HDC Project FV134 - Final Report

The rapid detection of *Botrytis allii* causing latent neck rot infection of onion bulbs

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(July 1995)

HDC FINAL REPORT

Final Report:	End of Year 3 July 1995
Project Number:	FV134
Project Title:	Rapid detection of *Botrytis allii* causing latent neck rot infection of onion bulbs.
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Location:	HRI Wellesbourne / Kirton
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Commencement:	1st July 1992
Completed:	1st July 1995
Key Words:	Onion, Neck rot, *Botrytis*, Detection

INDEX:

	Page
Relevance to growers and practical application	
Application	1
Summary	2
Experimental section	
Introduction	5
Materials and Methods	6
Results	12
Conclusions	19
Glossary	22
References	23
Appendices	24

APPLICATION

The project aim was to develop a serologically based method for the rapid detection and identification of latent infections of *Botrytis allii* in onions. The results show that it is possible to detect the pathogen at storage and predict the level of neck rot that will occur within stores post harvest. This work is currently being continued in a MAFF funded programme aimed at integrating the test in a simple format with the bacterial test for mushy rot of onions.

SUMMARY

Botrytis allii is the causal organism of neck rot of onions and can be responsible for severe losses of bulbs in store. Symptoms of neck rot are not detectable on grading lines and only become evident after onion bulbs have been in store for at least 8 weeks. Losses in excess of 50% have been experienced in some stores (Maude, 1988). Secondary sources of infection such as contaminated soil, onion dumps and the overlapping of autumn and spring grown crops may perpetuate the disease (Maude, 1983).

Current detection methods rely on culture and identification of the organism using standard mycological methods. This is both time consuming and requires a specialist knowledge of the fungus. Where latent infections are suspected, large numbers of samples need to be taken because viable spores or mycelium must be present for successful isolation. Subsequent identification is dependent on the growth of the fungus on agar medium which takes several days/weeks.

This project aimed to develop a rapid and sensitive serological technique to detect fungal antigens without the requirement for lengthy isolation procedures. Serological techniques are rapid, 1-24h and in an appropriate format require little operator knowledge or apparatus. In addition, the methods are highly sensitive and will detect antigenic substances diffusing from the fungus greatly increasing the probability of detection.

The development of a rapid detection method for *B. allii* would enable growers to assess the storage potential of stocks of onions prior to and during storage to facilitate improved store management.

Polyclonal antisera for the serological detection of *B. allii* were raised from cell wall and cytoplasmic extracts of the fungus, and from surface plate washings of two isolates of *B. allii*. These antisera were used to develop an indirect enzyme linked immunosorbent assay

(ELISA) for the detection of latent infections. The antisera did not react with healthy onion tissue and showed only minimal recognition of non-*Botrytis* fungi. Some cross-reactivity was observed with *B. cinerea* but not with other *Botrytis* species tested. Results from Western blots showed that there were areas of similarity between *B. allii* and *B. cinerea* but that by cross-absorption these could be removed to provide a specific polyclonal antibody to *B. allii*.

It was found that the ELISA could detect soluble antigenic material diffusing through apparently healthy tissue in advance of the infection (Linfield *et al.*, 1995). The results from in store sampling indicated that the progression of the fungus around the neck of the bulb may not be uniform in the early stages of infection, therefore multi-site sampling is required. In many instances following infection, fungal antigen could be detected earlier by ELISA than by direct culture.

In initial tests on bulbs from a commercial store, the level of *B. allii* was found to be 3.3% by both ELISA and direct plating, but this result was achieved 7 days sooner by ELISA. In field tests planted in March 1993, the indirect ELISA gave a good indication at harvest of the probable incidence during storage of neck rot in untreated and artificially inoculated plants, whilst culturing gave results consistently lower than the ELISA until the bulbs had been in store for several months, when results for ELISA and culturing were the same. Spores applied to plants during the growing season led to greater levels of neck rot than did infested seed, indicating that field debris and waste from cold stores may be more important sources of inoculum in some years than seed contamination. Previous work has shown that relative humidities in excess of 85% (wet early summer conditions) are highly correlated with neck rot development from infested seed. During June 1993 there were only four days at the latter part of the month when RH was 85% or greater and this may explain the low levels of neck rot found in infested seed plots.

The response by growers in 1994-95 to the HDC press release for commercial onion store samples was disappointing and additional samples were taken from Kirton and Stockbridge to provide adequate samples. The amount of neck rot in the commercial stores varied from 0-15% with most stores having 1-3% neck rot. Low levels of neck rot in commercial samples were readily detected by the ELISA.

The ELISA test developed is clearly able to detect *B. allii* antigen in advance of symptoms and is considerably more sensitive than isolation methods. In most instances during this work the test was able to predict the level of neck rot that would develop in a store. In two of the commercial stores though the predicted level was lower at storage than the actual level found by ELISA several weeks post storage. In one of these stores the observed level was similar to the harvest ELISA result and the higher post storage ELISA reading may have been due to an unusually high number of infected bulbs in the sample biasing the result. These problems highlight the importance of sampling the bulbs at the correct time and the problems of sample size with respect to the number of bulbs in a store. This aspect of the work is now being continued in a MAFF funded programme which considers sample size and time of sampling together with combining the ELISA for *B. allii* with that for *Pseudomonas gladioli* pv. *alliicola*. The procedure for detection of both pathogens uses an indirect ELISA format and can be run simultaneously on the same bulb sample. It is envisaged that the test will be formatted as a kit which could be utilised by crop consultants and growers.

Rapid detection of *Botrytis allii* causing latent neck rot infection of onion bulbs

EXPERIMENTAL SECTION

Introduction

Botrytis allii Munn (syn. *B. aclada* Fres.) is responsible for neck rot of onions and is the main cause of deterioration of stored bulbs and pickling onions in the UK (Maude & Presly, 1977). *B. allii* is carried within the seed and is viable for at least 3 years when stored at 10°C and 50% r.h. (Maude & Presly, 1977). Symptoms of neck rot are not detectable on grading lines and only become evident after onion bulbs have been in store for at least 8 weeks. Losses in excess of 50% have been experienced in some stores (Maude, 1988). Secondary sources of infection such as contaminated soil, onion dumps and the overlapping of autumn and spring grown crops may perpetuate the disease (Maude, 1983).

In the past, good control has been achieved using commercially treated seed. However, extensive losses due to neck rot can still occur in store. With the declining profitability of the onion crop in recent years, minimisation of losses from disease is essential. Predicting the likelihood of disease development and bulb losses in store would enable growers to assess the storage potential of onions prior to and during storage. Current detection methods rely on culture and identification of the organism. Serological techniques are generally faster than such isolation procedures and require less expertise. Due to their high sensitivity, it is often possible to detect antigen at low levels and when formatted for the multiwell plate ELISA system many samples can be handled.

The objective of this work funded by HDC was to develop a rapid immunological assay which could be used to detect the presence of *B. allii* in onion bulbs at harvest and during storage to facilitate improved store management.

Materials and Methods

Antigen preparation

Two isolates of *B. allii* were used for polyclonal antiserum (PAb) production. Strain 292 066, obtained from the International Mycological Institute (IMI, Egham, Surrey, UK), was grown on Petri plates containing prune, lactose, yeast, streptomycin and erythromycin (PLYSE) medium (Maude, 1963) for 14 days at 20°C. Spores were collected by washing them off the plates with sterile distilled water. Spore suspensions were autoclaved for 15 min at 121°C and freeze dried. Strain DOC-2 was isolated from an onion bulb naturally infected with *B. allii*. Mycelial mats were obtained after growth in Czapek Dox liquid medium for 14 days. Cultures were incubated at 20°C with 6 h orbital shaking at 100 revs / min and 8 h illumination (three 40W Philips cool white fluorescent tubes) each day. The mats were removed from the medium and excess moisture soaked up with filter paper. The mycelium was ground in liquid nitrogen to a fine powder in a cold mortar and pestle, and re-suspended in a small quantity of sterile distilled water. The mycelial suspension was centrifuged at 700 x g for 20 min. The supernatant was re-centrifuged at 25000 x g for 30 min and the liquid phase collected as cytoplasmic antigen. The original pellet containing cell wall was re-suspended in 2 ml of sterile distilled water. Both fractions were freeze dried.

Polyclonal antiserum preparation, sensitivity and specificity

Freeze dried spores (1 mg) and 10 mg of the cell wall and cytoplasmic extracts were each resuspended in 0.5 ml of sterile distilled water. For the initial injection, 0.5 ml of Freund's complete adjuvant was added and for subsequent administration 0.5 ml of incomplete adjuvant was emulsified with the immunogen. Female New Zealand White rabbits were bled to obtain pre-immune serum and each was given seven once-weekly subcutaneous injections either side

of the midline of the back. Each 1 ml dose was divided between two or three injection sites and on each week alternate sides of the animal were used. Three immune bleeds were taken, beginning 4 weeks after the initial injections, and a final bleed at 3 weeks following the last administration of antigen. Sera were separated from compacted clots and stored at -20°C in 250 µl aliquots after adding 0.02% sodium azide.

The sensitivity of the antisera was assessed using an indirect ELISA against the cytoplasmic antigen extract of DOC-2 adjusted to 2.5 µg protein/ml. Between each step the plates were washed with four cycles of 200 µl 0.01 M phosphate buffered saline pH 7.4, containing 0.01% thimerosal (Sigma T-5125) (PBST) with a 60 s soak period between cycles. Paired wells of a flexible 96 well PVC microtitre plate (M29, Dynatech) were loaded with 100 µl of the antigen and incubated at 4°C overnight. Unbound material was removed by washing and the wells were blocked for 1 h with 5% bovine serum prepared in PBST. Polyclonal antisera screened at serial dilutions 1:50 to 1:51200 in PBST were added to the wells (100 µl/well) and the plates agitated for 1 h on a Wellwarm 1 (Denley) microplate shaker. Following a further wash, 100 µl of goat anti-rabbit IgG whole molecule antiserum conjugated to alkaline phosphatase (Sigma A-8025) and adjusted to 0.75 enzyme units/ml, was added and the plate agitated for 1 h. Plates were developed by the addition of 100 µl of substrate containing 1 mg/ml p-nitrophenyl phosphate (Sigma N-2765) in 1 M diethanolamine buffer, pH 9.8. Absorbance values (405 nm) were read after 30 min incubation in the dark using an HT2 microplate reader (Anthos Labtec) with empty wells acting as an instrument blank; the means of the paired wells were calculated. Optimal enzyme concentration for the conjugate and the substrate incubation time had previously been determined from kinetic curves.

In order to determine if the antisera could be used to detect *B. allii* antigen in infected

onions, 1 g samples of diseased tissue from a number of naturally infected bulbs were ground in 3 ml of 0.1 M Tris - glycine buffer pH 8.2 containing 1.6% Triton X - 100 and 7 mg aprotinin (Trasylol 50000 K10, Bayer) (TGTT) centrifuged at 10000 x g for 10 min and the supernatant collected. This buffer has been shown to give high protein yields and reduced degradation of samples during storage (Linfield, 1993). A dilution series of the supernatant from 0 to 1:1024 was prepared in the same buffer and used as antigen in an indirect ELISA. Bulb infection with *B. allii* was confirmed by culture on PLYSE medium for 10 days.

To determine the possibility of non-specific cross-reactivity, PAb 1 and PAb 3 were tested against a range of other onion pathogens and soil-borne fungi. These were grown in Czapek Dox liquid medium amended with 0.5 g/litre of asparagine under the same conditions as described above. Mycelial mats were ground in liquid nitrogen and re-suspended in 0.1 M TGTT buffer. After centrifuging at 50000 x g for 90 min the supernatants were collected and the protein concentration adjusted to 2.5 μg/ml with buffer. Absorbance values were calculated as a percentage of the value for the standard isolate, *B. allii* DOC-2. Results are the means of four assays, using two replicates per assay.

In-vivo antigen detection

Where infection with *B. allii* occurs within a bulb soluble antigenic substances diffuse into the tissue ahead of the mycelial growth. To determine the distance of migration of these antigens, and the ability to detect them using serology, healthy bulbs were quartered longitudinally and the penultimate outer scales used for experimentation. A 10 μl drop of sterile distilled water containing 1 x 10^6 spores/ml was applied to one end of the ellipse of each of 60 scales. Scales were also inoculated with sterile distilled water as controls. After incubation for 24, 48, 72, 96, 168 and 192 h in a moist chamber at 20°C, 10 scales were cut

transversely at sites 1, 2 and 3 cm from the inoculation site. Each segment was weighed and ground in 2.5 x weight/volume of PBS. Two 100 µl aliquots of the homogenate were used in paired wells for indirect ELISA and 6 x 20 µl was inoculated onto PLYSE medium for detecting viable propagules of the fungus.

Bulb tests

A random sample of 90 bulbs from a store where neck rot symptoms had been found was collected. A 1 cm thick transverse section was cut from each bulb 1 cm below the neck and the ring of tissue comprising the outer scale was used for testing. From this ring of tissue six full thickness 3 mm core samples were taken, two for culture on PLYSE medium and the remainder for ELISA. The latter were incubated, unground, overnight at 4°C in wells of a microtitre plate (M129A, Dynatech) containing 100 µl of PBST . Indirect ELISA was carried out using PAb 3 prepared from plate washings of IMI strain 292 066 at a dilution of 1:400.

Field experiment

To determine whether the assay was able to detect the pathogen under field conditions a 0.5 acre split plot was drilled at HRI, Kirton, Lincolnshire. Three beds 1.83 m x 300 m consisting of five rows of onions spaced 30 cm apart were drilled with untreated onion seed cv. Hysam on 30 March 1993, giving an estimated plant population of 60 plants/m^2, with the central bed of five rows acting as an untreated guard. In addition, two beds were planted with infested seed with an untreated guard bed of five rows separating them from each other and the untreated plots. Seed was infested by immersing 250 g of untreated seed in three times its volume of a 3.0 x 10^6 spores/ml suspension of *B. allii* (DOC-2) in sterile distilled water. It was left in a covered flask overnight. Seeds were then removed and dried in a

single layer on a sheet of sterile cellulose wadding in a sterile air flow for 18 h. Beds with untreated and treated seed were randomly split into six plots and, on 14 July (106 days after drilling), to ensure neck rot development in the absence of infection from previously infested seed, three plots of plants from both untreated and infested seed were sprayed with 3 l of 1.5 x 10^4 spores/ml/plot of *B. allii* (DOC-2) in sterile distilled water.

On 14 July prior to treatment and on 23 September (177 days after drilling) prior to lifting, random samples of 50 bulbs from each of the 12 replicate plots were lifted for assessment. The brown outer scales of each onion were removed and a 1 cm section cut through the neck. Half of this section was split into four to six pieces and plated on PLYSE, and the remainder was ground in 1 ml of PBS-A and used in the ELISA. Bulbs were lifted on 23 September, dried and stored according to commercial recommendations (Anon, 1978). Subsequent random samples were taken on 11 Oct (18 days after harvest), 24 Nov (62 days after harvest), 1 Feb (131 days after harvest) and 8 Apr (197 days after harvest) and assessed as before.

Store samples

A press release was issued by HDC inviting growers to provide samples of onions from store for testing. Samples of 100 bulbs were taken from commercial stores at harvest and from onion crops at Kirton and Stockbridge. Five commercial stores were subsequently re-sampled during storage to determine whether the amount of neck rot had increased. Samples of some stores were also removed during the initial drying period and stored outside in ambient conditions to allow for maximum neck rot development. Tissue extracts were prepared from bulbs as previously described and isolations were made onto selective media. Polyclonal antiserum (PAb 4, antigen: plate washings) was prepared as previously described for PAb 3, this was then cross absorbed to remove cross reactivity with *B. cinerea*. Bulbs were tested

for the presence of *B. allii* antigen by indirect ELISA and comparisons made with culture results.

Results

All three antisera recognised DOC-2 antigen and gave absorbance values > 2.0 when tested in indirect ELISA. The highest antiserum titre was shown by PAb 3, raised to plate washings, followed by PAb 1 (cell wall) and PAb 2 (cytoplasm). The dilution series of infected bulb tissue showed that antibody/antigen recognition was possible even at high dilution of the infected tissue (Appendix Fig. 1). Pab 3 again gave the best response, with PAb 1, giving similar results. Recognition of *B. allii* infected onion tissue was lowest with PAb 2, and this antibody was not used in further studies.

Cross-reactivity tests (Table 1) indicate high specificity of PAb 1 and PAb 3 to *B. allii*, although both showed some cross-reactivity with *B. cinerea* (absorbance values of 30% and 65% of the standard isolate respectively). Absorbance values for isolate B4065, which proved non-pathogenic in bulb assays, were 10% and 9% of the standard isolate, similar to those for *B. byssoidea*, *B. porri* and *B. squamosa*.

In-vivo antigen detection

The detection of diffused antigen in bulb tissue and a comparison with recovery of viable propagules is shown in Table 2. Elevated ELISA absorbance values in the absence of growth of *B. allii* were obtained from four scale slices, and also from three slices where not all of the six drops of tissue homogenate applied to PLYSE medium yielded *B. allii*. In all instances where growth of *B. allii* occurred in all six drops, the absorbance values were above the negative cut-off level. The results show that the detection of the fungus within tissue by ELISA is possible before the fungus can be cultured.

Table 1. Reaction of two antisera with nineteen fungal isolates in indirect ELISA.

Identity	No.	Source	Host	Year	% Absorbance[a]	
					PAb 1	PAb 3
Botrytis allii	DOC2[b]	UK	Onion	1992	100.0	100.0
Botrytis allii	IMI 147 186[c]	UK	Onion	1970	66.5	56.9
Botrytis allii	IMI 292 066[c]	UK	Onion	1985	62.3	85.5
Botrytis allii	B4021[b]	Denmark	Onion	1987	83.9	90.9
Botrytis allii[d]	B4065[b]	UK	Onion	1988	9.7	8.6
Botrytis allii	B4068[b]	UK	Onion	1988	82.3	87.3
Botrytis allii	B4090[b]	UK	Shallot	1988	97.5	76.8
Botrytis byssoidea	B3726[b]	UK	Leek	1984	14.5	10.5
Botrytis byssoidea	B3730[b]	UK	Leek	1984	4.0	8.6
Botrytis cinerea	B1113[b]	UK	Salad onion	1978	30.6	65.0
Botrytis cinerea	B3506[b]	UK	Salad onion	1981	37.9	65.9
Botrytis porri	B3715[b]	UK	Leek	1984	12.1	14.1
Botrytis squamosa	B3808[b]	UK	Onion	1984	8.1	7.7
Botrytis squamosa	B3834[b]	UK	Onion	1985	12.9	14.2
Corticium rolfsii	IMI 328 618[c]	India	Mahogany	1988	4.8	11.4
Fusarium oxysporum f. sp. *narcissi*	80/26[b]	UK	Narcissus	1980	7.7	9.1
Penicillium sp.	93/07[b]	Mexico	Garlic	1992	2.6	10.0
Sclerotium cepivorum	9181[b]	UK	Onion	1981	13.1	10.0
Sclerotium cepivorum	16675[b]	UK	Onion	1981	27.5	27.7

[a] Plates were coated with mycelial extracts at 2.5 µg protein/ml and antiserum diluted 1:800. Absorbance values expressed as a percentage of standard isolate *B. allii* DOC2.

Isolates obtained from: [b] HRI Wellesbourne; [c] International Mycological Institute, UK

[d] Non pathogenic in bulb tests

Table 2. Determination of the distance diffusible antigen could be detected in advance of mycelial growth[a] on three onion scales.

	Incubation time (h)					
Site[b]	24	48	72	96	168	192
1	0.619	0.603 (6)	1.016 (6)	1.307 (6)	1.622 (6)	1.067 (6)
2	0.243	0.327	0.414 (2)	0.668 (6)	1.396 (6)	2.038 (6)
3	0.299	0.320	0.281	0.411	1.030 (6)	1.856 (6)
	Negative cut-off[c] = 0.355					
1	0.571 (6)	1.471 (6)	1.063 (6)	1.374 (6)	1.814 (6)	1.839 (6)
2	0.274	0.255	0.210	0.761 (1)	0.527 (6)	1.976 (6)
3	0.326	0.357	0.313	0.340	0.338	2.057 (6)
	Negative cut-off[c] = 0.391					
1	0.503 (6)	0.475 (6)	0.565 (6)	0.970 (6)	1.770 (6)	1.797 (6)
2	0.274	0.297	0.390	0.582 (6)	1.281 (6)	2.077 (6)
3	0.311	0.290	0.295	0.547	0.384 (2)	1.952 (6)
	Negative cut-off[c] = 0.343					

[a] Values are expressed as absorbance at 405 nm and the figures within brackets indicate how many of six drops inoculated onto PLYSE medium produced growth.

