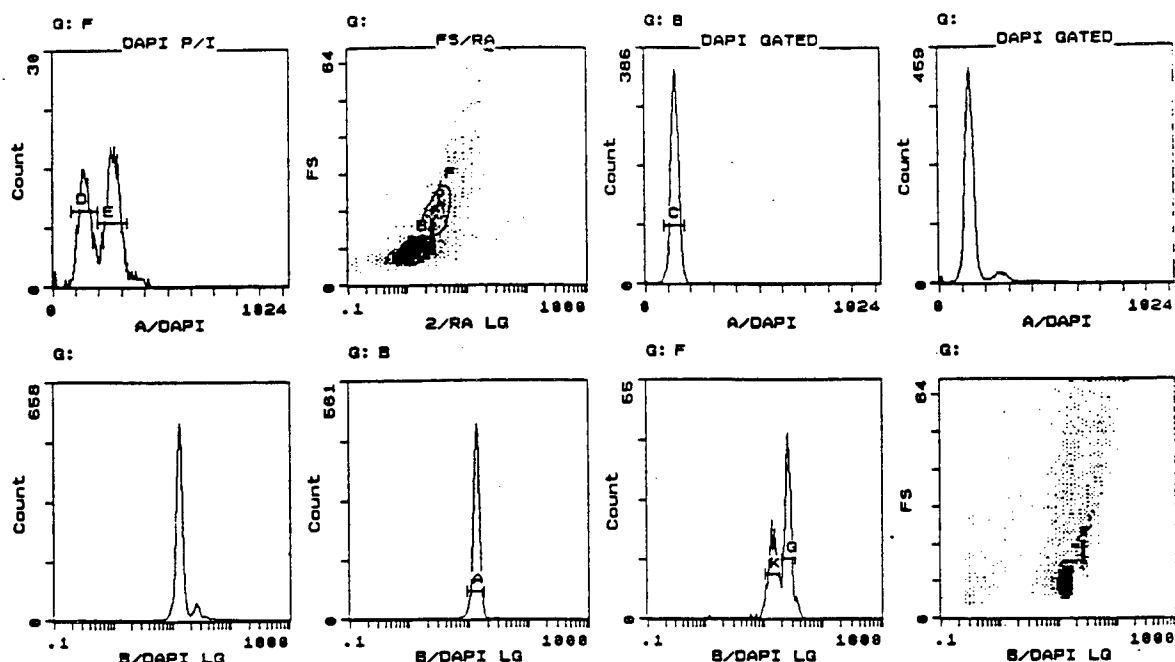
Cladosporium and Mucor. These spores have almost the same range of sizes. The plot of FS vs. RA does not readily distinguish two populations, but the plot of FS vs. DAPI LG, the logarithm of the DAPI value, does distinguish two populations. Overall, FS of the population indicates that this is quite a heterogenous sample in terms of size. Cladosporium is probably the population with the greater amount of fluorescence.



STATISTICS										
SINGLE PARAMETER STATISTICS										
ID	Pent	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
D	0.2	8	102	1	153.0	27.3	17.9	0.416	78	192
E	3.7	166	282	6	282.1	28.7	10.2	0.489	195	323
G	0.0	1	0.47	1	0.468	0.000	0.000	0.000	0.35	0.64
K	98.7	4377	62	49	72.4	33.3	46.1	2.53	21	238
DUAL PARAMETER STATISTICS										
ID	Pent	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
B	0.0	0	XXXX, XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX
F	35.5	4436	0.9, 24	52	9.09	3.19	35.0	23.1	6.6	28.7
L	2.3	290	24, 63	37	25.4	11.4	45.1	62.8	0.5	0.829

Figure 1b. Cladosporium and Heterobasidion.

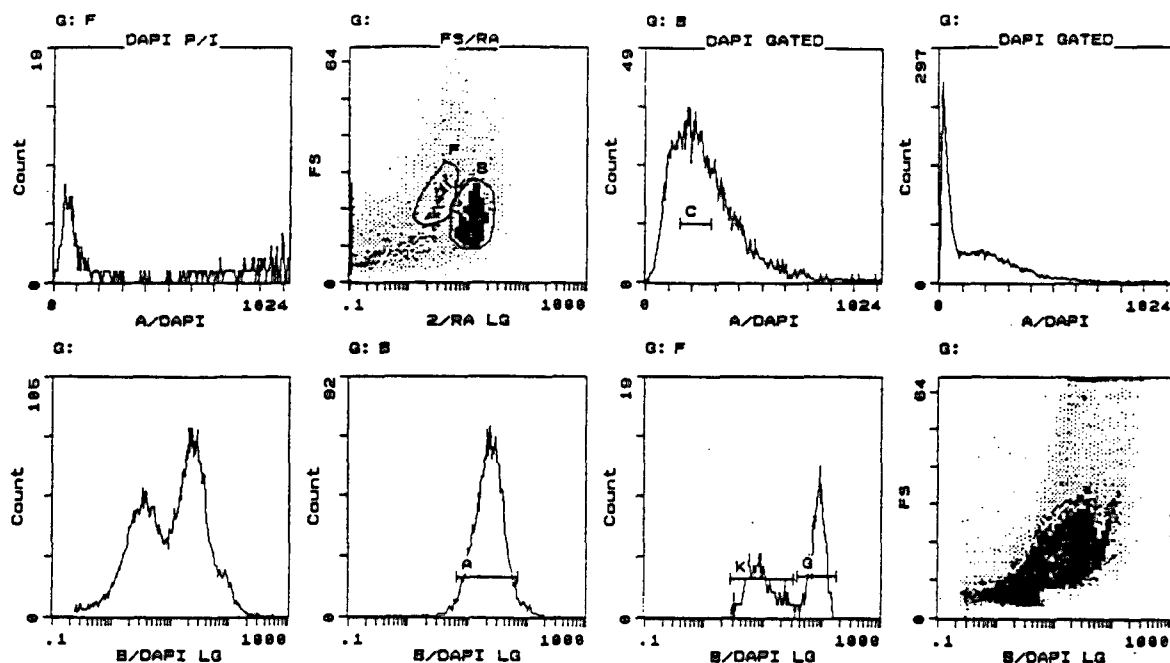
Cladosporium and Heterobasidion. Heterobasidion spores are expected to be slightly smaller than those of Cladosporium so that the smaller particle size (lower FS) group would be Heterobasidion. The position of Heterobasidion also indicates that it has a lower level of A-T than does Cladosporium. However, there is uncertainty about this location of the Heterobasidion spores as it compares to the interpretation of Figure 1d.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
			Peak.....	X Channel.....						
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	98.0	14385	14	561	13.4	1.54	11.5	8.78	9.4	18
C	98.4	14442	136	386	131.3	16.3	12.4	10.8	79	172
D	48.0	966	142	22	140.8	24.4	17.3	1.93	78	192
E	53.4	1289	254	38	268.8	28.1	18.8	8.769	195	323
G	55.2	1333	26	55	25.7	2.87	11.2	6.43	28	35
K	37.7	918	14	31	14.3	2.18	14.7	8.682	11	19
DUAL PARAMETER STATISTICS										
			Peak.....	X Channel.....				Y Channel.....		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
B	71.7	14683	1.2, 8	792	1.21	0.328	26.5	8.5	1.5	17.3
F	11.8	2415	1.8, 12	152	2.68	0.754	29.8	16.5	3.8	22.9

Figure 1c. Cladosporium and Penicillium.