[b] Distance from inoculation point (cm)

[c] Determined as the mean + twice the standard deviation of the absorbance values obtained from healthy control tissue

Bulb tests

Of the sample of stored bulbs, 87 produced no growth and ELISA absorbance values were below the negative cut-off point obtained from healthy tissue. Three bulbs gave positive ELISA results following substrate addition for 1 h, although the range of values obtained from the four core samples examined was wide. These were: 1.512, 0.065, 1.010, 0.773 for Bulb A; 0.371, 1.590, 1.183, 0.782 for Bulb B; and 0.548, 0.290, 1.295, 1.091 for Bulb C. *B. allii* was isolated and identified morphologically from the same three bulbs following incubation for 7 days on PLYSE medium. The identification was confirmed by pathogenicity tests.

Field experiment

The results of the field trial (Table 3) indicate that there was a dramatic rise over time in the number of occasions when the fungus could be isolated from stored onions, the later samples indicating substantially increased disease levels compared with those taken at harvest. In contrast, the ELISA results showed a high level of infection in the early samples which was maintained during later sampling. The amount of neck rot found at the end of storage on 8 April could be determined by ELISA at or within 3 weeks of harvest, whilst attempted isolations only gave these results following sampling 131 days after harvest. Infected seed alone gave very low levels of neck rot infection (Appendix Fig. 2), and it was only where spores had been applied as a spray that high levels of infection occurred.

Table 3. Percentage of neck rot found in onion bulbs before and at harvest and following storage. 150 bulbs tested per treatment by culturing and ELISA on each sampling date.

Sampling date in 1993/94	Untreated seed			
	Plants inoculated[a]		Plants not inoculated	
	Culture	ELISA	Culture	ELISA
14 Jul	0	0	0	0
23 Sep	30	60	0	10
11 Oct	41	65	2.5	10
24 Nov	37	55	5.0	10
1 Feb	70	75	7.5	12
8 Apr	80	80	12.5	15
	Infested seed			
14 Jul	0	0	0	0
23 Sep	35	70	10	20
11 Oct	47	65	10	20
24 Nov	49	65	10	21
1 Feb	75	80	22	25
8 Apr	80	80	20	30

[a] *B. allii* applied as 3 l of 1.5 x 10^4 spores/ml over an area of 1.83 m x 300 m

Store samples

The response from the HDC press release requesting samples was disappointing and consequently additional samples were taken from Kirton and Stockbridge to provide adequate materials. The results in Table 4 show that the ELISA detected a higher level of neck rot in the store than culturing. In two stores, I and M there was an apparent increase in % neck rot shown by the ELISA in the November sample that had not been predicted earlier. This result is further confused as the visible rots in trays was recorded as 18% compared with 7% (ELISA test) for store I and 2% in trays compared with 8% in the ELISA for store M. Results for the ELISA's for each of the commercial stores are given in the Appendix. Results for samples from Kirton and Stockbridge again indicated that low levels of neck rot (< 5%) could be readily detected by the ELISA and that these were often missed or appeared considerably lower in culture.

Table 4. Percentage of neck rot found in commercial store samples. 100 bulbs tested per store by culturing and ELISA.

Store	At harvest		Post storage	Tray storage*
	Culture	ELISA	ELISA	Visual
A	1	3	-	-
B	1	2	-	-
C	1	1	-	-
D	1	2	-	-
E	0	0	-	-
F	0	1	-	-
G	0	2	-	-
H	0	0	2	1
I	0	1	7	18
J	1	1	0	3
K	3	3	-	-
L	10	15	10	10
M	0	1	8	2
N	5	-	8	-
O	4	-	7	-
P	0	-	2	-
Q	0	-	0	-
R	8	-	10	-

* Visual assessment by D. O'Connor & Associates, 10 weeks post storage. These results are from samples which did not receive a full standard storage regime. These samples were taken from storage during the initial drying period and stored at ambient.

Conclusions

The results obtained in this study showed that two of the polyclonal antisera raised to fractions of *B. allii* detect the pathogen *in vivo* and *in vitro* and have clear potential for the rapid detection of infection in stored onion bulbs. An advantage of serological assays over conventional culture procedures is often the ability to detect antigenic substances that have been produced either as metabolites or following the liberation of cell contents after outer membrane lysis. In this work it was found that the ELISA could detect soluble antigenic material diffusing through apparently healthy tissue in advance of the infection (Linfield *et al.*, 1995). This overcomes the problem of conventional plate isolation where tissue containing the fungus as spores or mycelium is needed before culture is possible. The results from in-store sampling indicate that the progression of the fungus around the neck of the bulb may not be uniform in the early stages of infection. These results confirm the requirement for multi-site sampling from individual bulbs.

Screening of a range of isolates of *B.allii*, other *Botrytis* species and non-*Botrytis* fungi in an ELISA indicate that there is little cross reactivity with most *Botrytis* species or other onion pathogens, although there is recognition of *B. cinerea*. Results from Western blots have shown the areas of serological similarity between *B. allii* and *B. cinerea* but by cross-absorption these can be removed to provide a specific PAb to *B. allii*.

The results from the field experiment suggest that the ELISA is more sensitive in detecting the fungus than conventional isolation procedures. Levels of infection were determined earlier with the ELISA than with isolation although at the end of storage, results were similar for both methods. The low levels of neck rot found with artificially infested seed alone compared with plots sprayed with spores were perhaps surprising as earlier work

had suggested that seed infection is the most common cause of infection. Maude *et al.*, (1986) found that the most consistent relationship between weather conditions and disease incidence was that between log % neck rot and relative humidity towards the end of June. Relative humidities in excess of 85% over a period of 7 years between 1973-1980 were correlated ($R = 0.97$, $p<0.05$) with a high incidence of neck rot in the stored crop. Thus under wet early summer conditions seeds carrying similar levels of infection gave higher numbers of neck rot infected bulbs in store than those grown in drier summers. During June 1993 there were only 4 days on which the RH was 85% or greater and none of these was towards the latter part of the month. This suggests that conditions may not have been ideal for infection from seed. The results suggest that field debris, waste from cold stores and other spore sources may be more important in some years than seed contamination.

The results from the commercial stores and samples at Kirton and Stockbridge showed that low levels of neck rot could be readily detected in commercial samples. The amount of neck rot in the stores varied from 0 - 15% with most stores having 1-3% neck rot. Although visual assessments were made 10 weeks post harvest these were difficult to link with the culture and ELISA results because the samples were taken from storage during the initial drying period and stored outside in ambient conditions. These samples consequently had not received a full standard storage regime and therefore neck rot levels may be some what higher than those receiving full storage regimes. The ELISA developed is clearly able to detect *B. allii* antigen in advance of symptoms and is considerably more sensitive than isolation methods. In most instances during this work the test was able to predict the level of neck rot that would develop in a store. In two of the stores although the predicted level was lower at storage than the level found by ELISA several weeks post storage. In one of the stores the observed level was similar to the harvest ELISA result and the higher post storage ELISA

reading may have been due to an unusually high number of infected bulbs in the sample biasing the result. These problems highlight the importance of sampling the bulbs at the correct time and the problems of sample size with respect to the number of bulbs in a store.

This aspect of the work is now being continued in a MAFF funded programme looking at sample size and time of sampling together with combining the ELISA for *B. allii* with that for *Pseudomonas gladioli* pv. *alliicola*. The procedure for detection of both pathogens uses an indirect ELISA and can be run simultaneously on the same bulb sample. It is envisaged that the test will be formatted as a kit which could be utilised by crop consultants or large growers.

Glossary

Antigenic:-	The property of any substance (antigen) that will invoke an immune response in mammals with subsequent production of antibodies. An antigen may have many antigenic sites.
Antiserum:-	The blood product of an animal that contains antibodies.
ELISA:-	(Enzyme-linked Immunosorbent Assay) A sensitive test that uses antibodies to detect antigen which its indicated by a colour reaction.
Polyclonal Antisera:-	(PAB) Antisera containing a variety of antibodies produced in response to different antigenic sites on an antigen.
Serological:-	Any test or immune response associated with antibodies.

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Appendices

Fig. 1. Antisera (▲ PAb 1, cell wall; ● PAb 2, cytoplasm and ■ PAb 3, plate washing) tested for sensitivity by indirect ELISA against extract of infected onion tissue. Extract prepared by grinding 1 g tissue in 3 ml PBS-A buffer. The dotted line shows the negative cut-off, calculated as mean + twice the standard deviation of healthy tissue.

Fig. 2. Comparison of indirect ELISA results on onion bulb samples taken at the end of storage. Untreated seed, plants not inoculated (A); untreated seed, plants inoculated (B); infested seed, plants not inoculated (C); and infested seed, plants inoculated (D). Inoculated plants were sprayed with 3 l of 1.5 x 10^4 spores/ml/plot on 14 July. Values are the means of two wells for each sample; a positive test is indicated by $P < 0.05 = 0.632$.

Figs. A-R. Absorbance values for 100 bulbs from each of the commercial stores tested. Values are the means of two wells for each sample. Values above the cut off line are +ve for neck rot.

Fig. 1

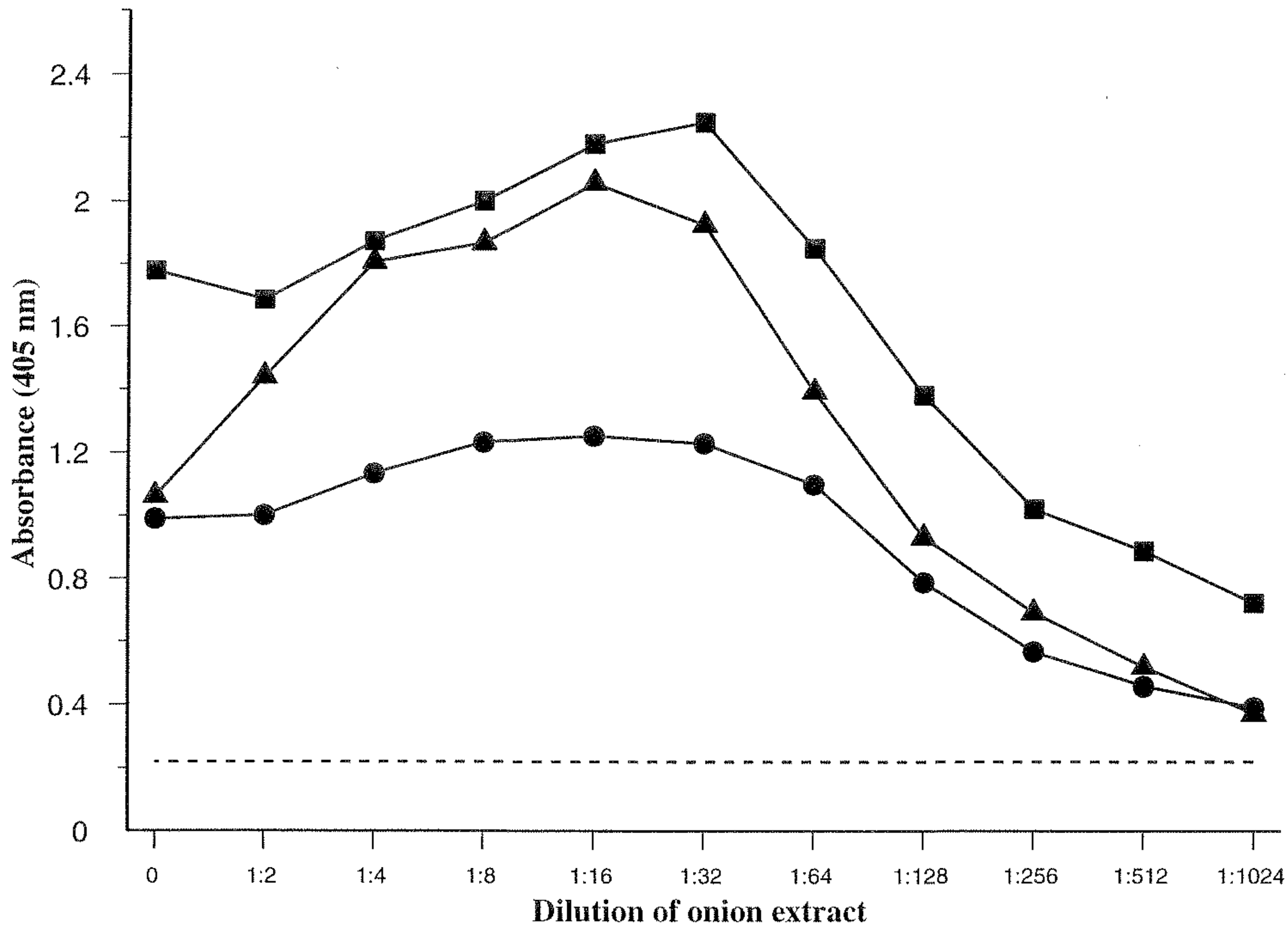
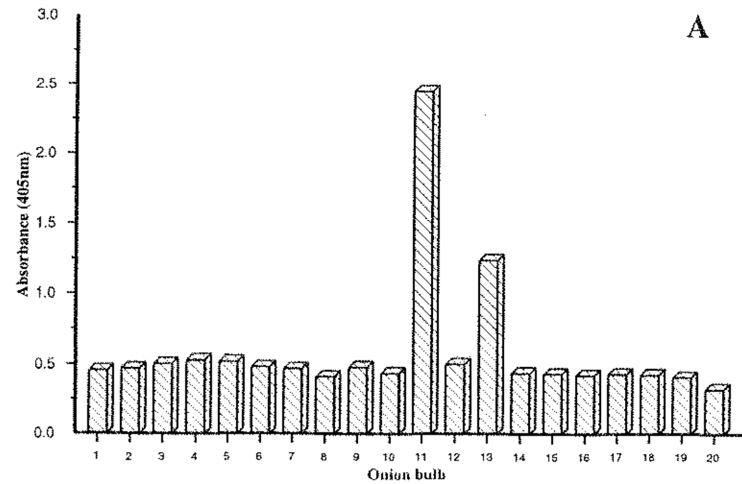
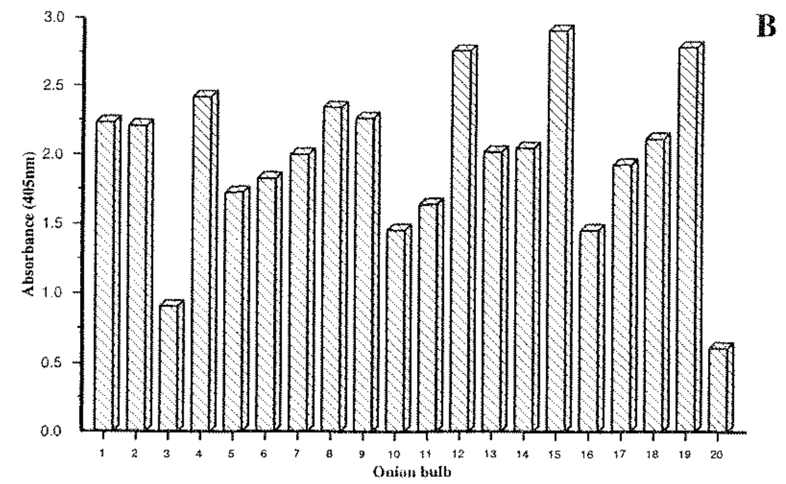
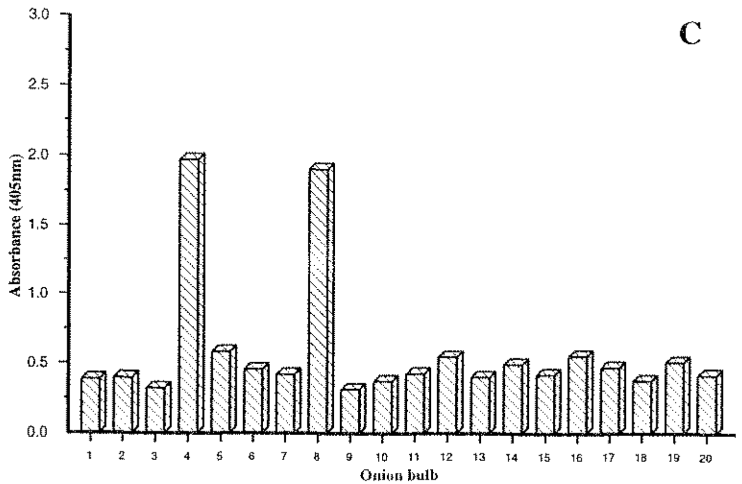
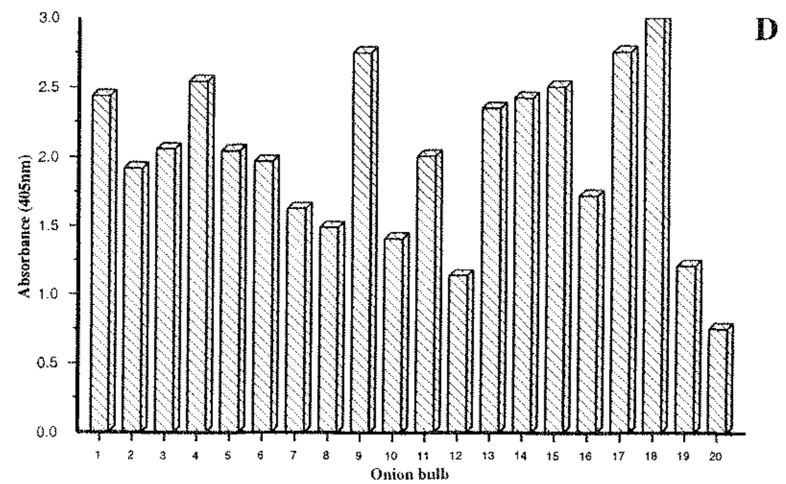