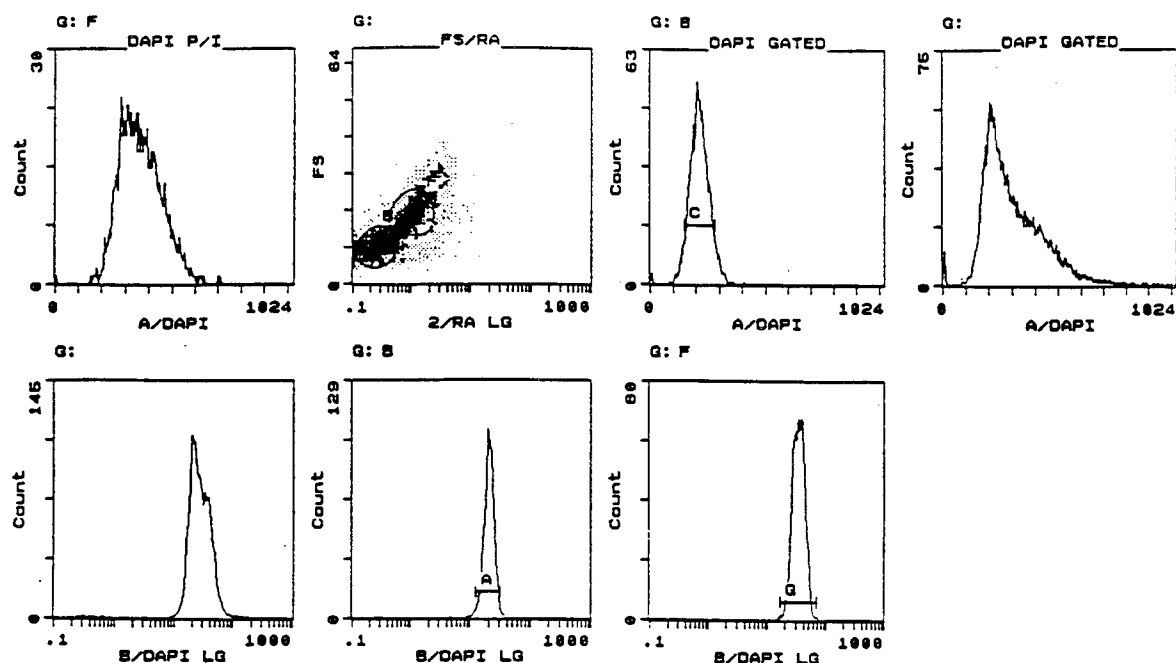
Cladosporium and Penicillium. Penicillium spores are much smaller than those of Cladosporium. The number of Cladosporium spores is very low compared to the number of Penicillium spores in this mixed sample. In the histograms of Count vs. DAPI LG, gates A and K probably represent the Penicillium population, and gate G probably represents the Cladosporium population.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
ID	Pcnt	Area	Peak.....	Position	Height	Mean	SD	FullCV	HalfCV	Min Max
A	94.9	9598		21	92	22.2	11.2	50.5	14.8	6.3 78
C	39.3	3968		242	49	211.2	38.8	17.4	3.76	149 280
G	62.7	720		93	19	83.2	22.1	26.6	0.561	38 173
K	44.6	610		7.4	9	9.94	5.13	56.8	0.573	2.8 32
DUAL PARAMETER STATISTICS										
ID	Pcnt	Area	Peak.....	Position	Height	X Channel.....	Mean	SD	CV	Y Channel.....
B	44.7	10109		12, 14	171	10.7	3.68	34.4	16.4	4.7 28.8
F	6.0	1367		2.4, 17	26	2.96	1.21	40.8	22.1	4.5 20.4

Figure 1d. Mucor and Heterobasidion.

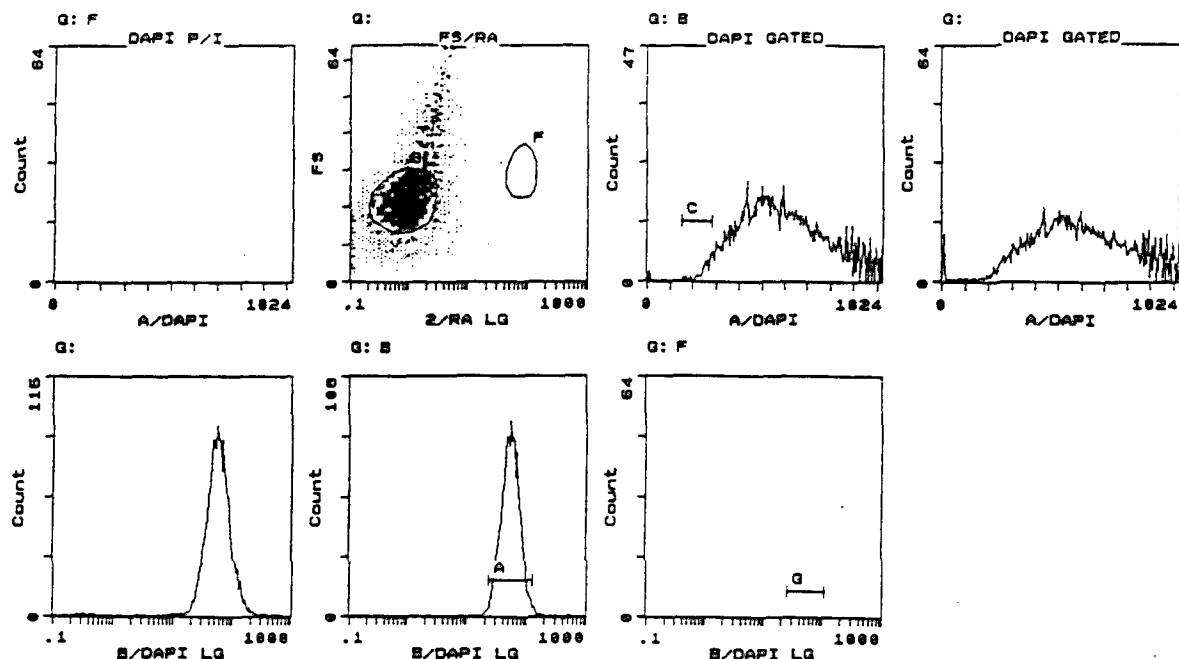
Mucor and Heterobasidion. The histogram of Count vs. DAPI LG is difficult to interpret since the fluorescence peak to the left may represent either Mucor, if the interpretation of Figure 1a is correct, or Heterobasidion, if the interpretation of Figure 1b is correct. However, Mucor spores are expected to be the larger particles (greater FS), and may be the population with proportionately greater DAPI fluorescence, and thus, be located further to the right in this histogram.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	97.4	4755	21	129	21.0	3.64	17.4	9.84	13	32
C	87.9	4293	284	63	213.1	31.4	14.7	9.31	149	288
G	99.3	4719	37	88	34.0	7.69	22.6	21.4	17	69
DUAL PARAMETER STATISTICS										
ID	Pcnt	Area	Position	Height	Mean	SD	CU	Mean	SD	CU
B	44.9	4882	0.43, 12	132	0.279	0.118	42.2	10.5	2.1	20.1
F	43.7	4750	0.66, 15	117	0.899	0.354	39.4	17.5	2.9	16.8

Figure 1e. Heterobasidion and "Yeast 1."

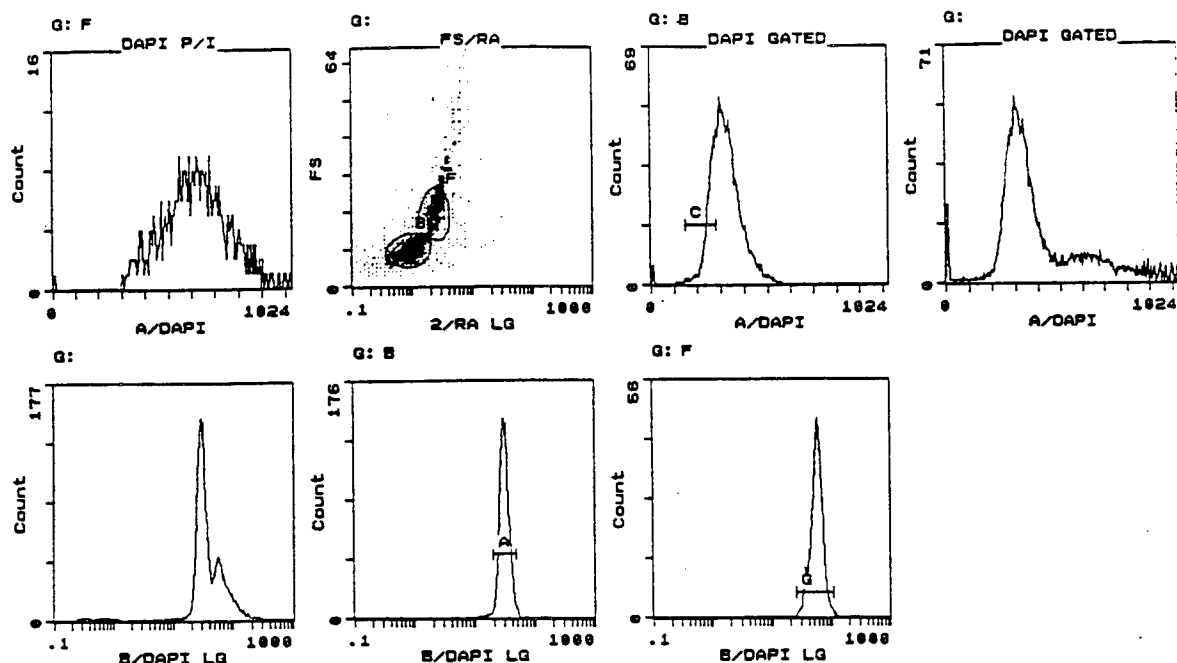
Heterobasidion and "Yeast 1." Two populations are showing in the histogram of the population count vs. DAPI LG. The yeast were expected to be more heterogenous in size than Heterobasidion. Although the yeast spores may reach the same length (10 mm) as the diameter of the Heterobasidion spores, the Heterobasidion spores are overall larger, and assumed to have proportionately more A-T binding sites to react with the fluorescent dye than would the yeast cells.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
ID	Point	Area	Position	Peak Height	Mean	SD	FullCU	HalfCU	Min	Max
A	98.2	7834	54	188	54.2	17.9	33.1	21.6	23	127
C	2.3	183	246	7	248.9	28.1	11.3	0.187	149	280
DUAL PARAMETER STATISTICS										
ID	Point	Area	Position	Peak Height	Mean	SD	CU	Mean	SD	CU
B	78.5	7981	9.89	21	104	0.914	0.462	50.6	21.5	3.7
F	8.8	0	****	****	****	****	****	****	****	****

Figure 1f. Heterobasidion and "Yeast 2."

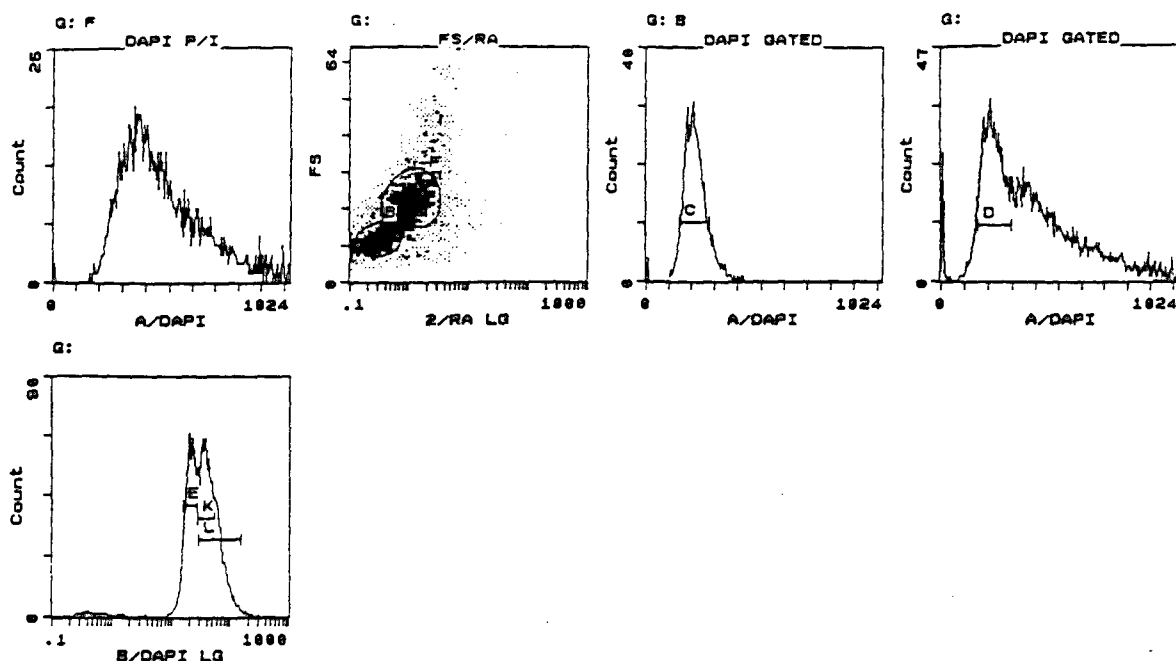
Heterobasidion and "Yeast 2." Heterobasidion spores are larger than this sample of yeast cells. However, only one population is apparent. This may be the result of too few spores of one kind, both types of particles being highly heterogenous and overlapping in their properties, or the sample being too few in numbers of particles to arrive at the average characteristics of the two populations.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
ID	Pcnt	Area	Peak..... Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	96.6	6917	32	176	38.7	5.83	16.4	12.8	28	48
C	24.6	1754	270	68	251.8	24.5	9.73	16.2	149	288
G	99.1	2592	68	66	65.6	13.8	23.4	6.49	25	118
DUAL PARAMETER STATISTICS										
ID	Pcnt	Area	Peak..... Position	Height	Mean	SD	CV	X Channel..... Mean	SD	CV
B	64.9	7151	1.8, 9	381	8.948	8.343	36.2	9.5	1.9	19.5
F	23.7	2615	1.6, 12	93	2.11	0.567	26.9	18.4	3.8	28.6

Figure 1g. Heterobasidion and Aspergillus.

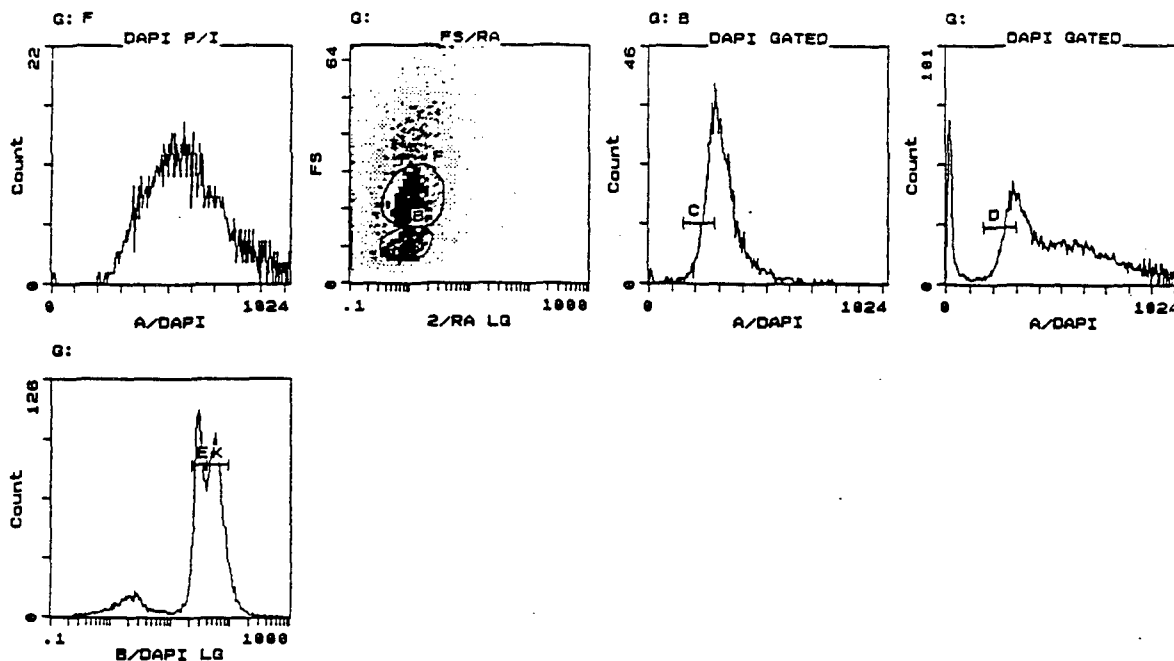
Heterobasidion and Aspergillus. Aspergillus spores are much smaller (lower FS) than Heterobasidion spores. They are easy to collect, thus their high count. The population with less fluorescence is likely that of Aspergillus spores due to their smaller size and proportionately smaller DNA.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
ID	Pcnt	Area	Position	Height	Mean	SD	FullCU	HalfCU	Min	Max
C	83.2	2629	292	40	210.7	32.3	15.3	4.74	149	280
D	35.3	3836	208	47	223.7	37.5	18.8	7.85	158	297
E	32.8	3386	21	90	21.2	3.13	14.8	9.55	16	28
K	39.0	4924	38	80	39.1	7.00	17.9	22.9	29	55
L	55.7	5845	38	80	40.9	17.5	35.0	22.9	30	150
DUAL PARAMETER STATISTICS										
ID	Pcnt	Area	Position	Height	Mean	SD	CU	Mean	SD	CU
B	38.7	3161	0.43, 12	71	0.329	0.147	44.5	11.3	2.3	28.0
F	55.6	5735	0.89, 18	80	0.991	0.472	47.6	20.4	3.6	17.5