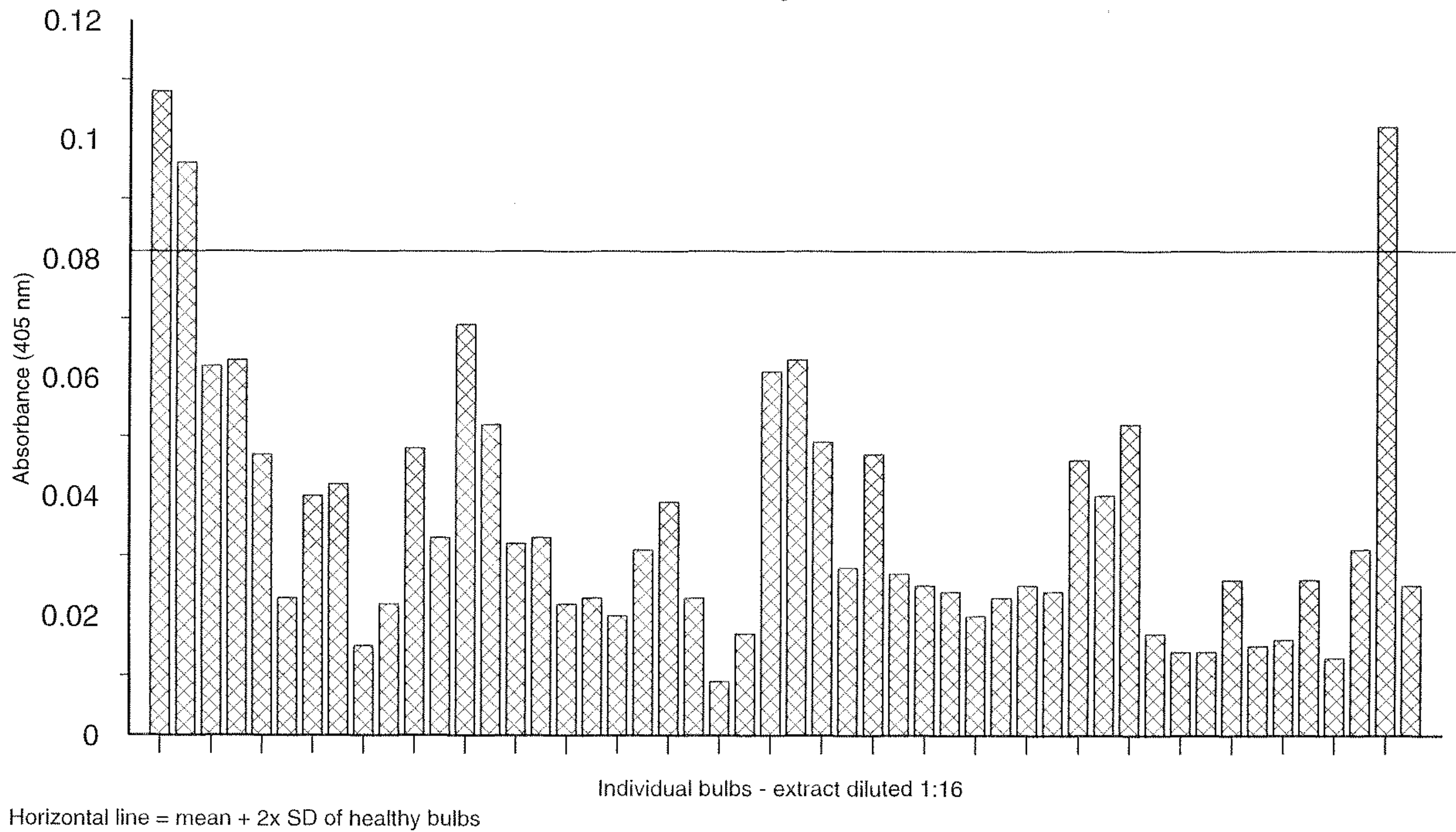
Fig. 2



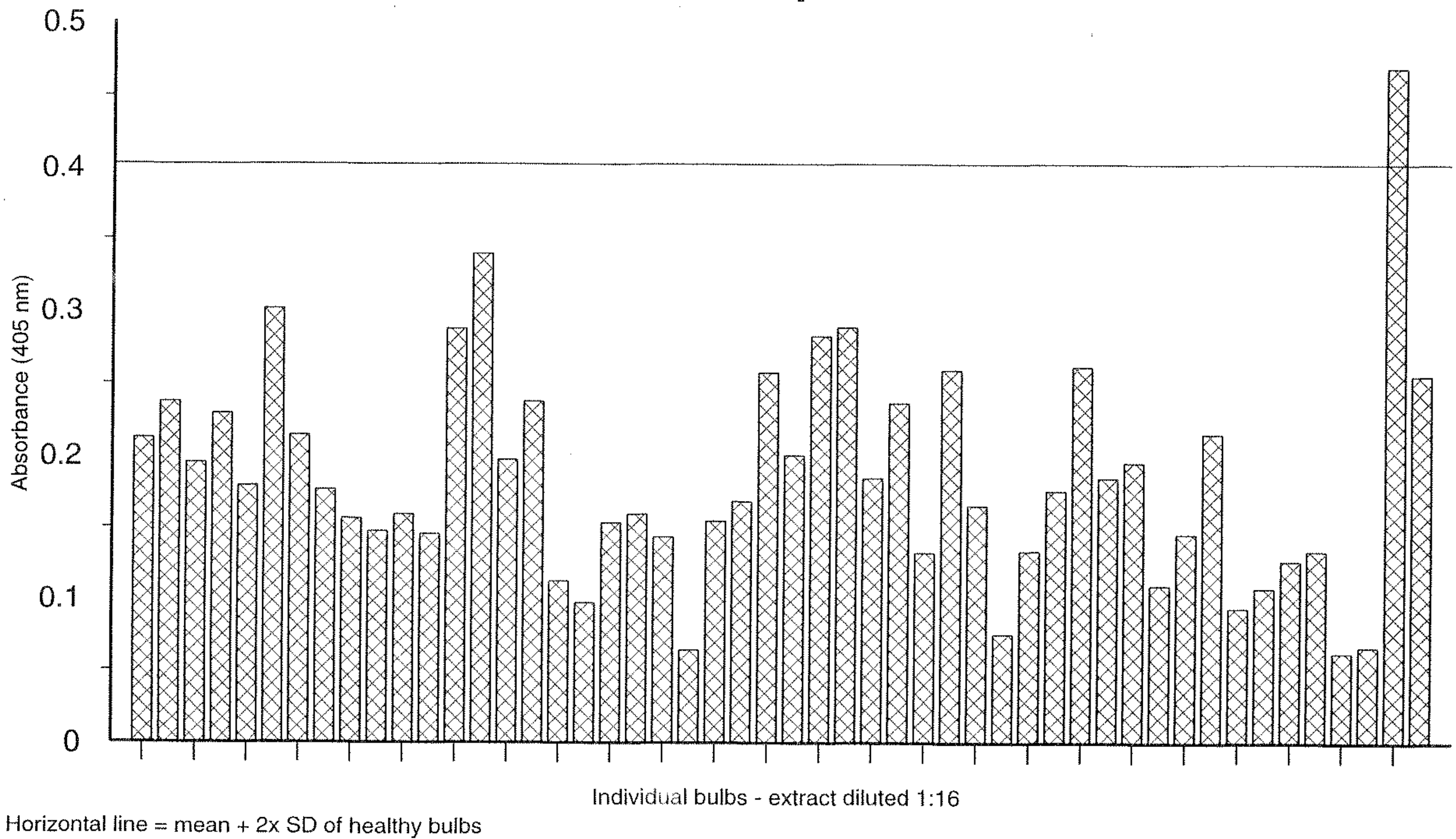


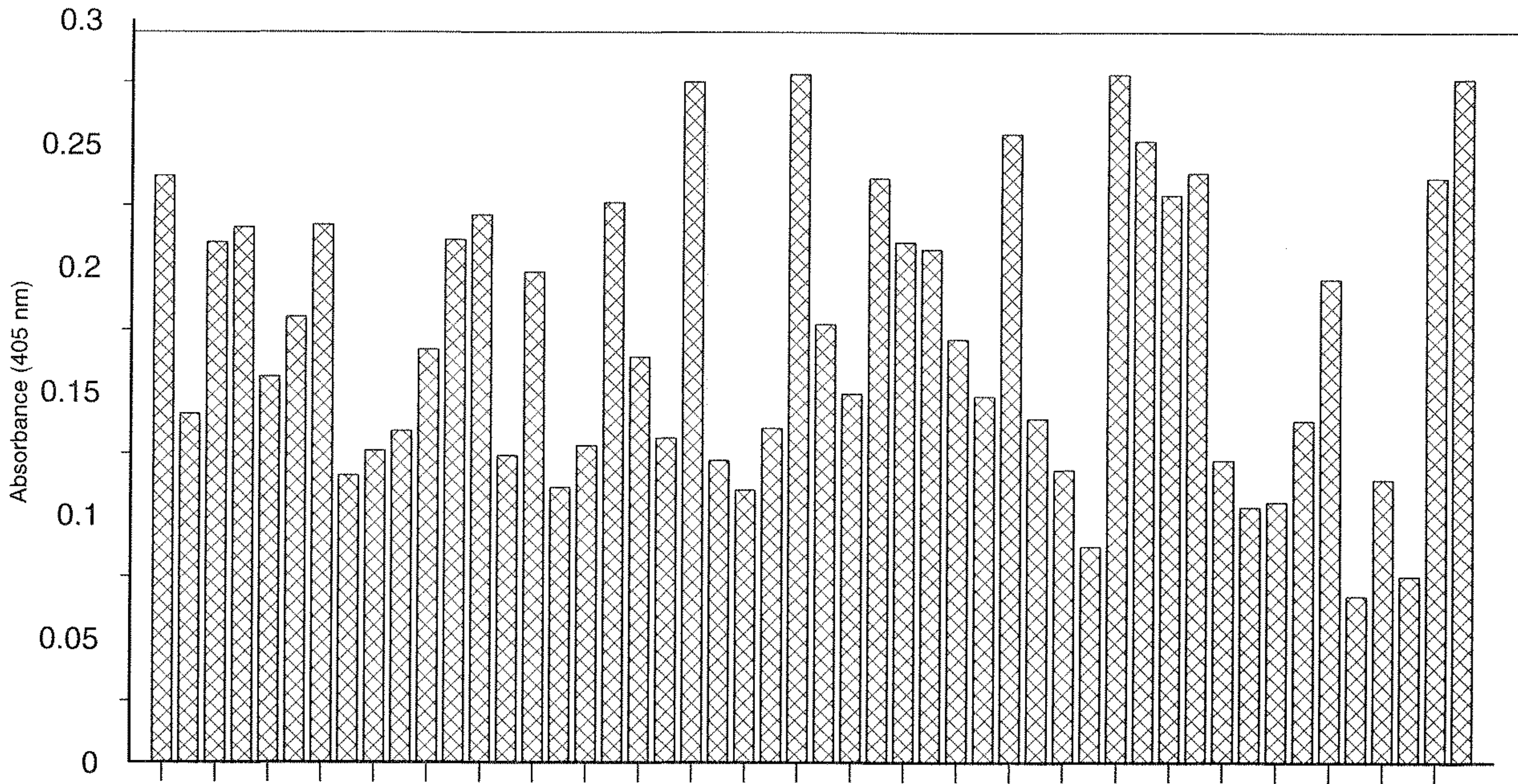


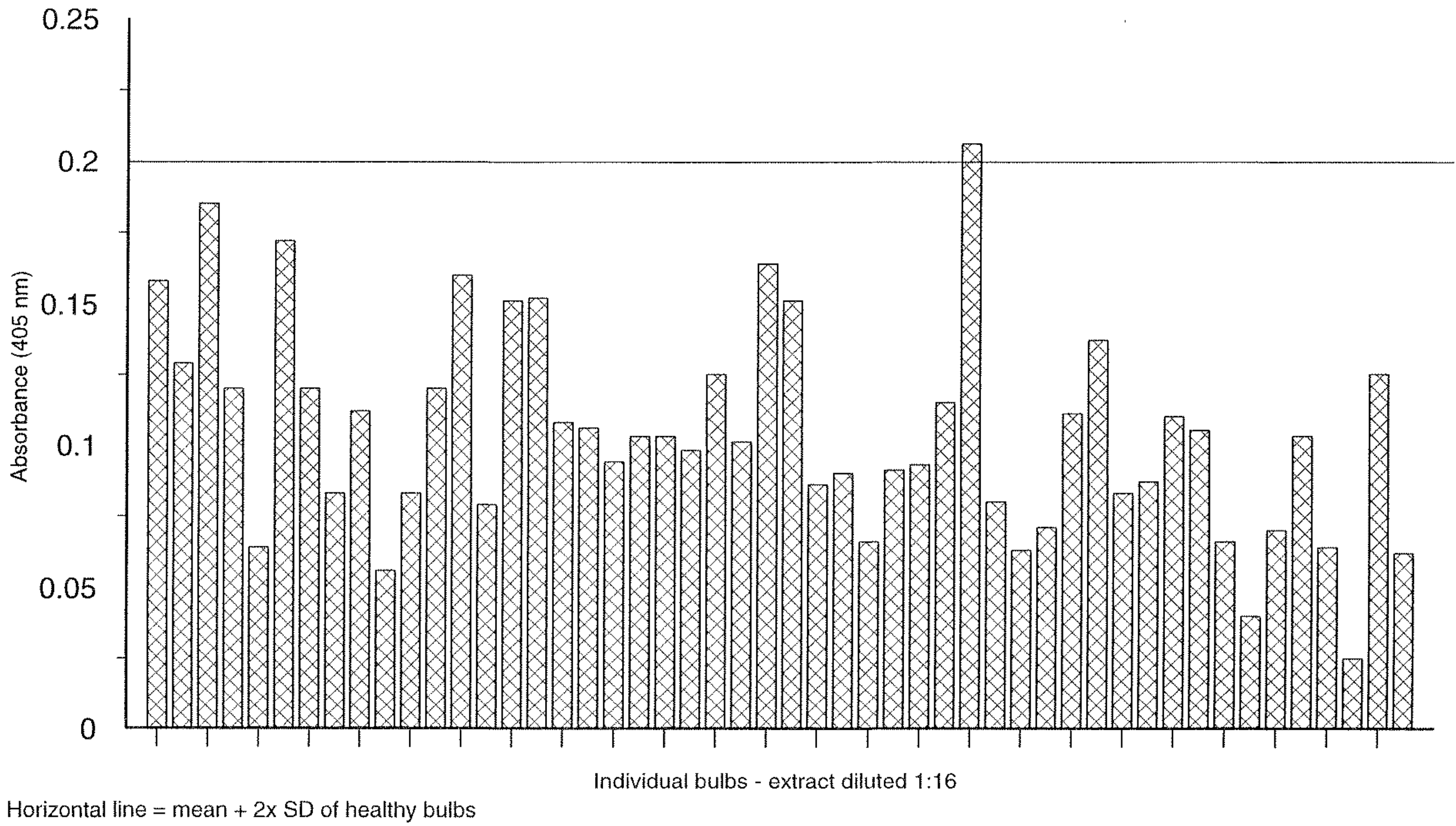


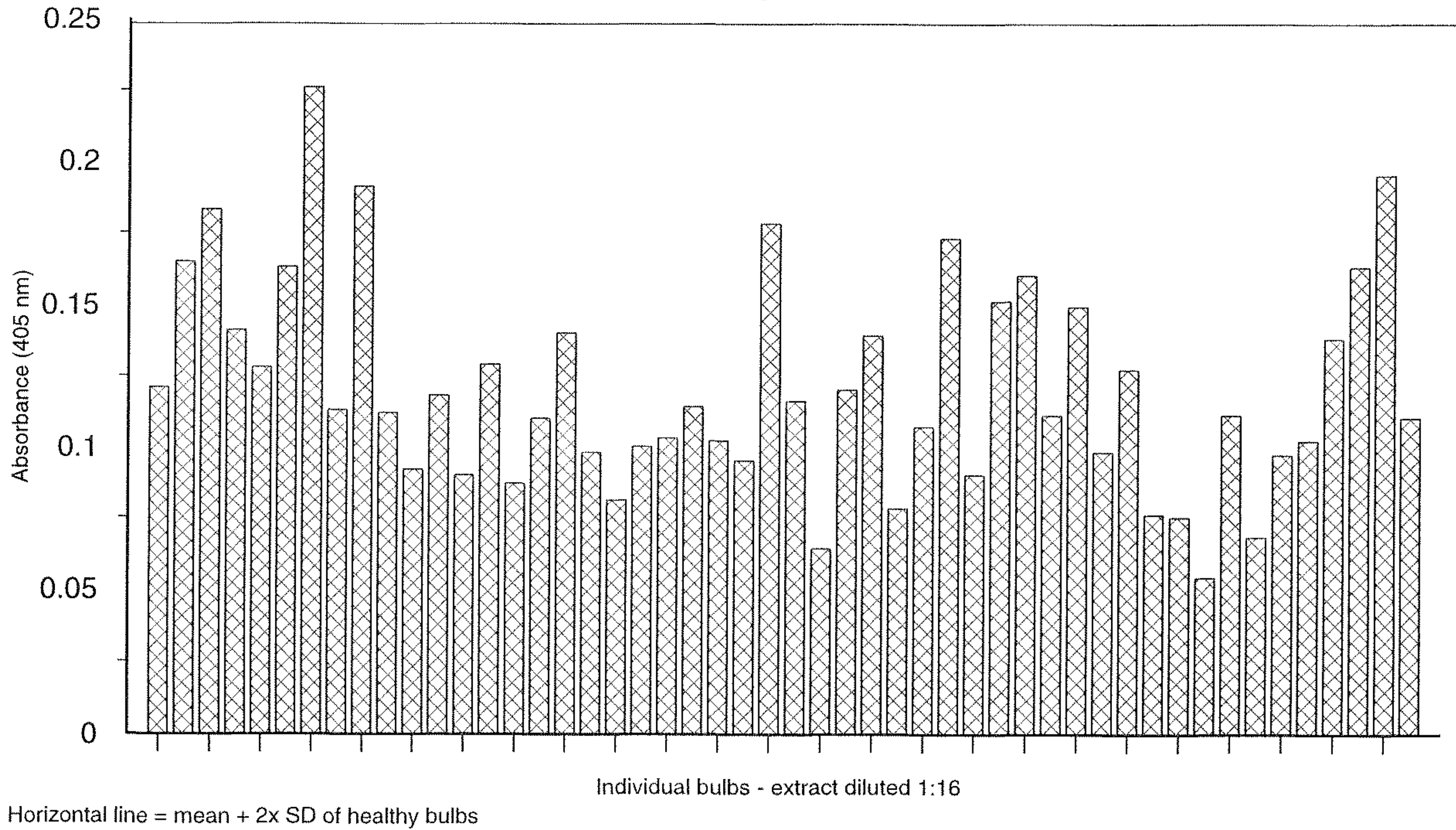


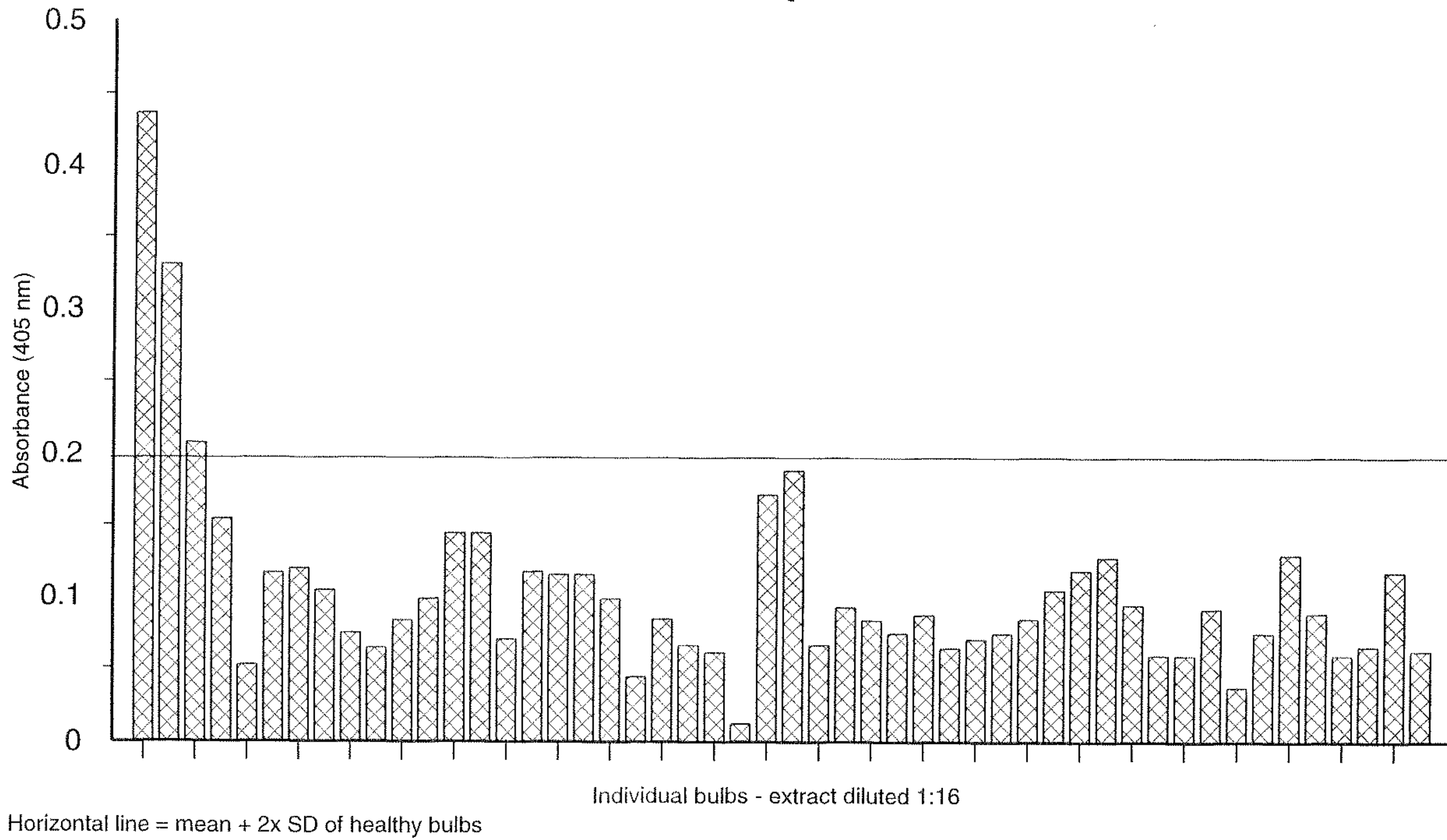
HDCFV134 Detection of latent Botrytis allii
Commercial sample A - 1-50
Absorbance (405 nm)
0.12
0.1
0.08
0.06
0.04
0.02
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