Figure 1h. "Yeast 1" and "Yeast 2."

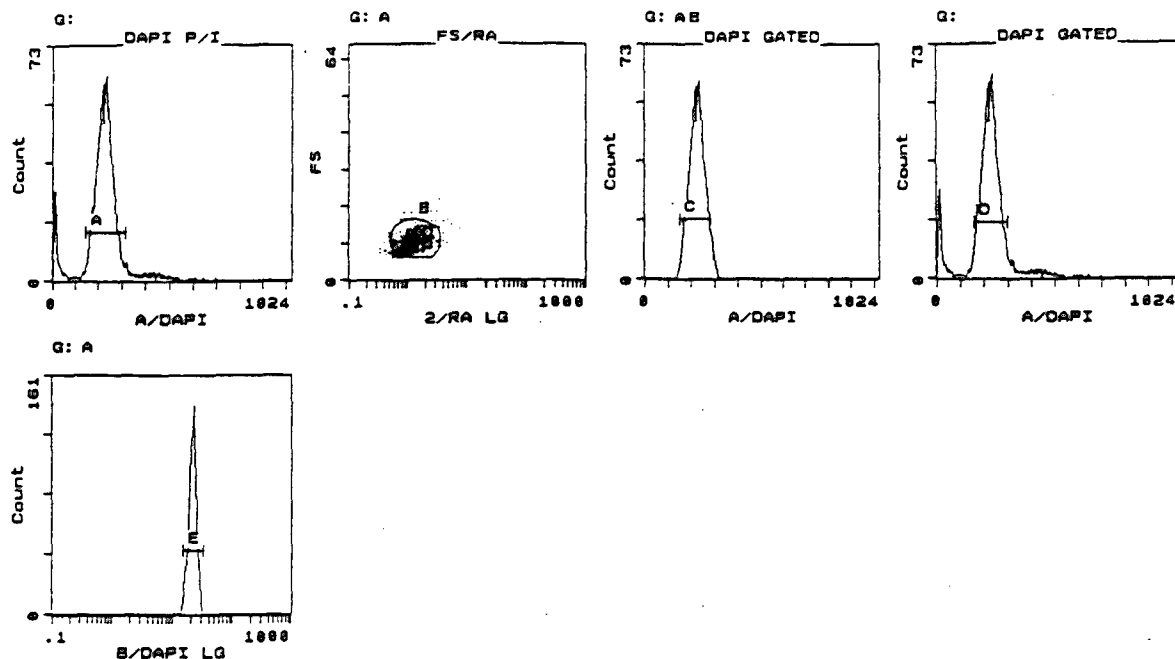
"Yeast 1" and "Yeast 2." The histogram of Count vs. DAPI LG illustrates the formation of two peaks. However, based on size, a population of "Yeast 2" was expected to fall in the middle of a population of "Yeast 1." Within gate B (as shown in the Count vs. DAPI histogram for gate B only), two, slightly delineated peaks appear; the smallest cells (but not the debris particles) could be those of "Yeast 1," and the peak to the right may represent a group of "Yeast 2" cells which are causing a bit of a spike within the gradient of sizes to be found in the total population.



----- STATISTICS -----											
SINGLE PARAMETER STATISTICS											
ID	Pent	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max	
C	32.6	1528	289	46	247.6	27.2	11.0	5.28	149	288	
D	16.8	2565	283	51	256.2	31.6	12.3	5.94	158	297	
E	29.9	4578	26	126	29.2	3.66	12.6	14.0	22	37	
K	43.4	6625	56	117	57.8	12.5	21.6	21.2	40	93	
DUAL PARAMETER STATISTICS											
ID	Pent	Area	Position	Height	Mean	SD	CV	Mean	SD	CV	
B	38.7	4686	0.89, 10	119	0.881	0.351	39.9	9.7	2.3	24.3	
F	34.1	5292	1.0, 21	55	1.02	0.534	52.4	22.0	4.4	19.8	

Figure 1i. "Yeast 2" and Aspergillus.

"Yeast 2" and Aspergillus. Aspergillus spores are smaller than the yeast cells. Two populations are separated out in the histogram of the logarithm of the DAPI value. There appears to be a fair population of debris particles as well, which appears against the y-axis in the scatterplot of FS vs. RA LG and in the histogram of Count vs. DAPI.



----- STATISTICS -----										
SINGLE PARAMETER STATISTICS										
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	81.6	5006	242	73	225.4	33.4	14.8	8.18	142	313
C	94.2	4599	242	73	221.8	29.3	13.2	7.47	149	289
D	79.1	4659	242	73	225.3	31.8	13.8	8.18	158	297
E	99.3	4978	23	161	22.1	3.88	13.9	9.84	15	33
DUAL PARAMETER STATISTICS										
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
B	97.5	4883	1.2,	9	197	1.25	0.443	35.3	9.7	1.8

Figure 1j. Penicillium and "Bacteria."

Penicillium and "Bacteria." Bacteria cells are much smaller than (by a factor of 10) fungal spores or yeast cells. The number of bacteria cells collected was relatively high. It is not certain if the peak to the left in the Count vs. DAPI histogram represents bacteria or debris. These small particles were treated as debris and gated off. Alternatively, the bacterial cell may contain as much DAPI dye as the Penicillium spore, and thus may be within the population gated off as A. The single peak hints of two populations in the plots of Count vs. DAPI.

sorting a population is done by gating the population of interest shown on the flow cytometer monitor display. The gate parameters are determined by the computer, and the cells are sorted electronically. Sorting was attempted on a monoculture sample of Penicillium spores to determine if three apparent populations were caused by clumping of the spores. Sorting was unsuccessful, because the spores were too small to be sorted with the equipment on this flow cytometer.

Enumeration of the particles passing through the flow cytometer is possible. The particle count can be related to the volume injected and time elapsed in the run. The rapid enumeration of bacterial concentrations in pure cultures has been demonstrated (Pinder et al., 1990).

Particle size (FS) was more useful in analyzing these samples than was internal complexity (RA). The combination of FS and DNA level (DAPI or DAPI LG) was even more useful in characterizing the makeup of the samples, and it should have been plotted for all samples. It can be seen that FS is proportional to the amount of DAPI stained in the particles (Figures 1a, 1b, 1c, 1d).

This assumes that the A-T content is approximately the same in each type of culture that was analyzed. The fluorescent dye, DAPI, has high DNA specificity and intense fluorescence. DAPI, like the commonly used Hoechst dyes, is adenine-thymine (A-T) specific, that is, the dye's molecular structure and properties enable it to bind to the outer groove of the DNA molecule at the location where a sequence of three adenine and thymine pairs, two of the nitrogenous bases that comprise DNA, in the DNA helix.

For greater characterization of biological particles, two dyes that could stain DNA differentially could be applied to future samples for flow cytometry analysis. Differential fluorescent staining of DNA may be accomplished by using one dye with affinity to A-T and another dye with affinity to guanine-cytosine base pair (G-C), the other two nitrogenous base pairs that comprise DNA, if they are excited at different wavelengths.

In human cells, the ratio of (A-T) to (G-C) is close to one; in bacteria the G+C content of DNA varies from less than 25% to more than 85% (Shapiro, 1988). The use of (A-T)- and (G-C)-specific dyes aids in measuring total DNA content, and allows a flow cytometric fingerprint of the biological particle to be plotted. This has been demonstrated in the studies by Allman et al. (1992), using mithramycin and ethidium bromide, by Miller and Quarles (1990), using fluorescein isothiocyanate and propidium iodide, and by Sanders et al. (1990) using Hoechst 33258 and chromomycin.

There were not enough samples analyzed on the flow cytometer to evaluate the reproducibility of the technique. The four (above-mentioned) trial runs of pure culture samples were fair; after the first run, general placement of a sample population in the scatterplots and its distribution along channels could be predicted, but only in a broad area. The differences in the exact location of the population in the trials may be accounted for by the unequal electronic settings and variability in the samples, such as clumping of spores and cells, differences in refractive index, and the stage of development, for example, budding yeast.

4. RESULTS AND DISCUSSION OF GAS CHROMATOGRAPH ANALYSIS

A simple procedure of sample preparation was preferred for the characterization of the spores of the Mucor, Cladosporium, Aspergillus and Penicillium species, Heterobasidion annosum, two yeast and one bacteria samples by gas chromatography (GC). The techniques of sample preparation in GC studies have traditionally consisted of multi-step derivatization or extraction with a variety of solvents. Since time is of importance in detecting and identifying the biological particles, the fewest steps possible in the extraction of volatiles was desired. Also, the use of easily disposable solvents was preferred.

Chromatogram results of the GC analysis of only four fungal spore samples extracted by 95% ethanol and two blank samples are shown in Figures 2a to 2f. These chromatograms were copied from thermal paper recordings, and the axes were added. Only ethanol extraction and the analysis of four spore samples were possible due to subsequent technical difficulties with the GC.

Each sample also contained a PTFE filter on which the samples were collected. The samples were not heated. The peak width of the blanks was 0.01 mm. The peak width of the samples was 0.04 mm. In each chromatogram, the tick marks above and below the baseline indicate peak starts and/or stops and valley points between merged peaks, respectively. The numerous tick marks in the blank samples are the result of having set a narrower peak width as the criteria for detecting peaks.

These results using ethanol as the extractant are not particularly detailed. After the solvent peak, three to six peaks were detected in each sample. The two blanks contained only ethanol, but they produced different chromatograms. The identities of the peaks have not been determined, as the trial on this GC served only to develop a preliminary temperature program, and to see if peaks could be obtained. (Also, the GC-mass spectrometer (MS) that was initially proposed for this analysis was awaiting repairs.)

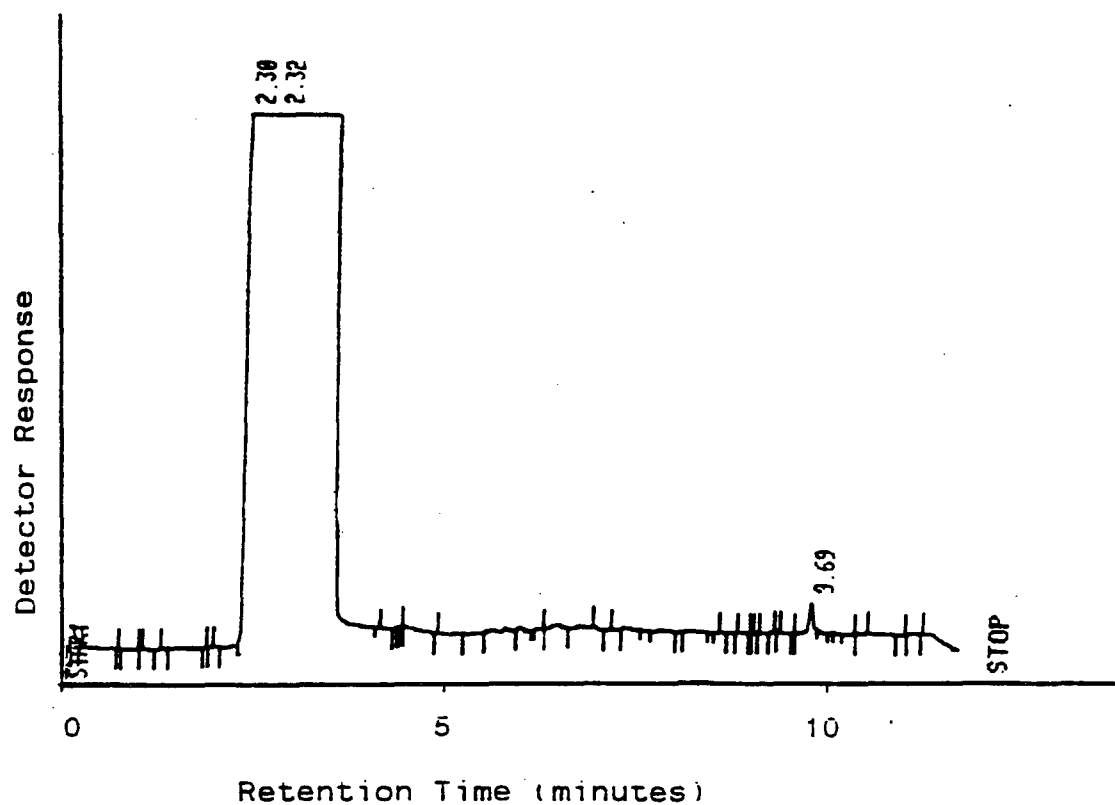


Figure 2a. "Blank 1."

The 95% ethanol solvent peak in sample "Blank 1" was reported as two solvents on the chromatogram, due to splitting of the solvent peak by an overloaded flame ionization detector. The solvent sample contained an unknown component at retention time 9.69 minutes.