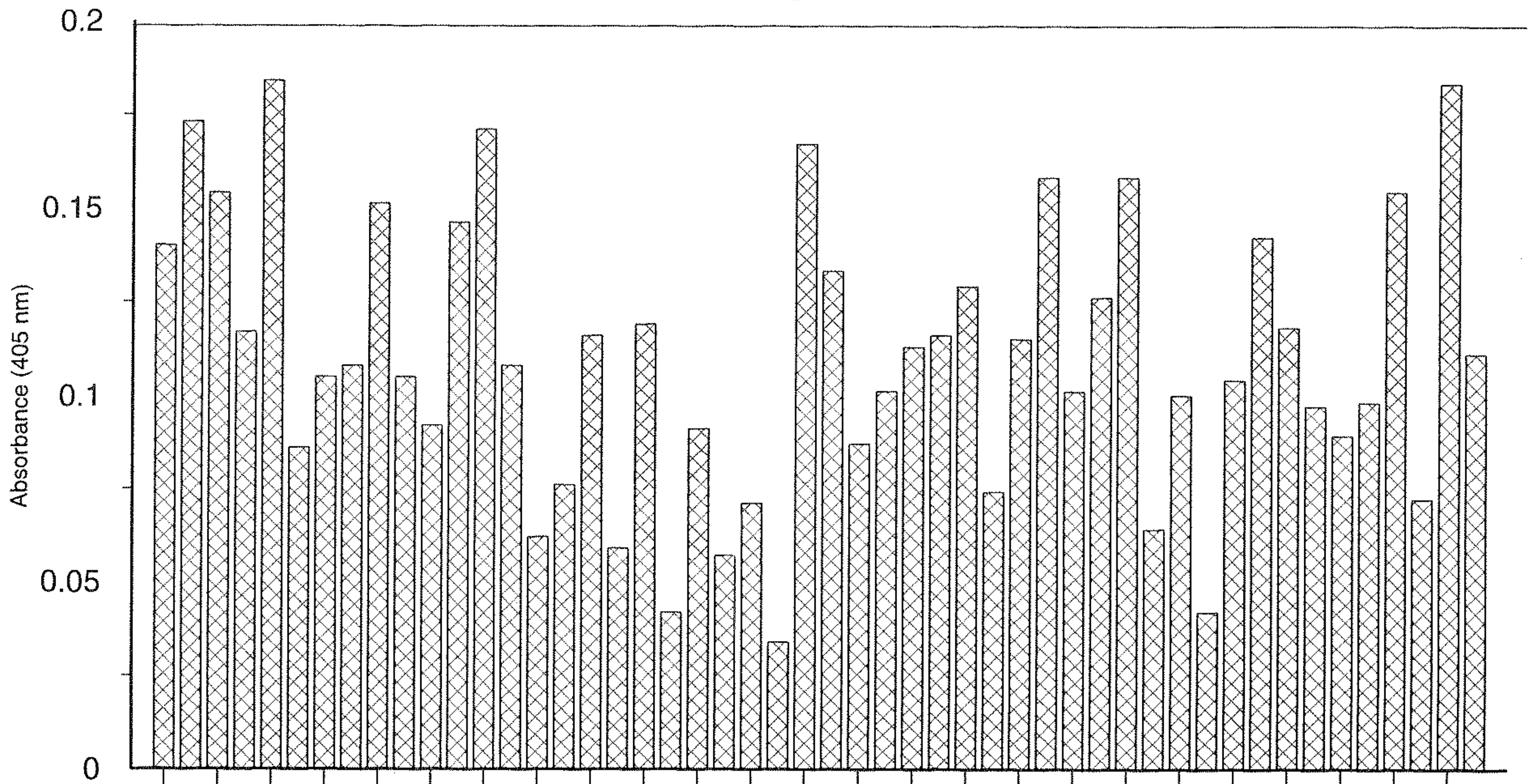

HDCFV134 Detection of latent Botrytis allii
Commercial sample I - 1-50
Absorbance (405 nm)
0.5
0.4
0.3
0.2
0.1
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample I - 51-100
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

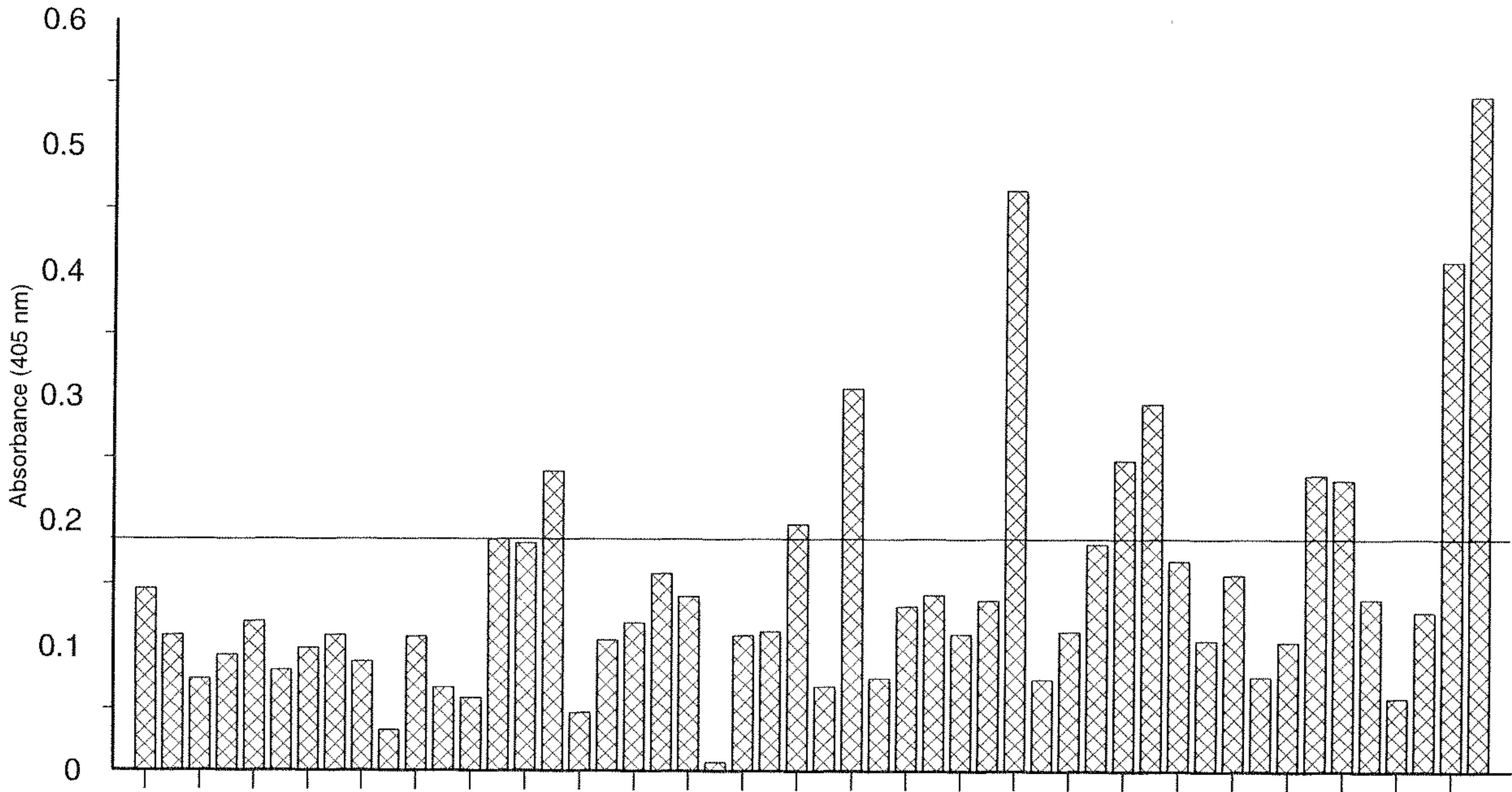

HDCFV134 Detection of latent Botrytis alliii
Commercial sample J - 1-50
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

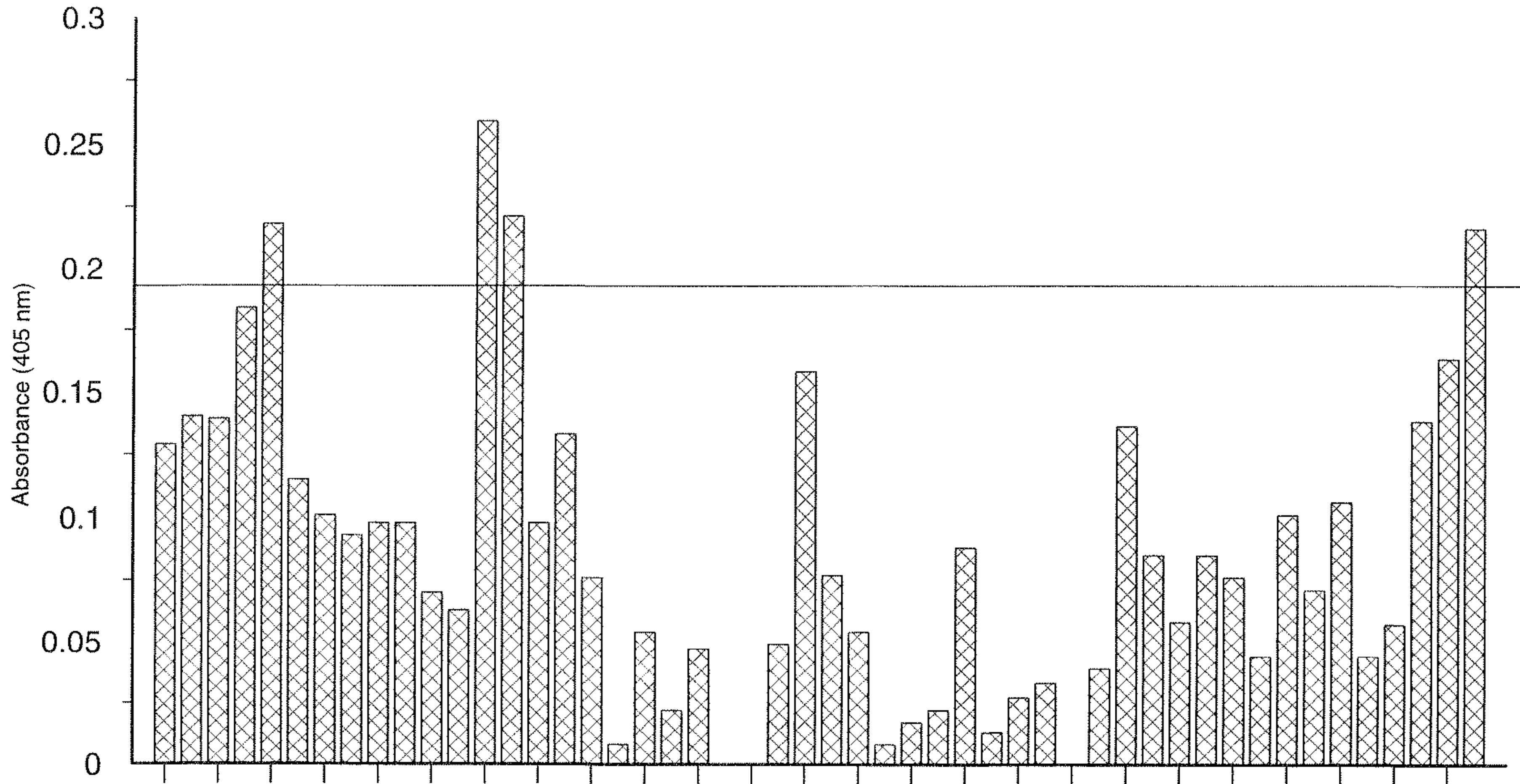

HDCFV134 Detection of latent Botrytis allii
Commercial sample J - 51-100
Absorbance (405 nm)
0.25
0.2
0.15
0.1
0.05
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

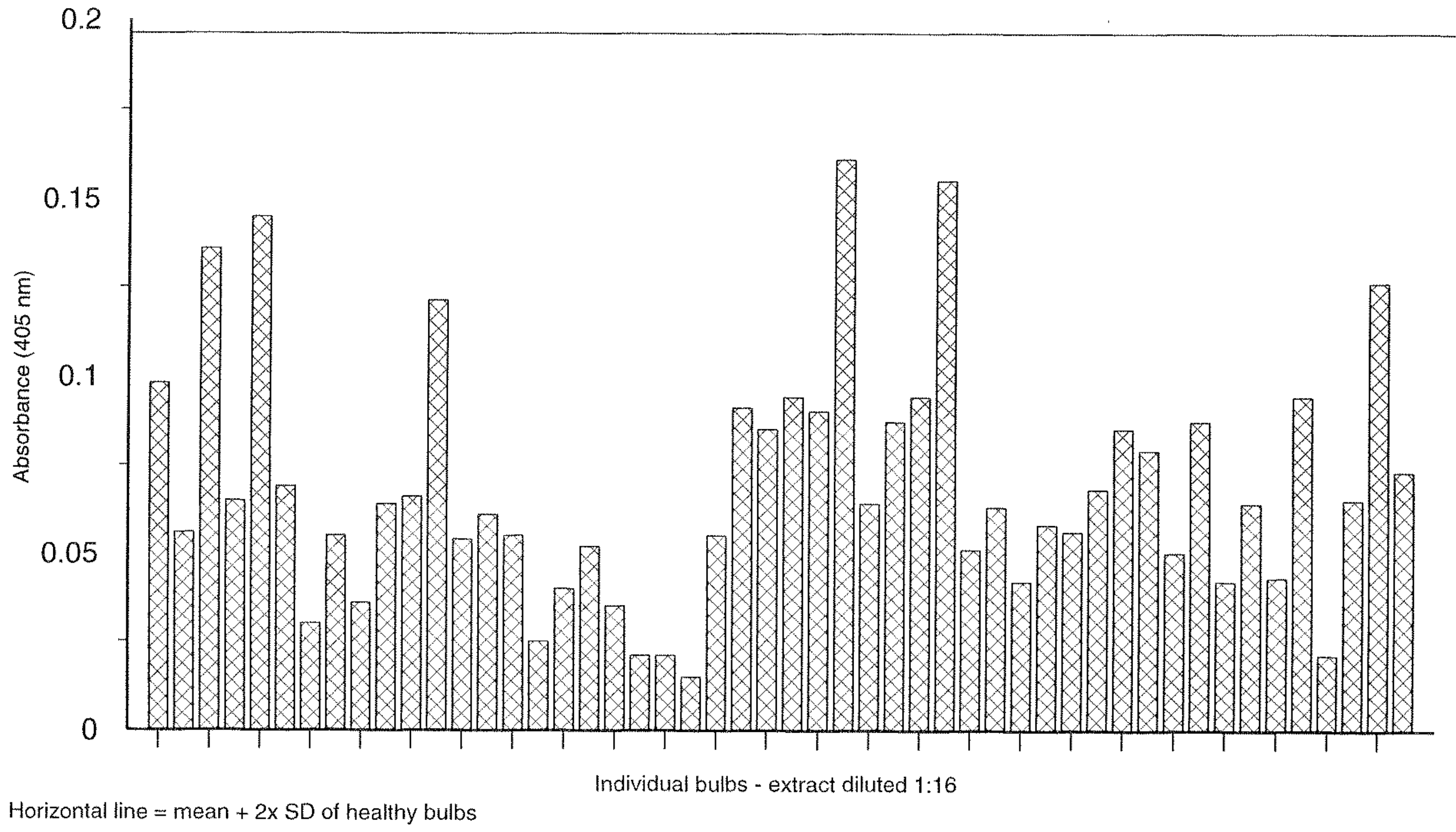

HDCFV134 Detection of latent Botrytis allii
Commercial sample K - 1-50
Absorbance (405 nm)
0.5
0.4
0.3
0.2
0.1
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