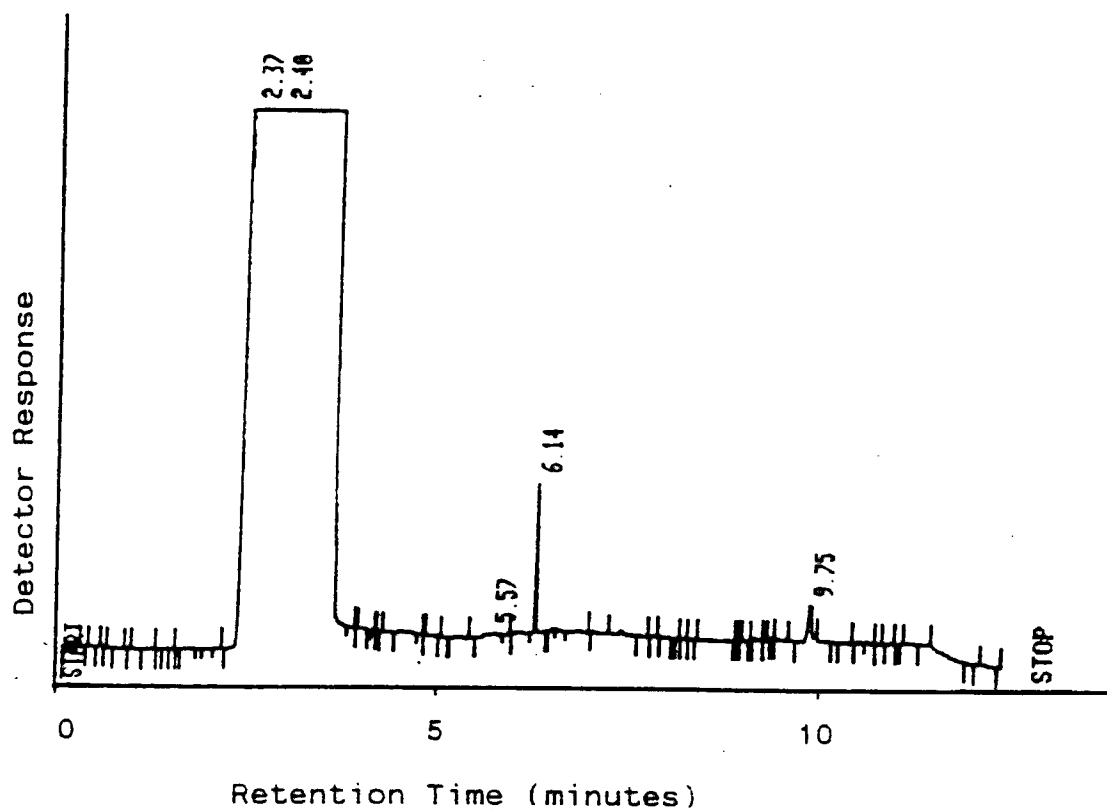


Figure 2b. "Blank 2."

The 95% ethanol solvent peak in sample "Blank 2" was reported as two solvents on the chromatogram, due to splitting of the solvent peak by an overloaded flame ionization detector. This solvent sample varied from the previous blank (Figure 2a) and contained two additional unknown components.

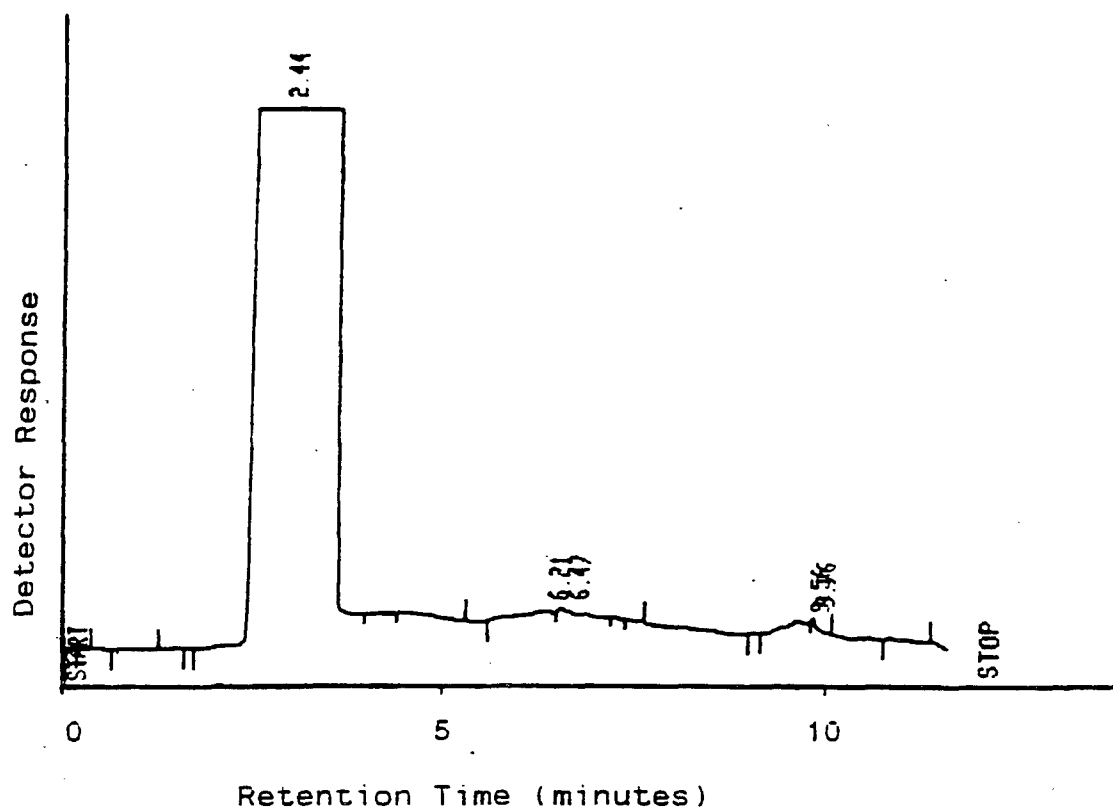


Figure 2c. Micor Spores.

This sample possesses one peak at 9.56 that is not found in the other samples. The peak at 6.47 appears to be the same as that found in the Penicillium sample at 6.42.

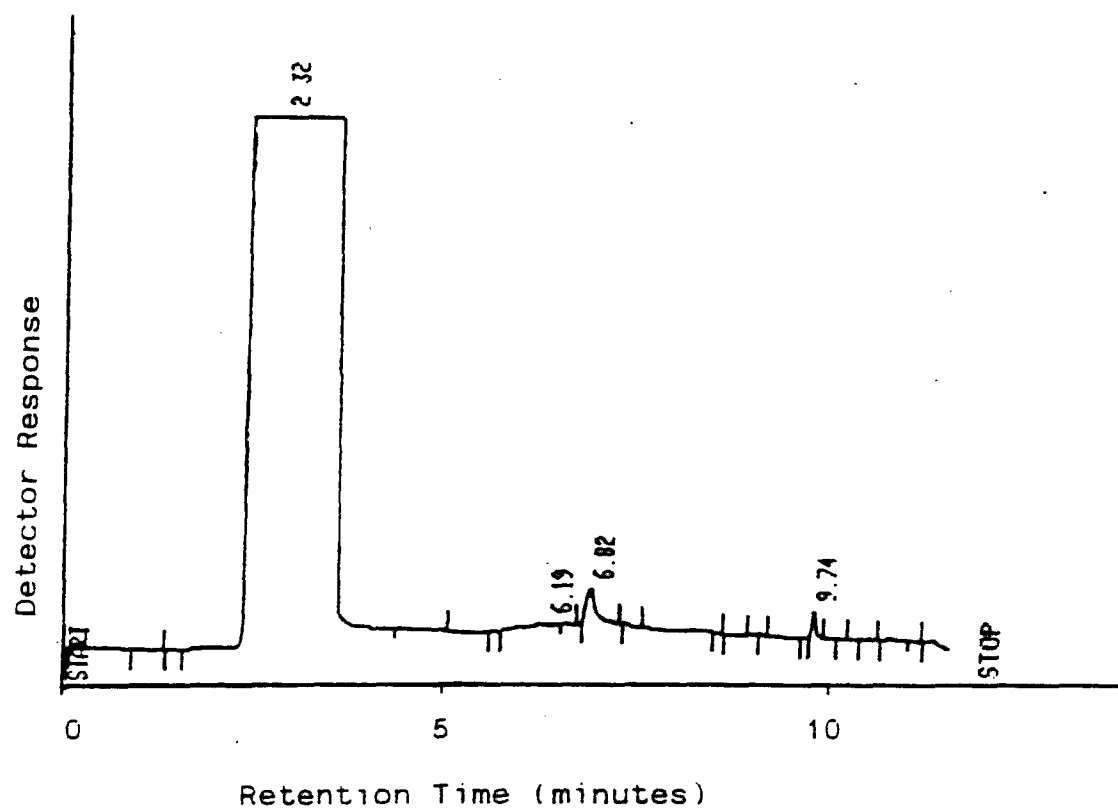


Figure 2d. Aspergillus Spores.