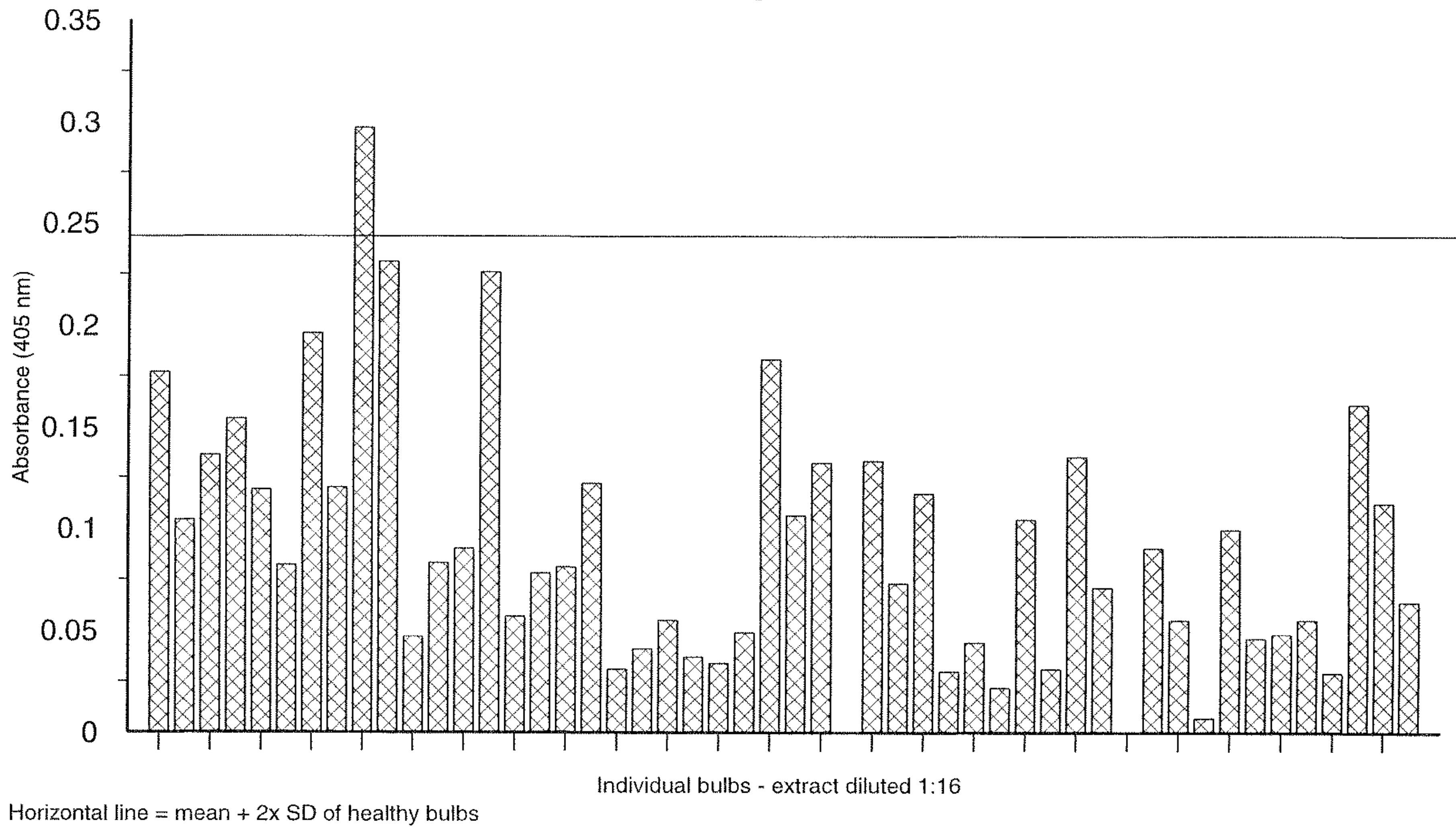


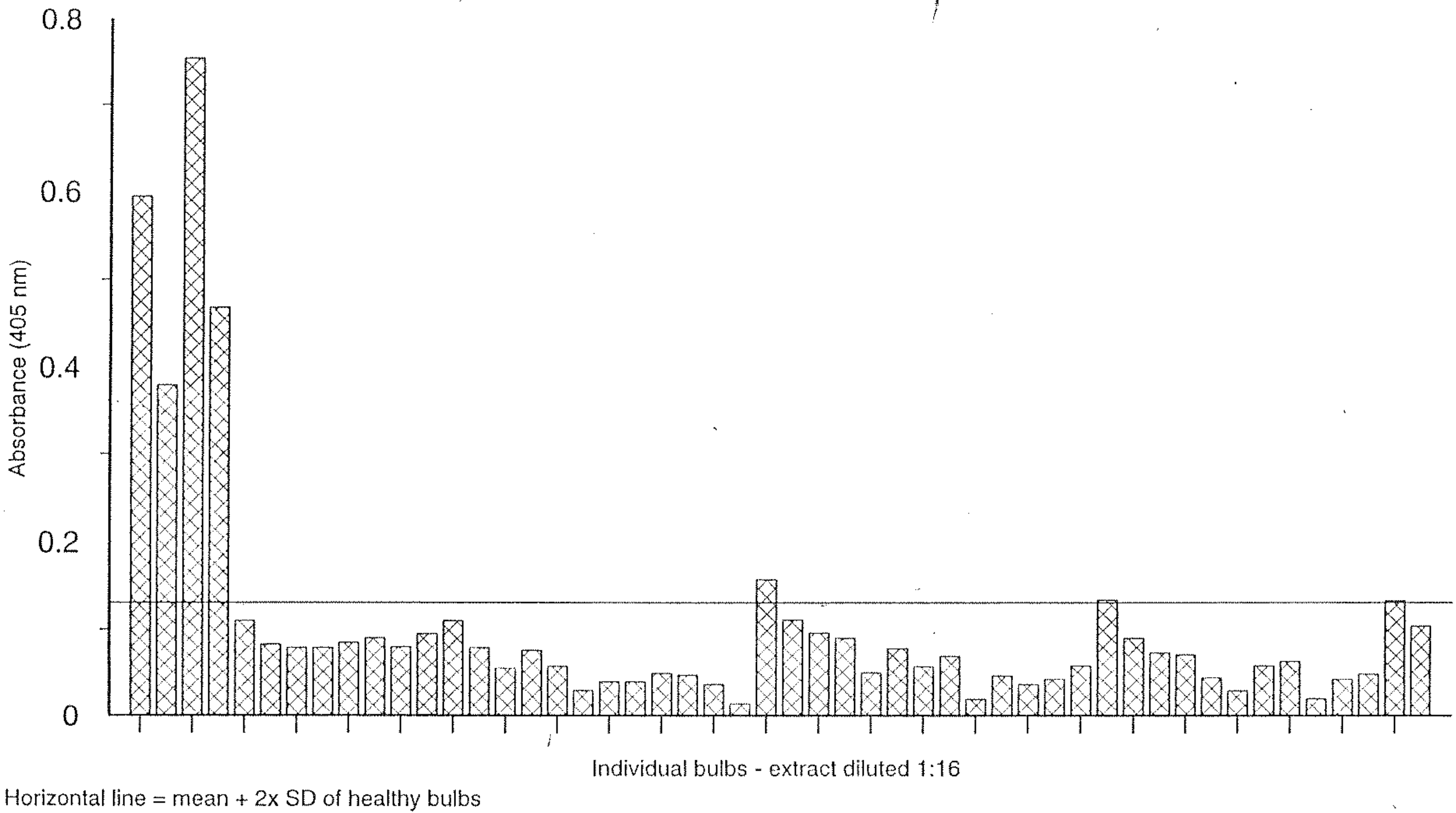
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample L - 1-50
Absorbance (405 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample L - 51-100
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample M - 1-50
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis alliii
Commercial sample M - 51-100
Absorbance (405 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample N - 1-50
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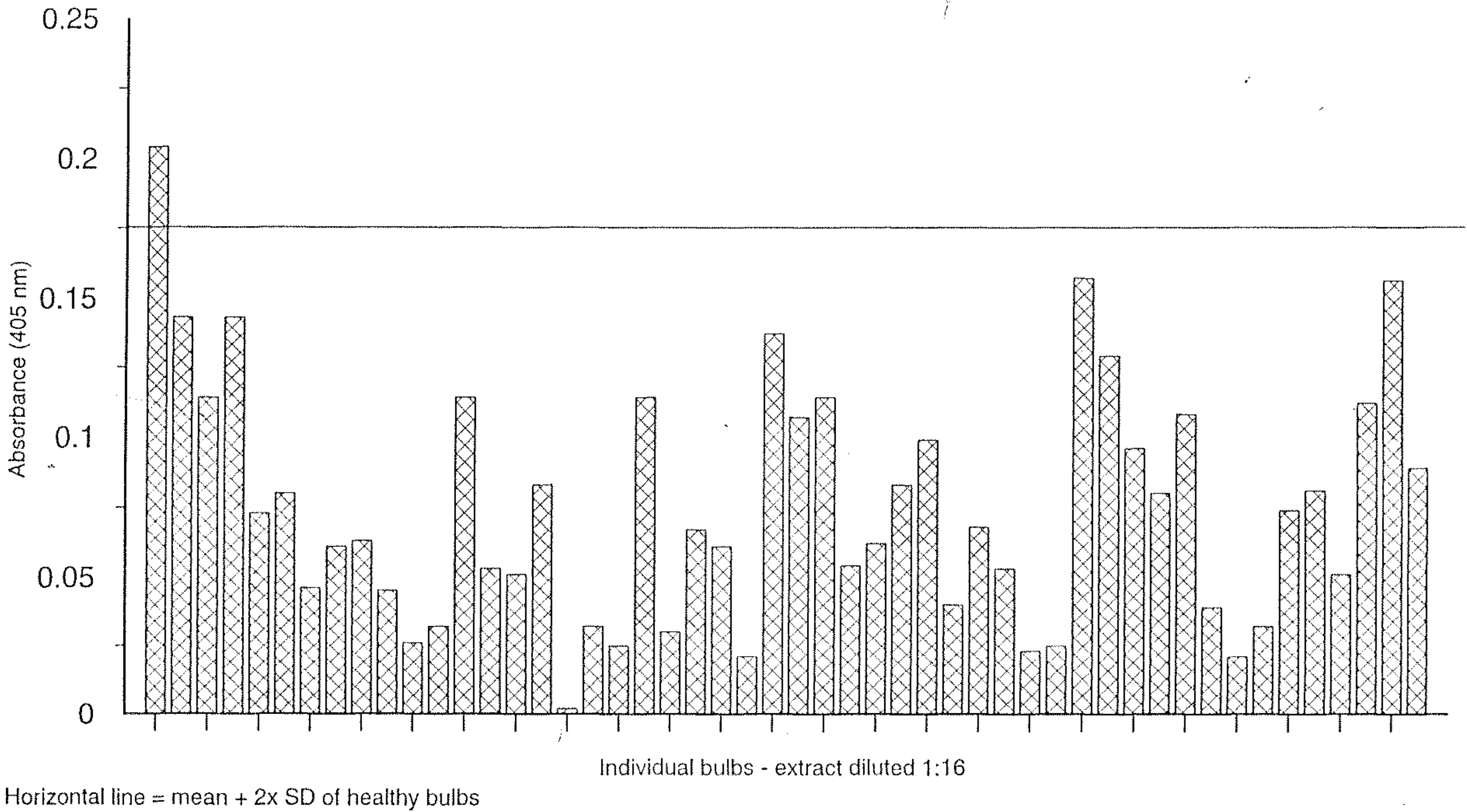
0.4
0.2
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample N - 51-100
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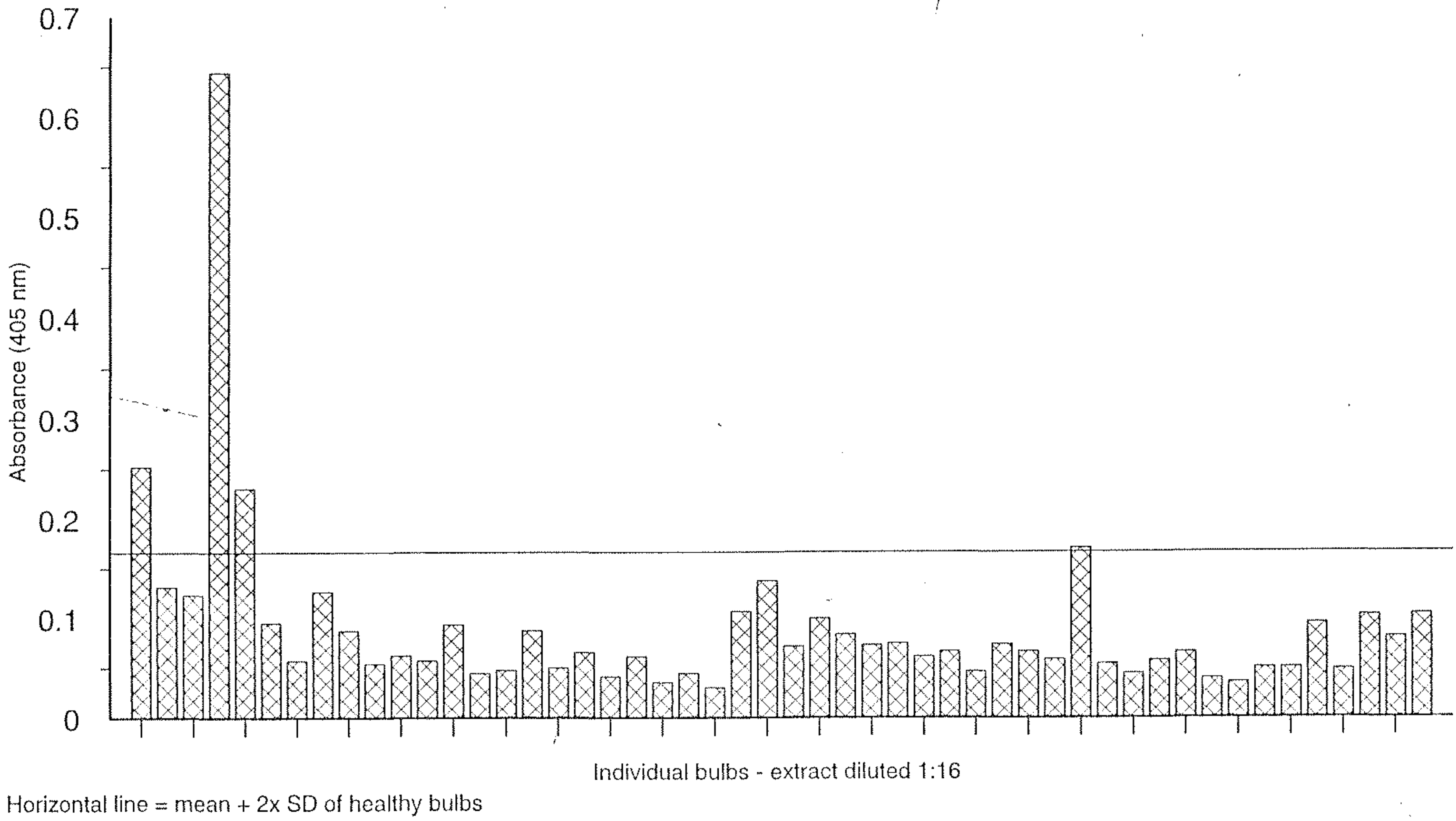
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample O - 1-50
Absorbance (405 nm)
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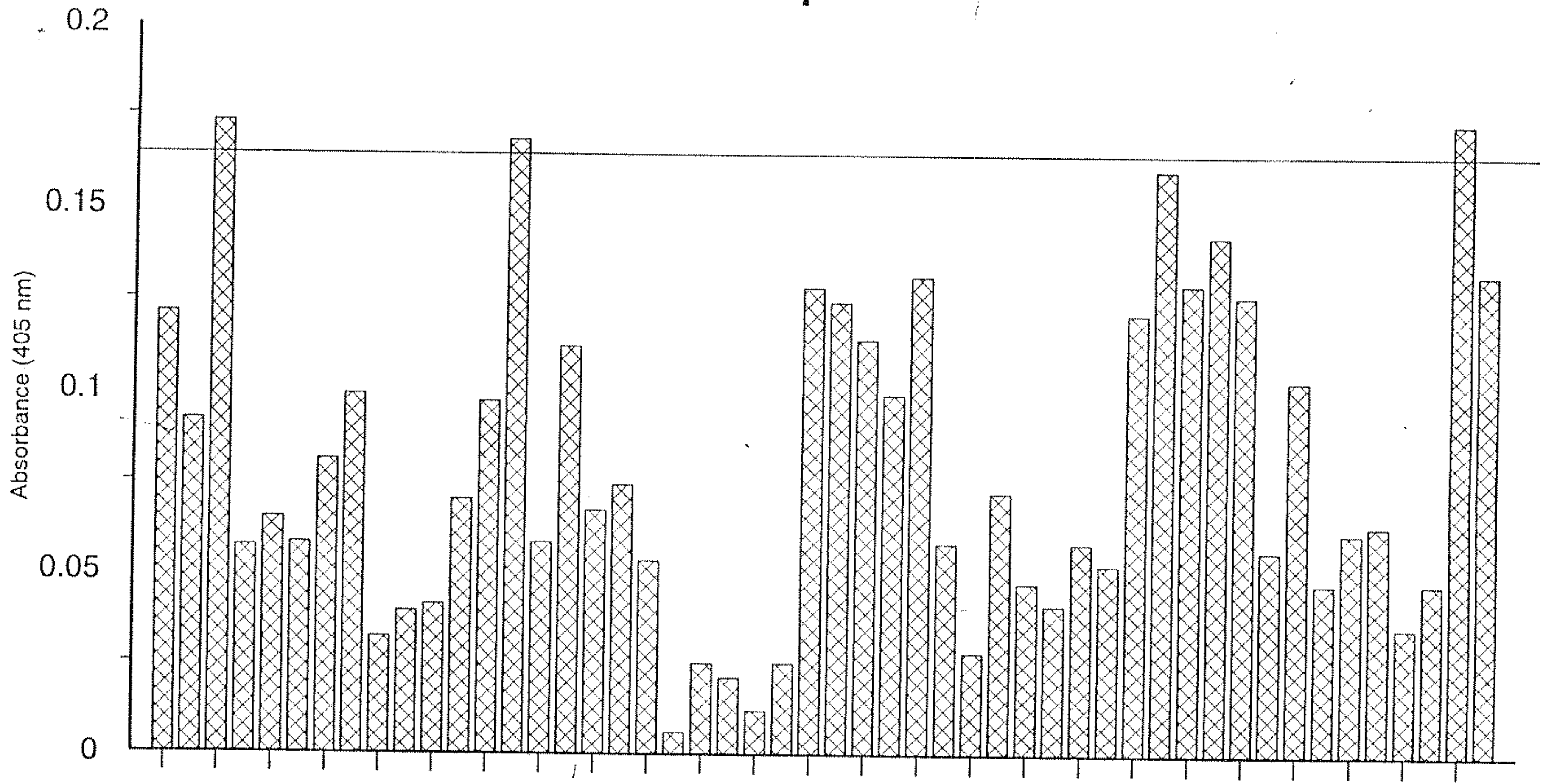
0.6
0.5
0.4
0.3
0.2
0.1
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs



HDCFV134 Detection of latent Botrytis allii
Commercial sample O - 51-100
Absorbance (405 nm)
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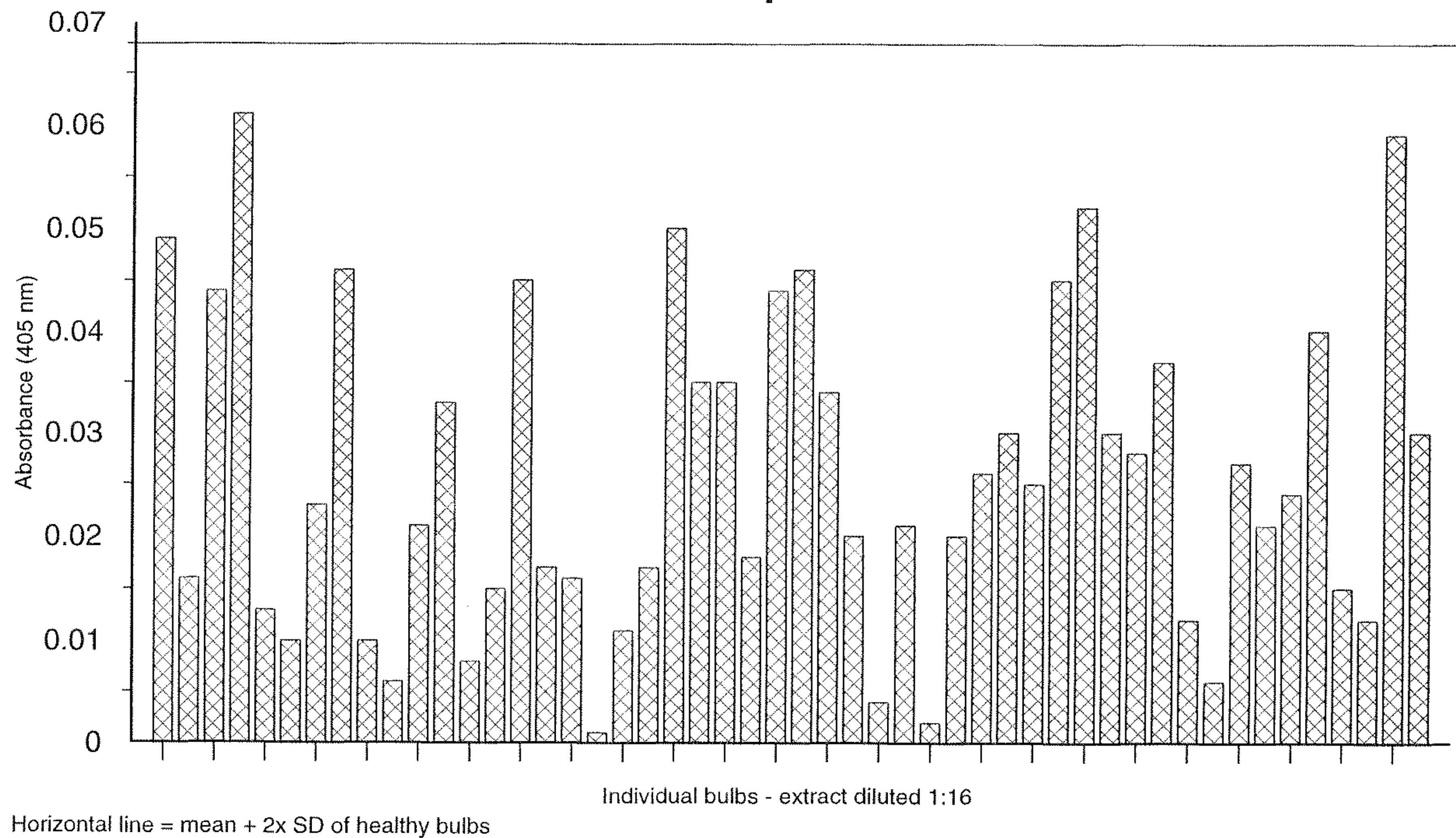
0.15
0.1
0.05
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample A - 51-100
Absorbance (405 nm)
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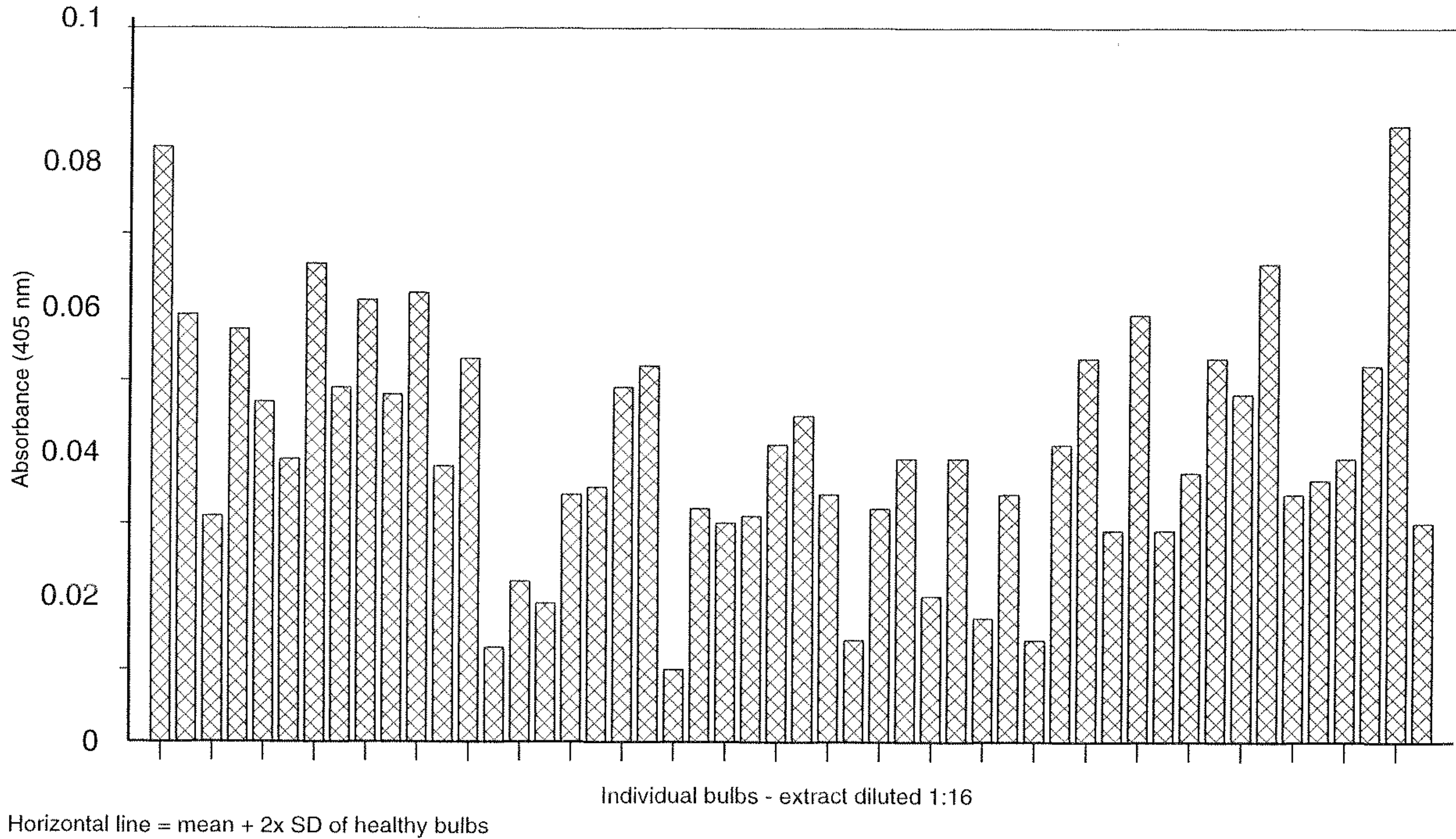
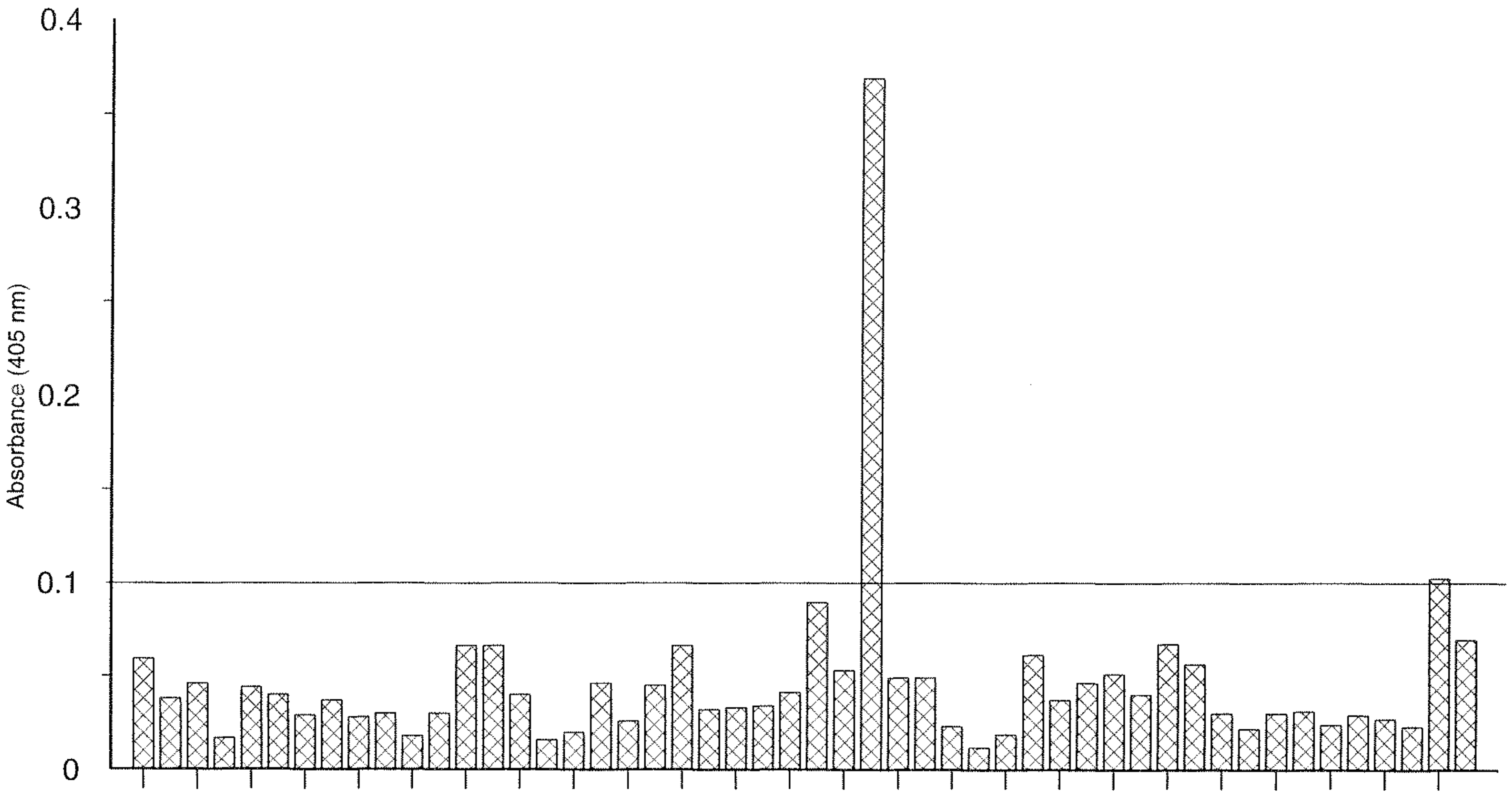
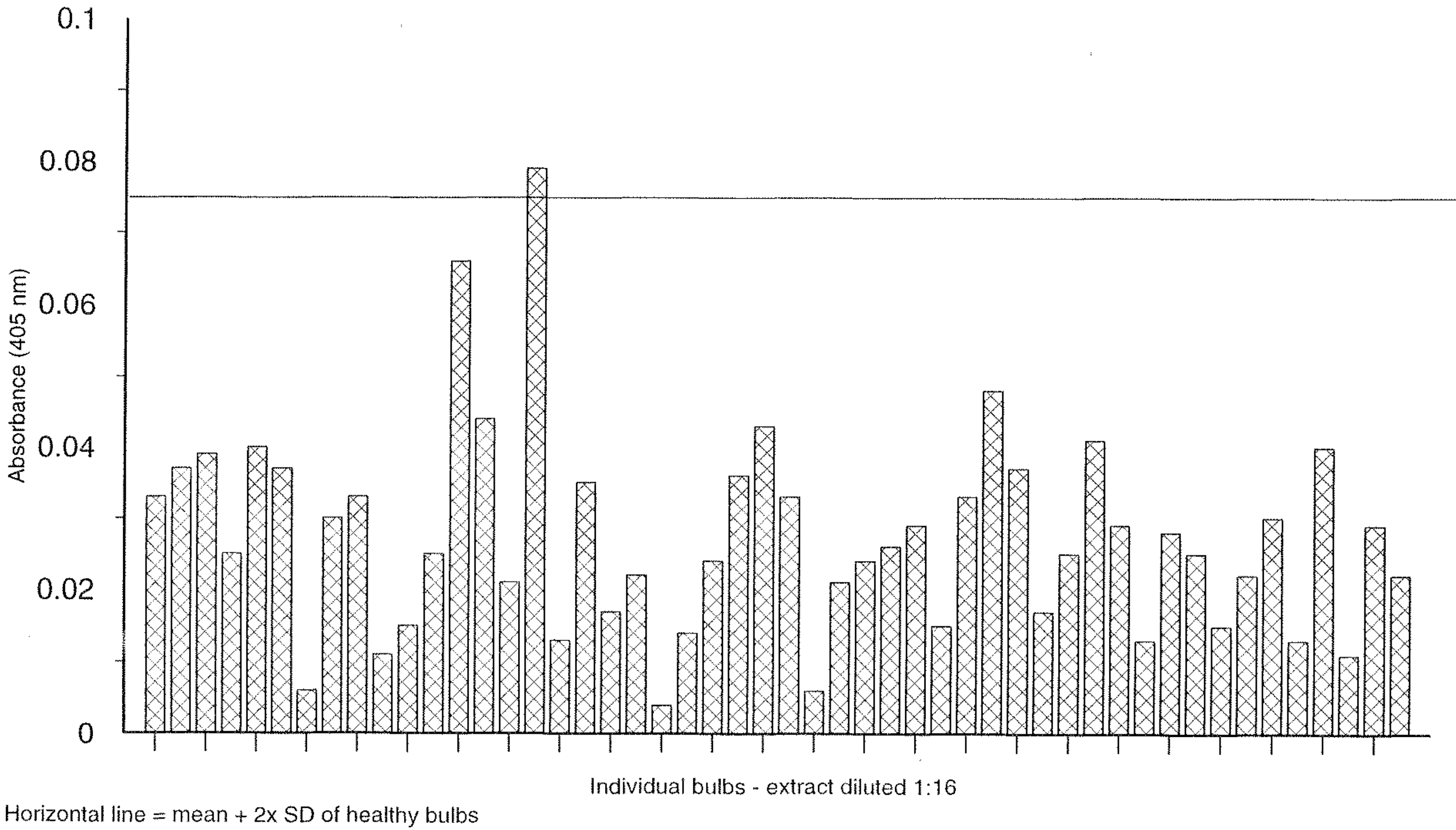
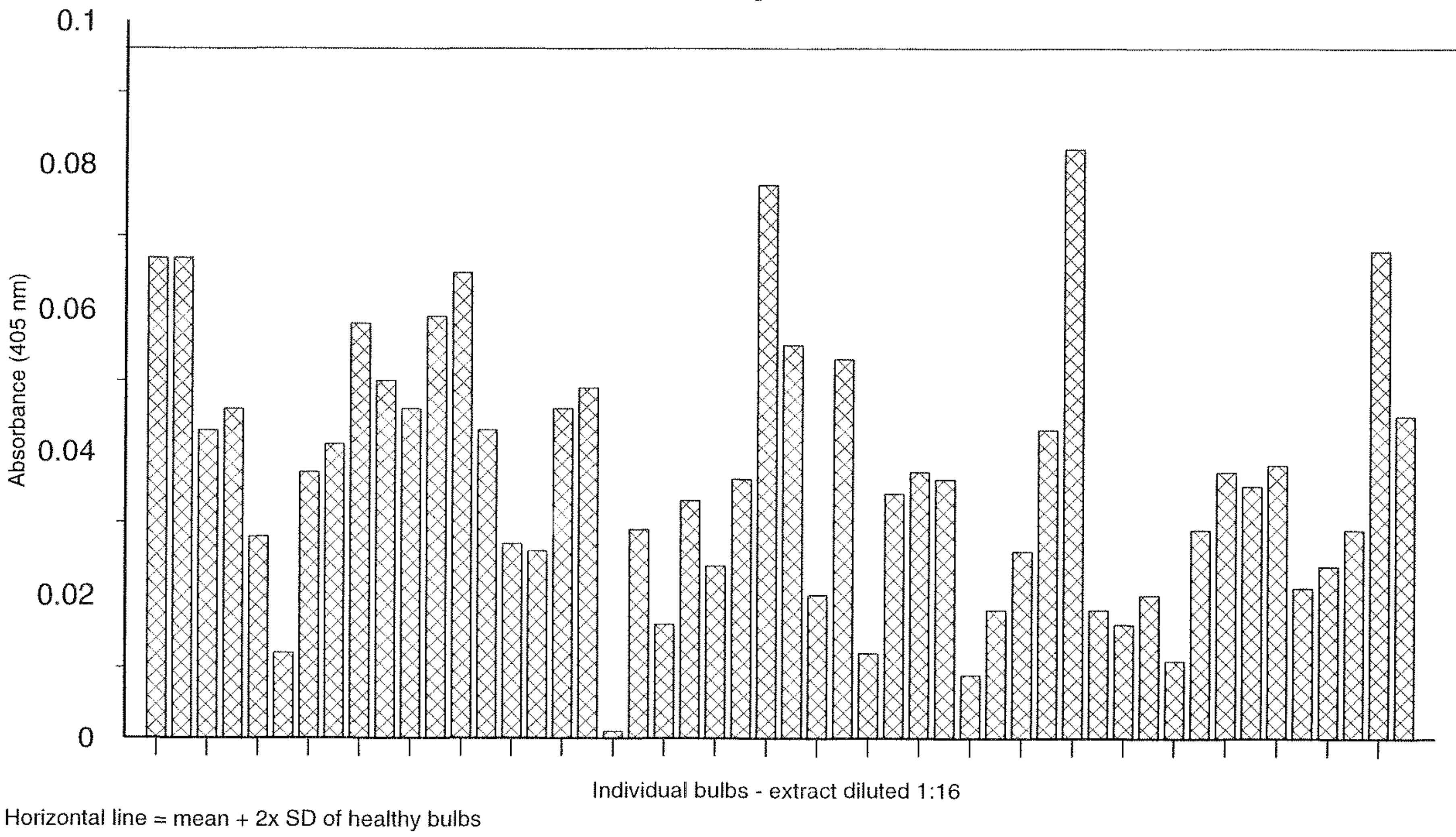
0.06
0.05
0.04
0.03
0.02
0.01
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

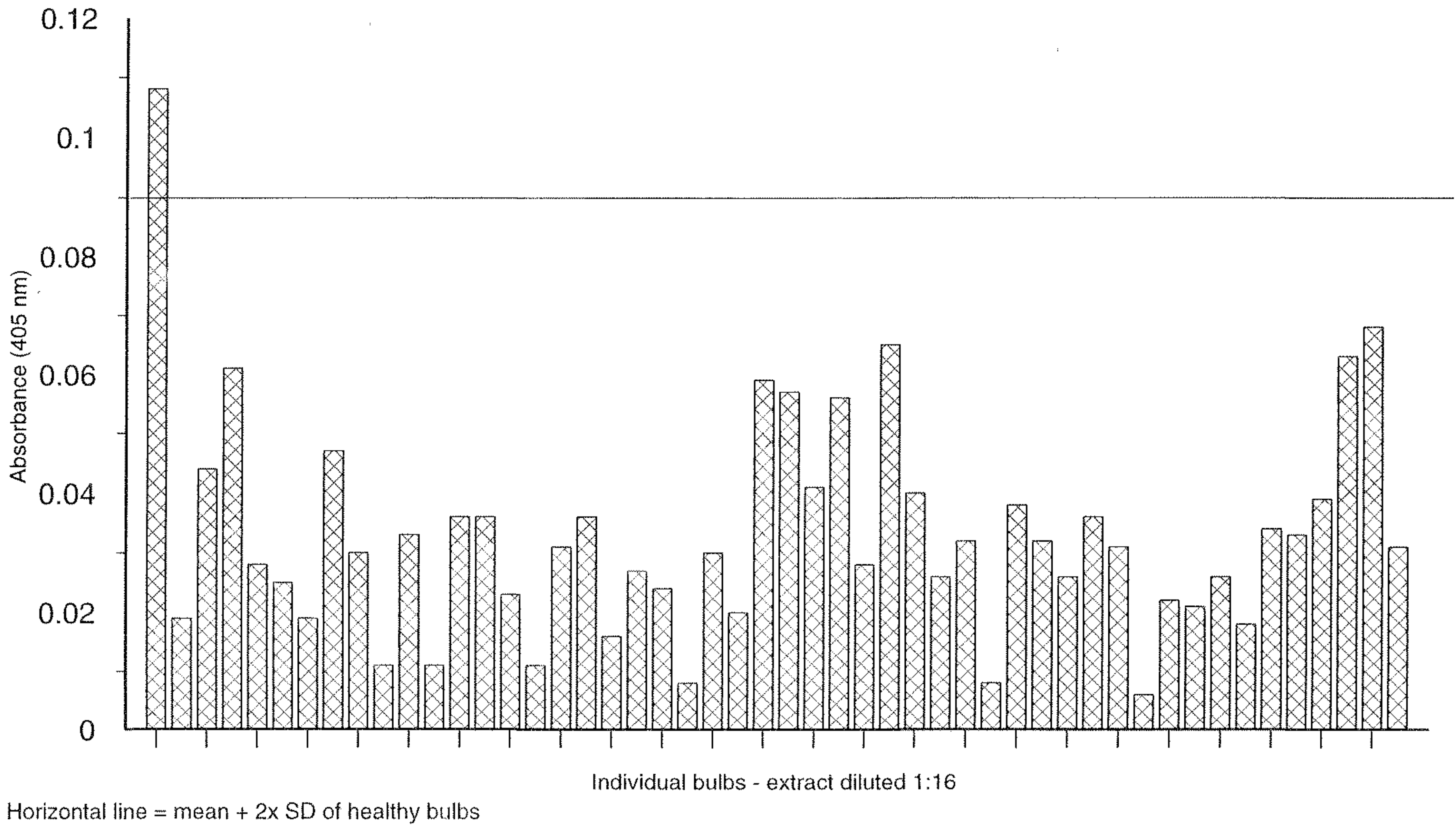

HDCFV134 Detection of latent Botrytis allii
Commercial sample B - 1-50
Absorbance (405 nm)
0.1
0.08
0.06
0.04
0.02
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample B - 51-100
0.4
0.3
0.2
0.1
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

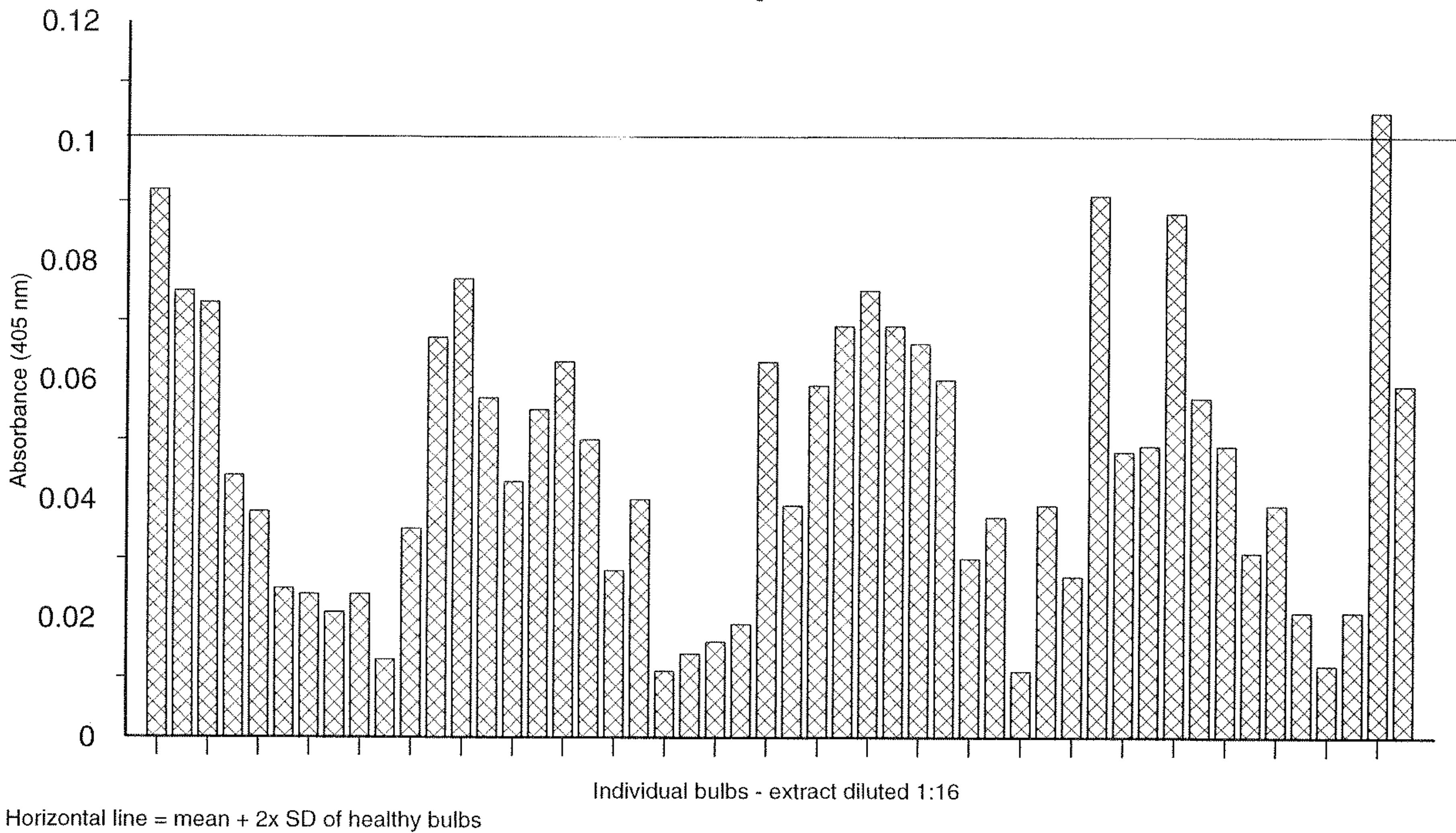

HDCFV134 Detection of latent Botrytis allii
Commercial sample C - 1-50
0.1
0.08
0.06
0.04
0.02
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