The two peaks at 6.19 and 6.82 appear to be unique to this sample.

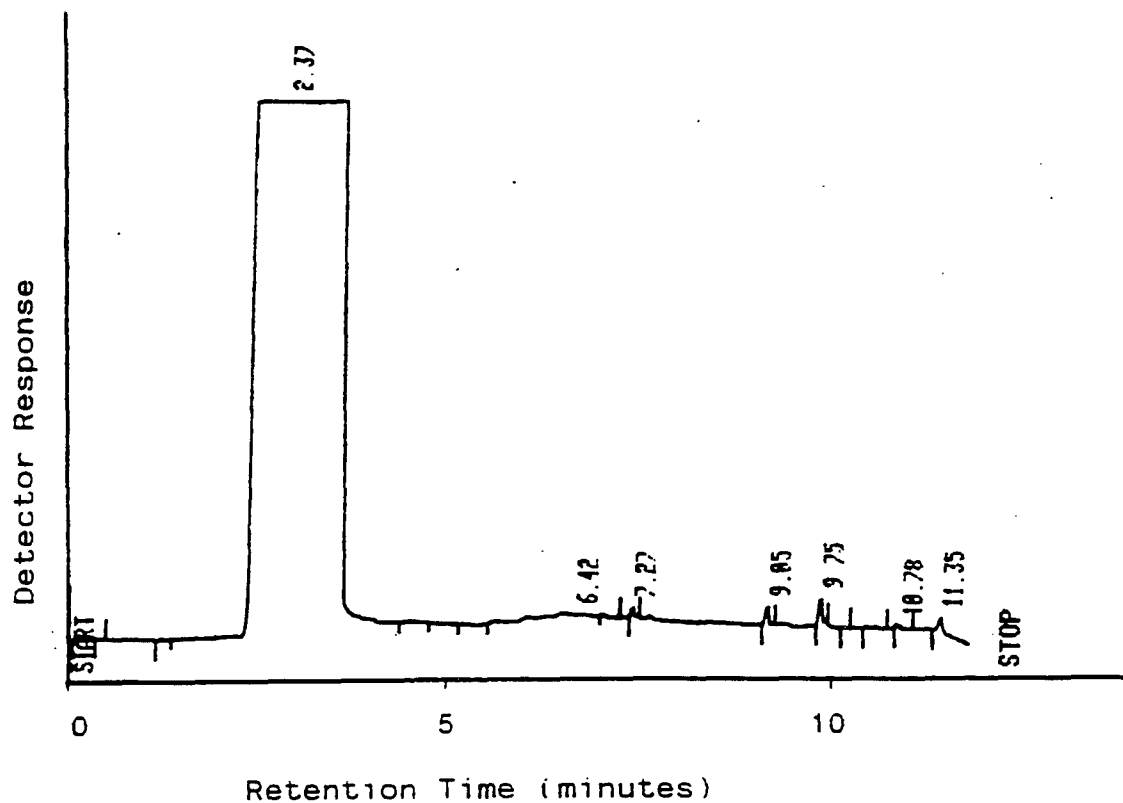


Figure 2e. Cladosporium Spores.

The peaks at 6.42, 7.27, 9.05 and 11.35 appear to be unique to this sample. The peak at 10.78 may be the same as that found in the Penicillium sample at 10.79.

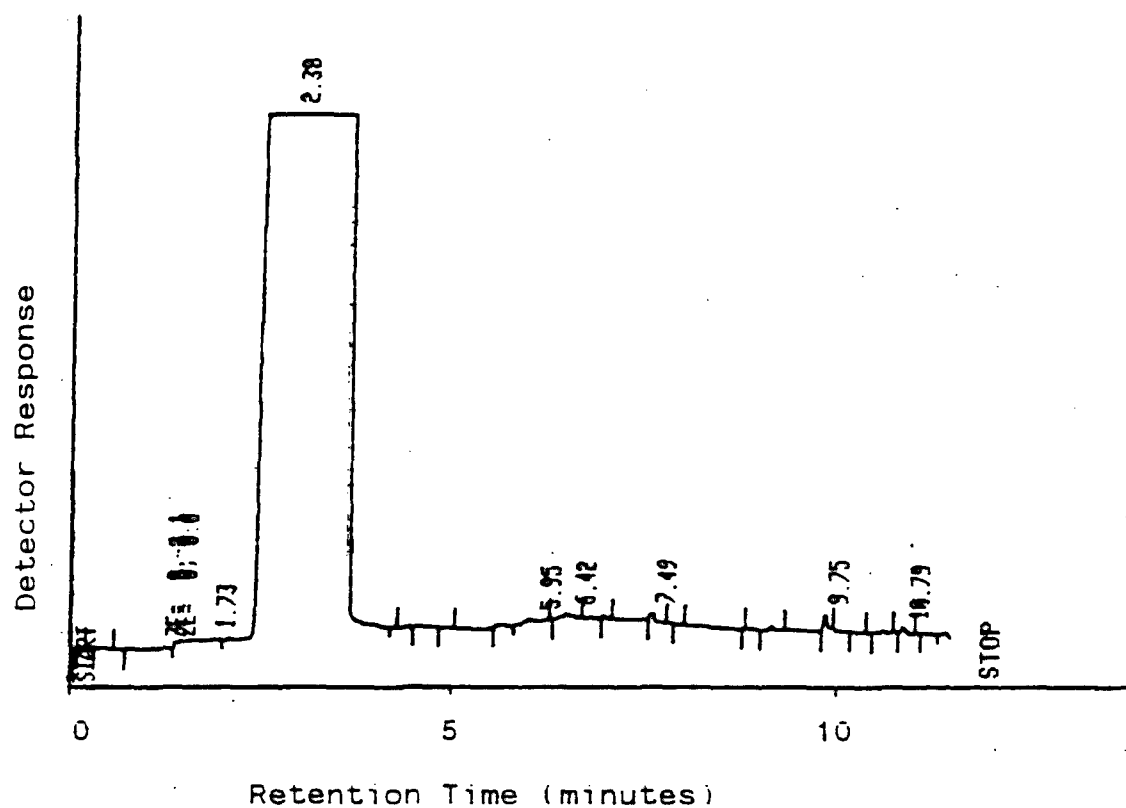


Figure 2f. Penicillium Spores.

The peaks at 5.95 and 7.49 appear to be unique to this sample. The peaks at 6.42 and 10.79 may be the same as that found in the Mucor sample at 6.47 and in the Cladosporium sample at 10.78, respectively. (The printing of "ZE=0.01" and "ZE=0.00" was the result of zeroing the baseline during the analysis.)

A summary of the retention times of detected peaks is presented in Table 2 for the two blanks and the four samples. To compare the retention times of peaks between samples, the time of the solvent peak was subtracted from the times of the detected peaks. The comparison is presented in Table 3. The matching of the peaks as indicated by the superscript in Table 3 only estimates which peaks may be a component common in each of the samples. The occurrence of a peak at 1.73 in the Penicillium sample (Table 2) may indicate that the temperature program was not carried out long enough to purge all of a sample before the introduction of the next sample into the GC.

Table 2. Summary of Retention Times of Detected Peaks During Gas Chromatography Analysis Using 95% Ethanol Extractant. The Retention Times in Parentheses are Regarded as Errors.

Sample	Solvent peak	Detected Peaks
Blank 1	2.30, (2.32)	9.69
Blank 2	2.37, (2.40)	5.57, 6.14, 9.75
<u>Mucor</u>	2.44	6.21, 6.47, 9.56, 9.76
<u>Aspergillus</u>	2.32	6.19, 6.82, 9.74
<u>Cladosporium</u>	2.37	6.42, 7.27, 9.05, 9.75, 10.78, 11.35
<u>Penicillium</u>	(1.73), 2.38	5.95, 6.42, 7.49, 9.75, 10.79

Table 3. Comparison of the Times of the Detected Peaks as Occurring After the Time of the Solvent Peak (Retention Time of Detected Peaks Minus Retention Time of Solvent Peak). The Superscripts Estimate Which Peaks May be the Same Component in Each of the Samples.