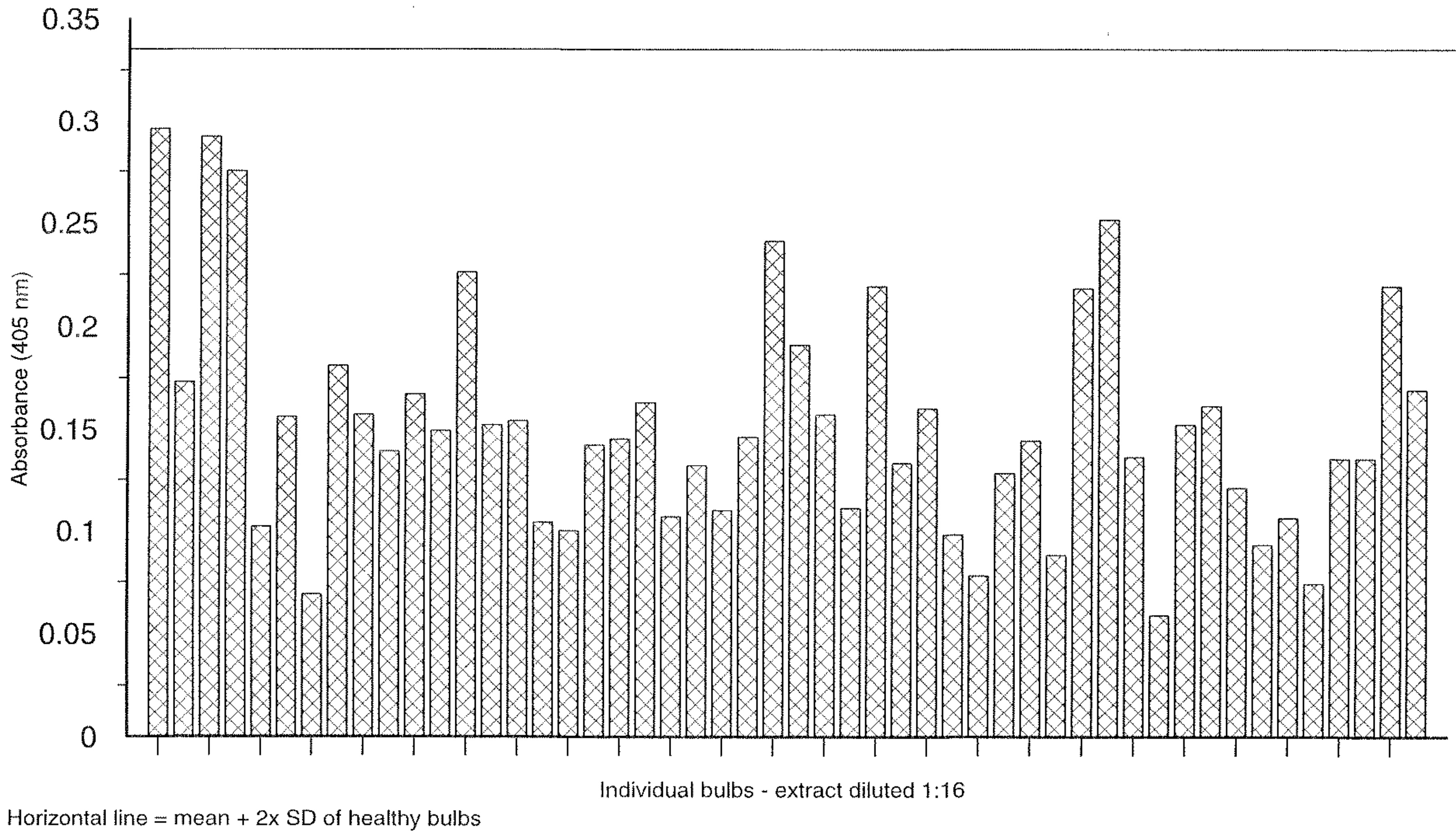

HDCFV134 Detection of latent Botrytis allii
Commercial sample C - 51-100
0.1
0.08
0.06
0.04
0.02
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

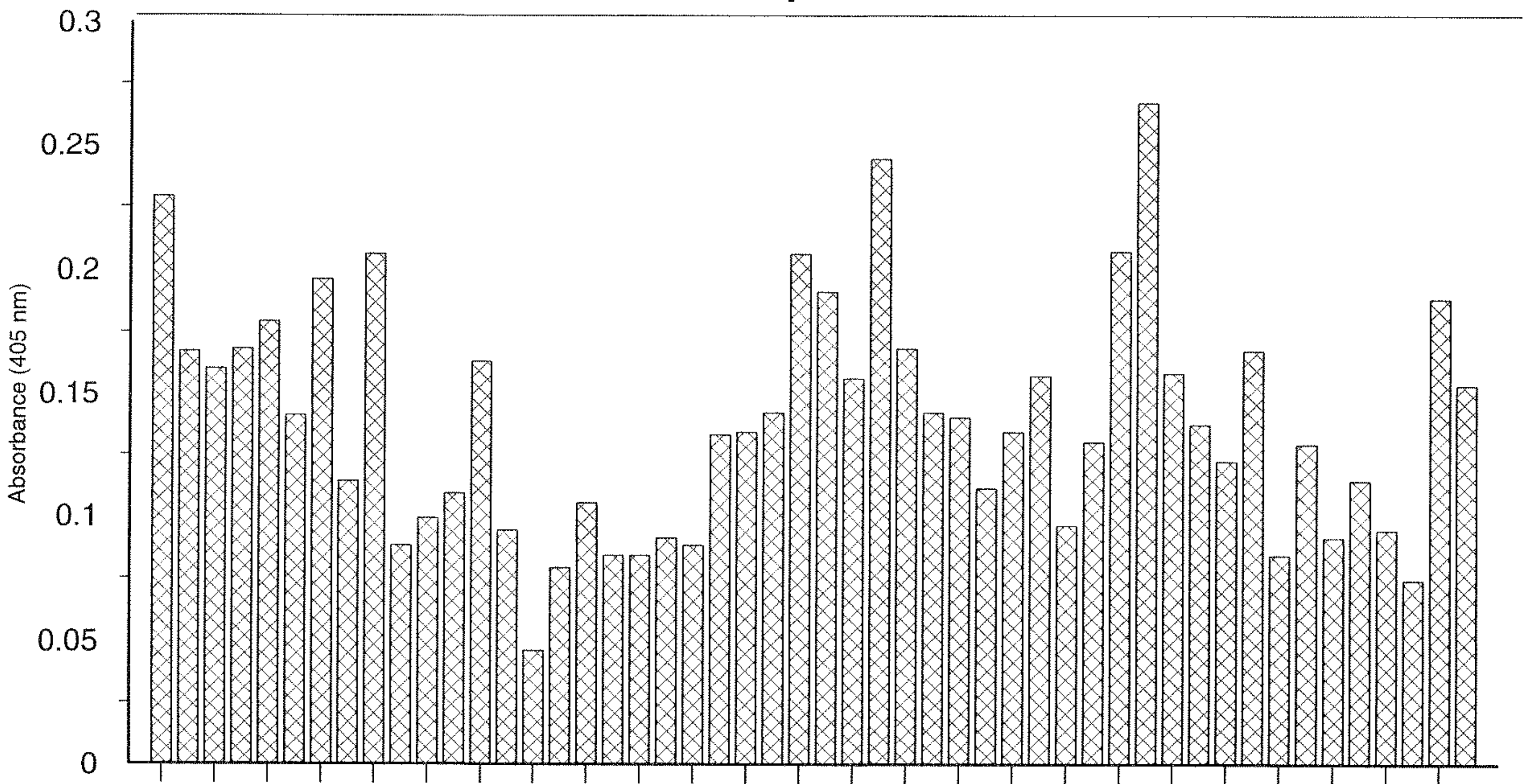
HDCFV134 Detection of latent Botrytis allii
Commercial sample D - 1-50

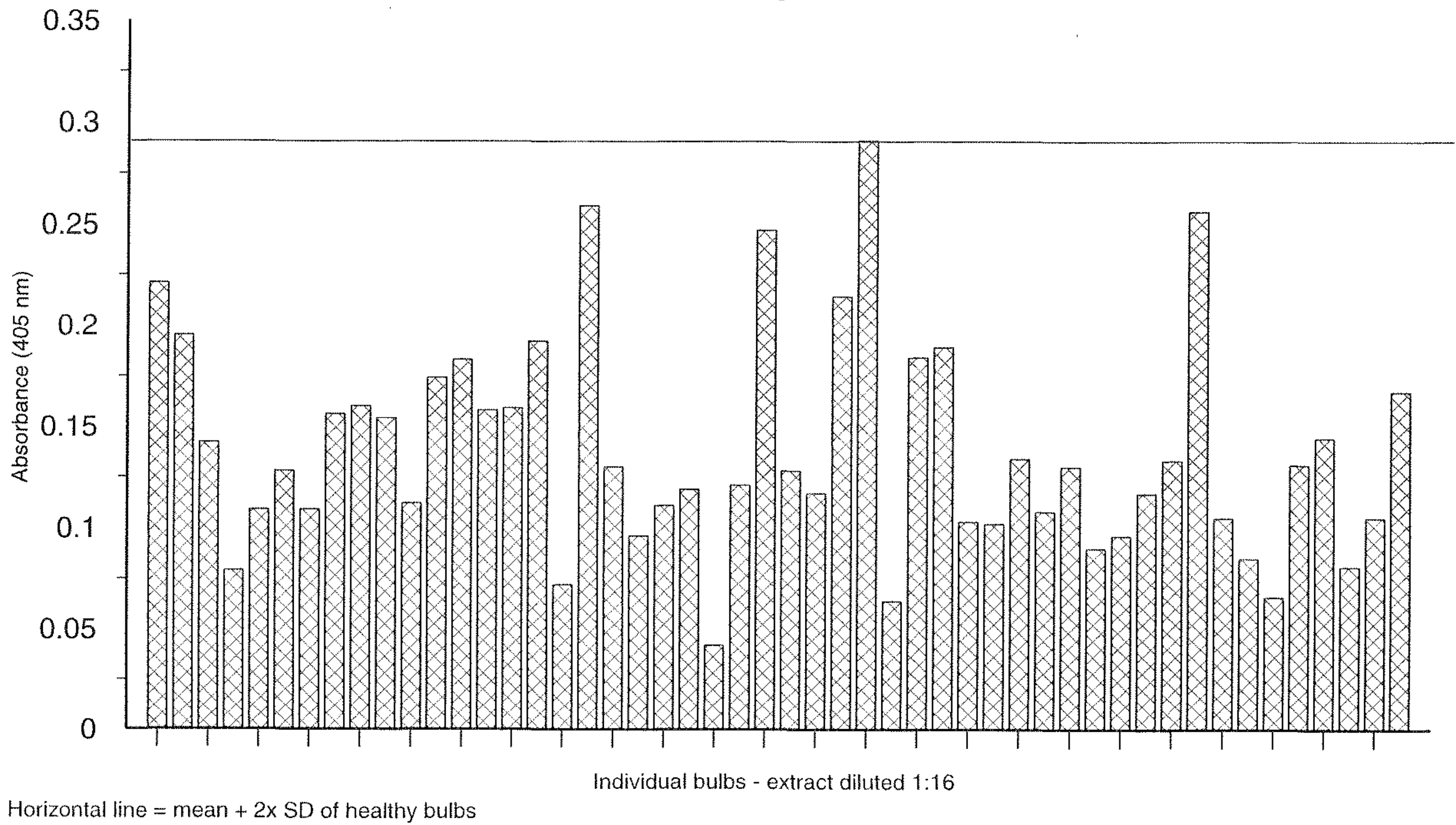


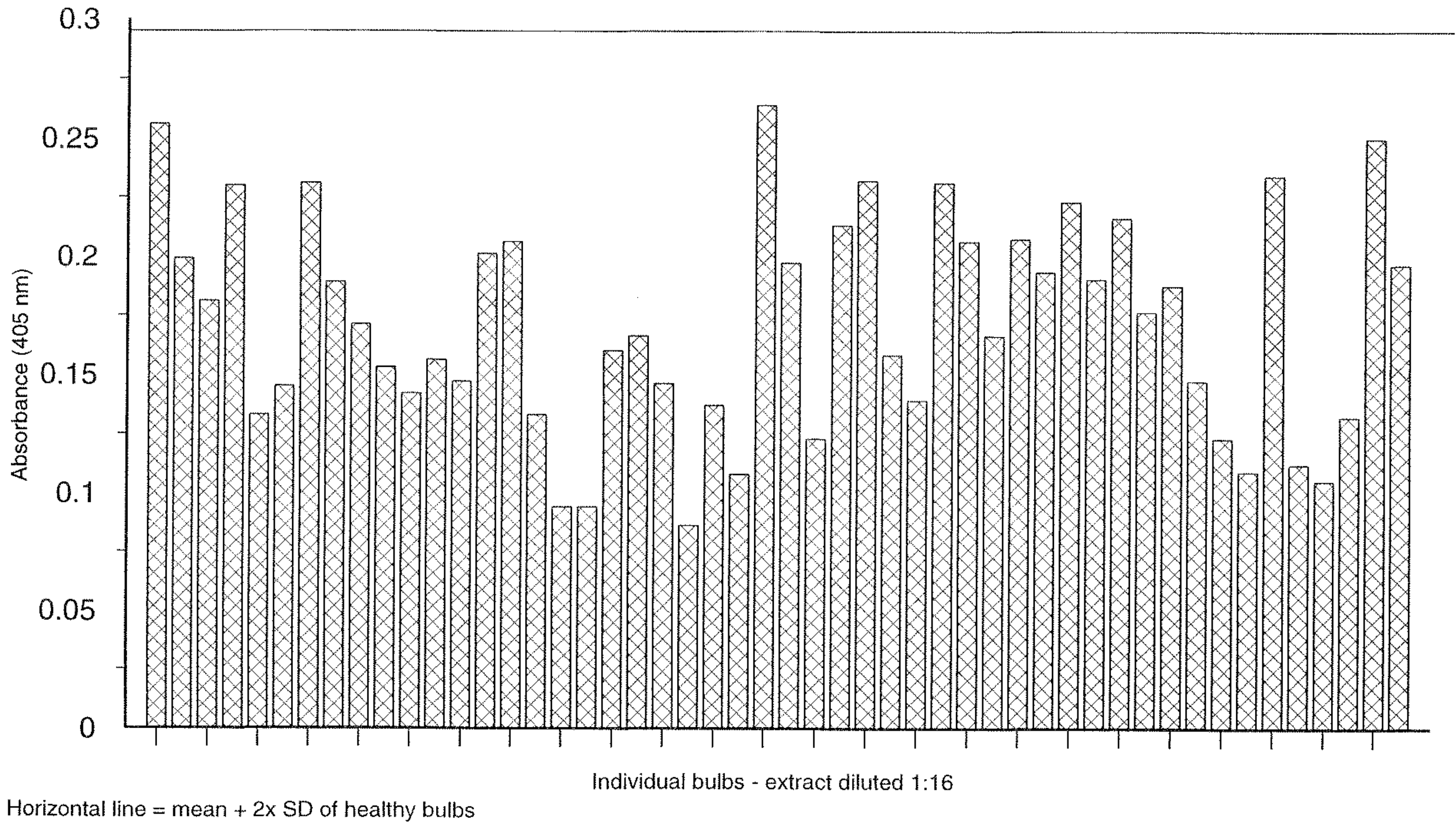
Horizontal line = mean + 2x SD of healthy bulbs

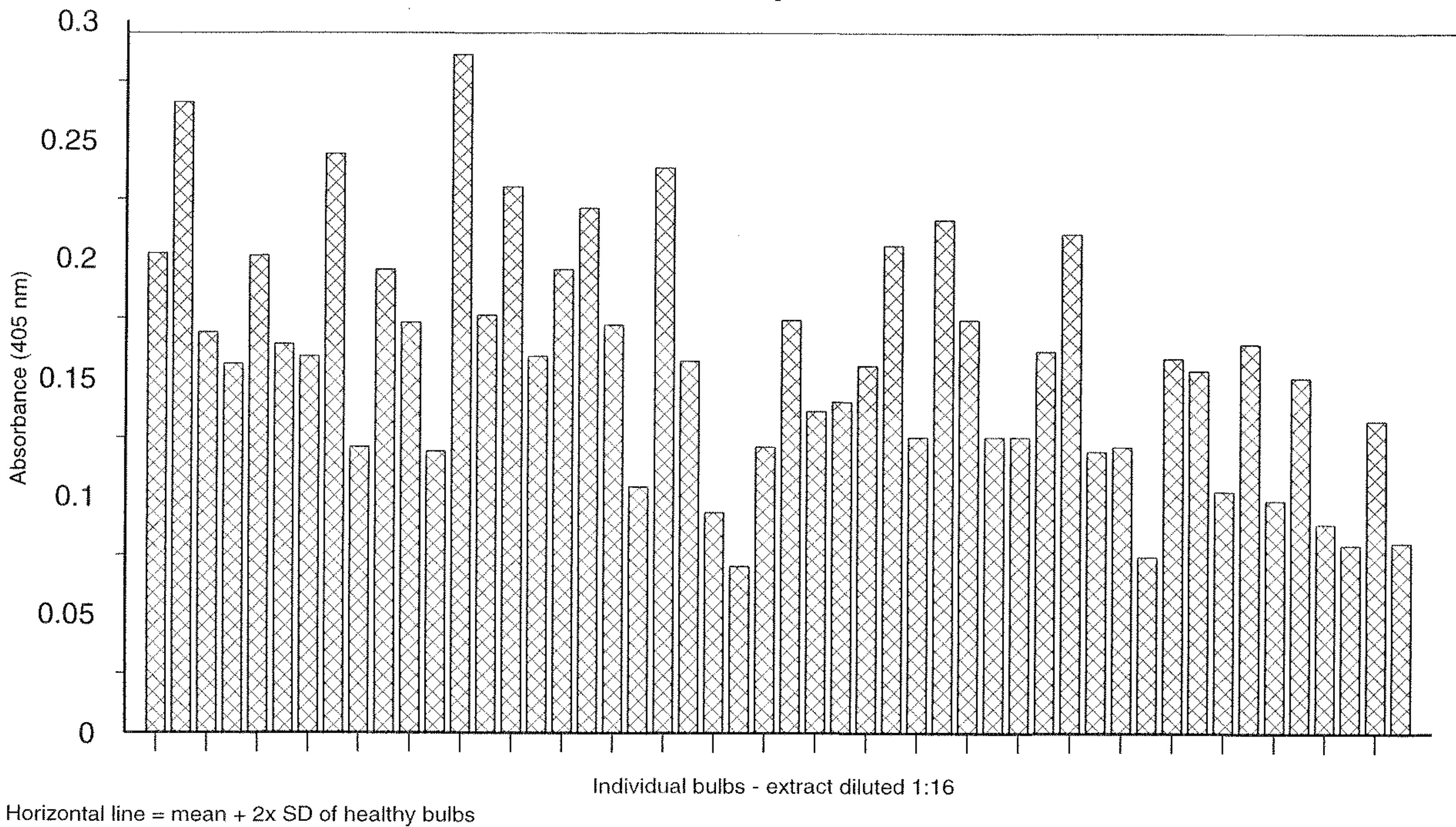

HDCFV134 Detection of latent Botrytis allii
Commercial sample D - 51-100
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample E - 1-50
Absorbance (405 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