Sample	Detected peaks
Blank 1	7.39(a)
Blank 2	3.20, 3.77(b), 7.38(a)
<u>Mucor</u>	3.77(b), 4.03(c), 7.12, 7.32(a)
<u>Aspergillus</u>	3.87, 4.50, 7.42(a)
<u>Cladosporium</u>	4.10, 4.95, 6.73, 7.43(a), 8.46(d), 9.03
<u>Penicillium</u>	3.57, 4.04(c), 5.11, 7.37(a), 8.41(d)

The analysis of fungal spores by GC is not common. Removal of the resistive spore coat was considered, but it would involve developing another set of procedures. When the spores and yeast cells were heated and analyzed, one small peak, if any, was produced in most of the samples, but this peak was not reproducible when a replicate sample was analyzed.

Chromatographic profiles of the derivatives of fatty acids have been a in the analysis of aquatic algae with GC-MS (Cojocar, 1988) and Gram-negative anaerobic bacilli with gas-liquid chromatography (Stoakes *et al.*, 1991). Sonesson *et al.* (1988) found that no hydroxy fatty acids were found in Gram-positive bacteria (*S. faecalis*, *S. mutans*, *S. viridans*, *C. perfringens*) or yeast (*C. neoformans*, *C. albicans*, *T. glabrata*).

Augustyn *et al.* (1992) and Botha and Kock (1993) conclude that fatty acid analysis is not a generally applicable yeast identification technique, but it is a reliable method to characterize yeast strains in some species. Welch (1991) thoroughly reviews the application of fatty acid analysis. Any identification of filamentous fungi on the basis of cellular fatty acid composition has not been described (Welch, 1991).

In addition to extracting volatile fatty acid compounds, the derivatization of sugars and carbohydrates, such as muramic acid, has been studied with some bacteria (Fox *et al.*, 1983; Fox *et al.*, 1984) and some actinomycetes (Goodfellow *et al.*, 1990). The methods used in these studies are lengthy. To obtain good sugars analysis results with methods as simple as those proposed (below) for this study may not be possible.

Extracting the fatty acids and sugars from plant matter is commonly practiced in the analysis of plant compounds by GC. This approach generally uses acetone for the extraction of volatile sugars while methylene chloride, and methylene chloride with a silanizing reagent are used for the extraction of fatty acids. The extractions are single- or two- step, with or without heating of the sample. Examinations of the liquid phase and headspace were considered to be the next steps in the GC analysis.

5. RESULTS AND DISCUSSION OF GENETIC PROBE ANALYSIS

There are two types of nucleic acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The genetic material that organisms inherit from their parents consists of DNA. Written in the structure of DNA are the instructions that program all of the cells activities. RNA functions in the actual synthesis of the proteins specified by DNA. RNA is single stranded, but it contains local regions of short complementary base-pairing. The RNA found in the ribosomes, the molecular machines that coordinate the interplay of transfer RNA (tRNA), messenger RNA

(mRNA) and proteins in the process of protein synthesis, is called ribosomal RNA (rRNA). rRNAs are highly conserved (Olsen et al., 1986) constituents of the subunits into which ribosomes can be dissociated, and they are classified by their sedimentation coefficients, that is, 5S rRNA, 16S rRNA and 23S rRNA are in bacteria, and eukaryotes commonly contain a fourth rRNA, 5.8S (Olsen et al., 1986).

To produce a nucleic acid fingerprint using an oligonucleotide probe, one or more restriction enzymes, also called restriction endonucleases, are used to cleave RNA or DNA molecules into specific fragments. The enzymes recognize specific nitrogenous base sequences in the RNA and cleave at specific places. The fragments are separated by gel electrophoresis, and the pattern of such fragments can serve as a fingerprint of an RNA molecule. The restriction fragments are denatured to form single-strands, transferred to a nitrocellulose sheet, and identified by hybridizing it with a labeled DNA probe strand which is revealed by autoradiography.

The procedure requires a large sample of rRNA. Nucleic acid amplification is achieved by the technique called polymerase chain reaction (PCR). A polymerase enzyme catalyzes the formation of DNA or RNA from precursor substances in the presence of preexisting DNA or RNA acting as a template. All RNA is synthesized by RNA polymerase according to instructions given by DNA templates.

An alternative to the use of a probe is using RNA as a template for reverse transcriptase enzyme to form a complementary strand of DNA which in turn functions as a template for the synthesis of a second complementary chain of DNA, yielding a double-stranded DNA molecule as the final product to be used to amplify, through replication with DNA polymerase, the sequence fragment.

The PCR is allowed to run for the desired number of cycles and is followed by gel electrophoresis. Staining, with ethidium bromide, for example, which fluoresces orange when it is bound to double-stranded DNA fragments, permits quick viewing and evaluation of the fragments. This approach does not require a library of DNA probes with which to hybridize the unknown, cleaved fragment. Instead, a database of fragment mappings of species is required.

Universal consensus PCR primers for the differentiation of fungi from bacteria were considered. In an air sample, populations of fungal and bacterial particles may be enumerated or monitored with the use of these primers. A primer is composed of nucleotides of RNA which are added to the end of an already existing DNA strand to start replication, and it may be used in

the amplification of all fungal DNA. For example, there exists a PCR primer for angiosperm and conifer DNAs that does not amplify fungal or algal DNAs (Cullings, 1992). Consensus sequences are base sequences of DNA promoter sites, which determine where transcription of DNA into RNA by RNA polymerase begins. The sites are not all identical, but they do possess common features.

For example, each base in the consensus sequence TATAAT is found in a majority of prokaryotic promoters (Stryer, 1988). PCR is applied to amplify the DNA. However, universal consensus PCR primers for fungi could not be found in the literature, library CD-ROM databases, or computer network molecular biology databases (Genetics Computer Group, European Molecular Biology Library (EMBL), GenBank) at this point; the search will continue.

Sequences of fungal and bacterial rRNA subunits are listed in GenBank's network-accessible electronic data publishing system. For example, a brief search found sequences of the 5S rRNA for Aspergillus flavus, A. nidulans, A. niger, Penicillium chrysogenum and P. patulum, the 16S rRNA for A. fumigatus and Mucor racemosus, and the 17S rRNA of a yeast, Cryptococcus neoformans. The databases are continually expanding. Also, sequences have been published for the 5.8S rRNA of Heterobasidion annosum (Kasuga, 1993).

Knowing the sequences of the rRNA may help to determine the specific restriction endonuclease enzyme(s) that will be useful. The number of fragments in the map could be determined, based on the number of locations where cleaving may occur. Unique or slightly different base pairs may be targeted.

6. CONCLUSIONS

The trial results presented in this report are encouraging, and the characterizations of airborne microbial particles can be achieved to a greater degree with further investigation and refinement of the flow cytometer, gas chromatography and genetic mapping methods. Flow cytometry has given the best results at this point. Work is required to make the results clearer and reproducible. Staining differentially with a second DNA dye, and analyzing mixed samples of more than two cultures could also be tested. It must be possible to sort particles of these dimensions and to study bacteria on this flow cytometer.

Gas chromatography results will be less uncertain and more meaningful if the samples can be analyzed on a GC-MS supervised by a trained operator. Carbohydrates and sugars and cellular fatty acids of the microbial particles can be analyzed by the application of various chemical compounds as extractants of volatile materials, or in making derivatization products for analysis. The methods for genetic mapping are widely published.

Suitable endonucleases and reagents can be chosen, and mapping can proceed if electrophoresis equipment is available for the preliminary mapping. The establishment of databases of fingerprints and profiles of airborne biological particles, obtained from these methods under standardized procedures, is important reference for identifying the microbes.

Blank

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