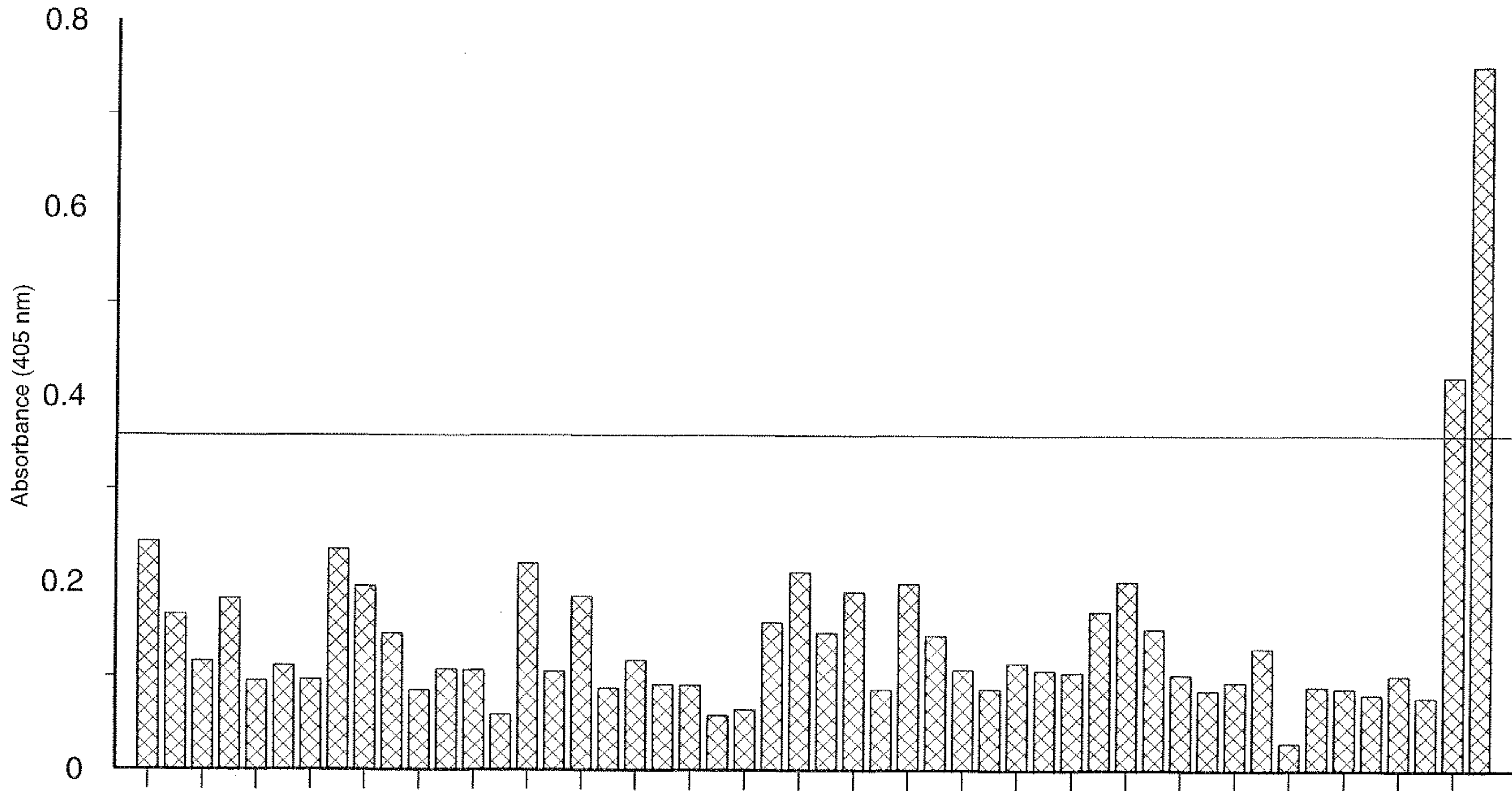

HDCFV134 Detection of latent Botrytis allii
Commercial sample E - 51-100
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample F - 1-50
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

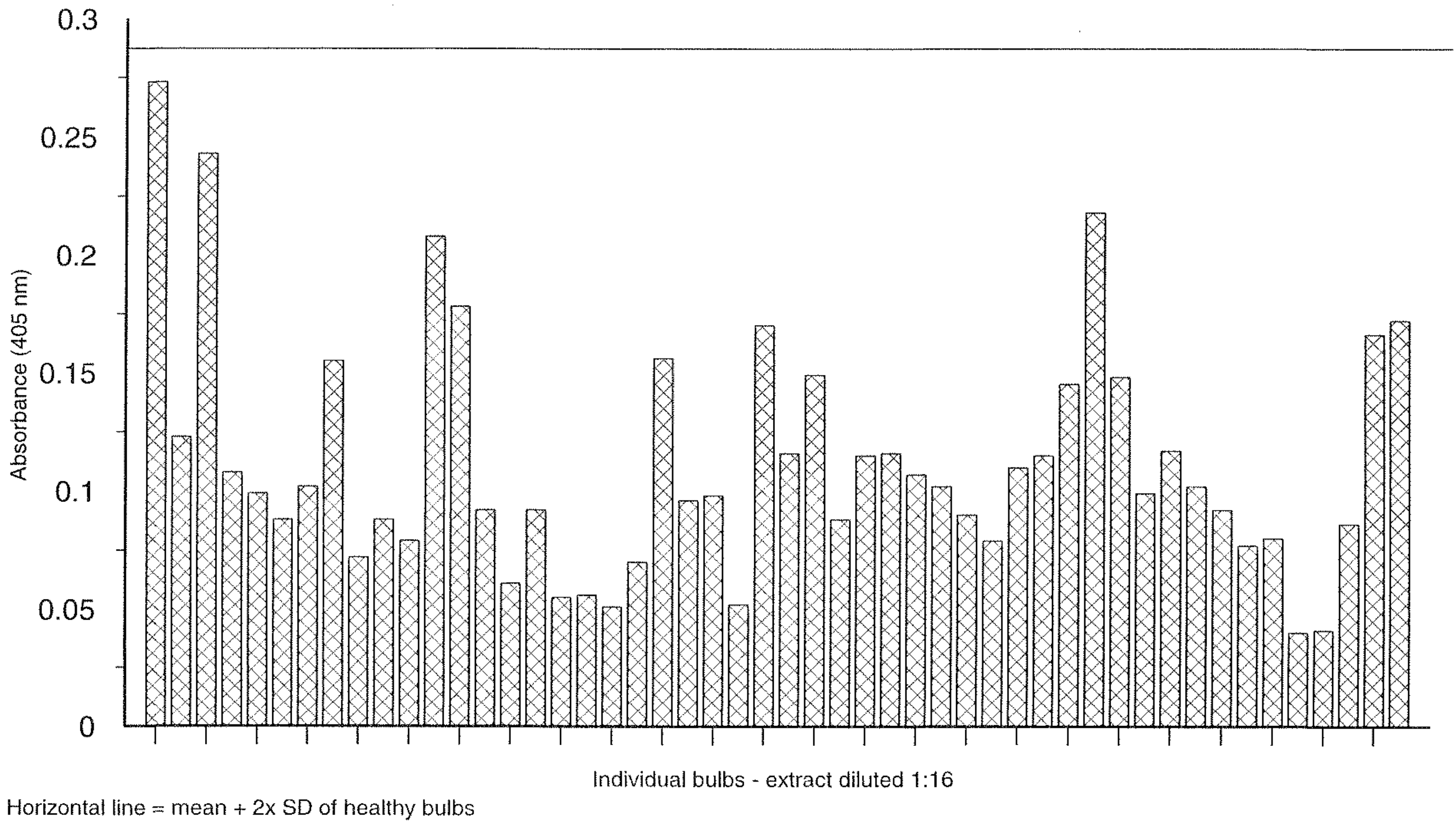

HDCFV134 Detection of latent Botrytis allii
Commercial sample F - 51-100
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample G - 1-50
Absorbance (405 nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

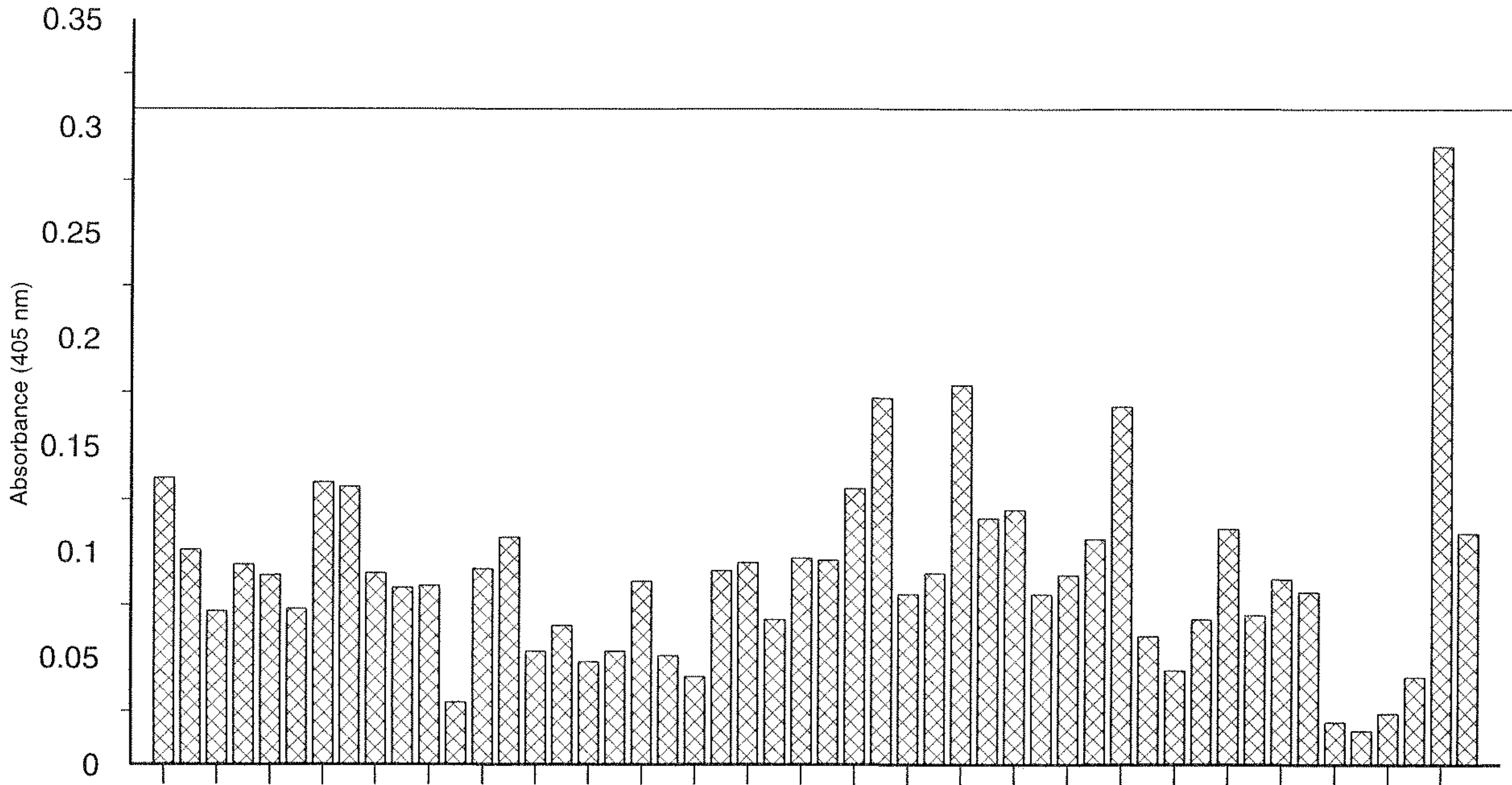
HDCFV134 Detection of latent Botrytis allii
Commercial sample G - 51-100



Horizontal line = mean + 2x SD of healthy bulbs

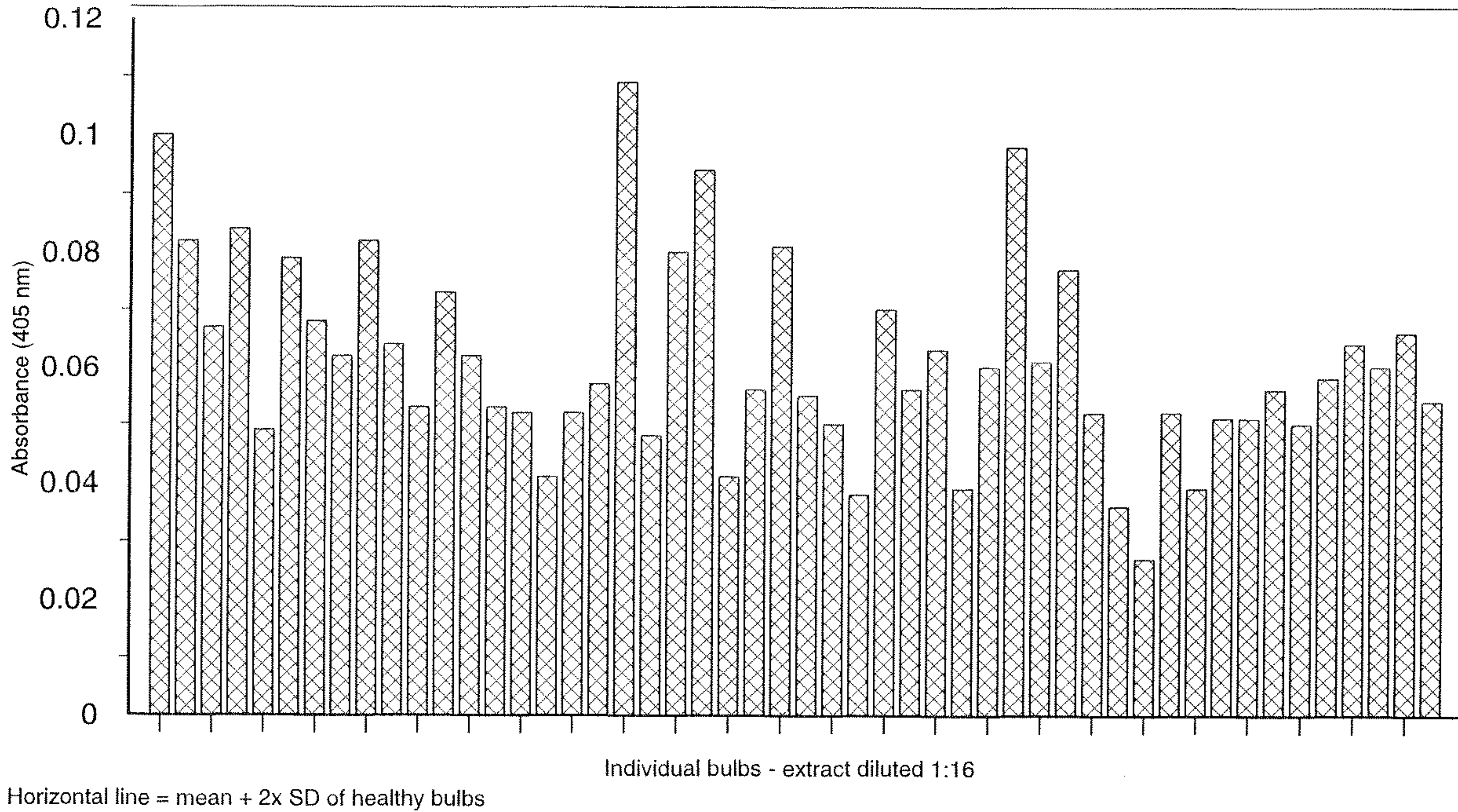


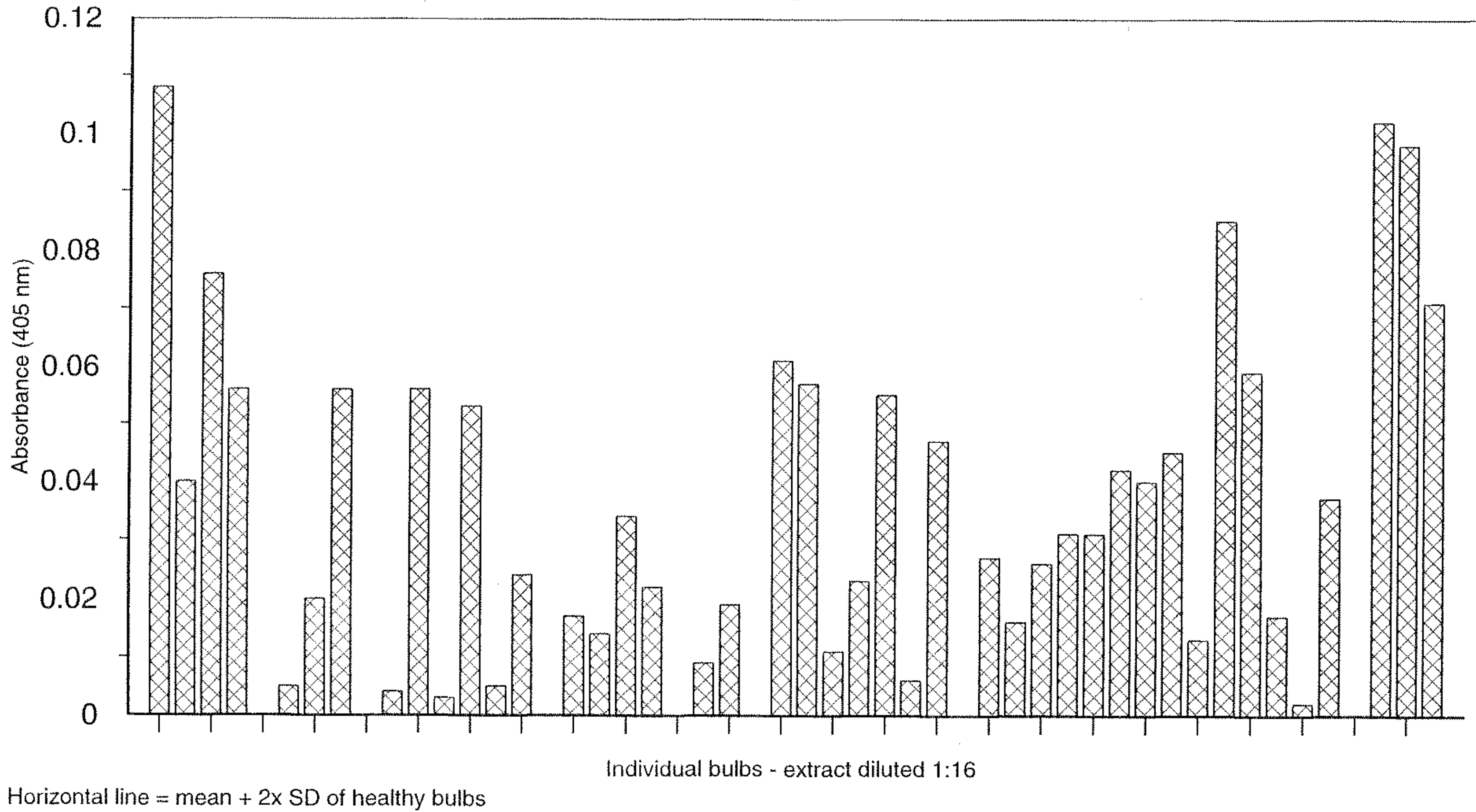
HDCFV134 Detection of latent Botrytis allii
Commercial sample H - 1-50
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (405 nm)
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

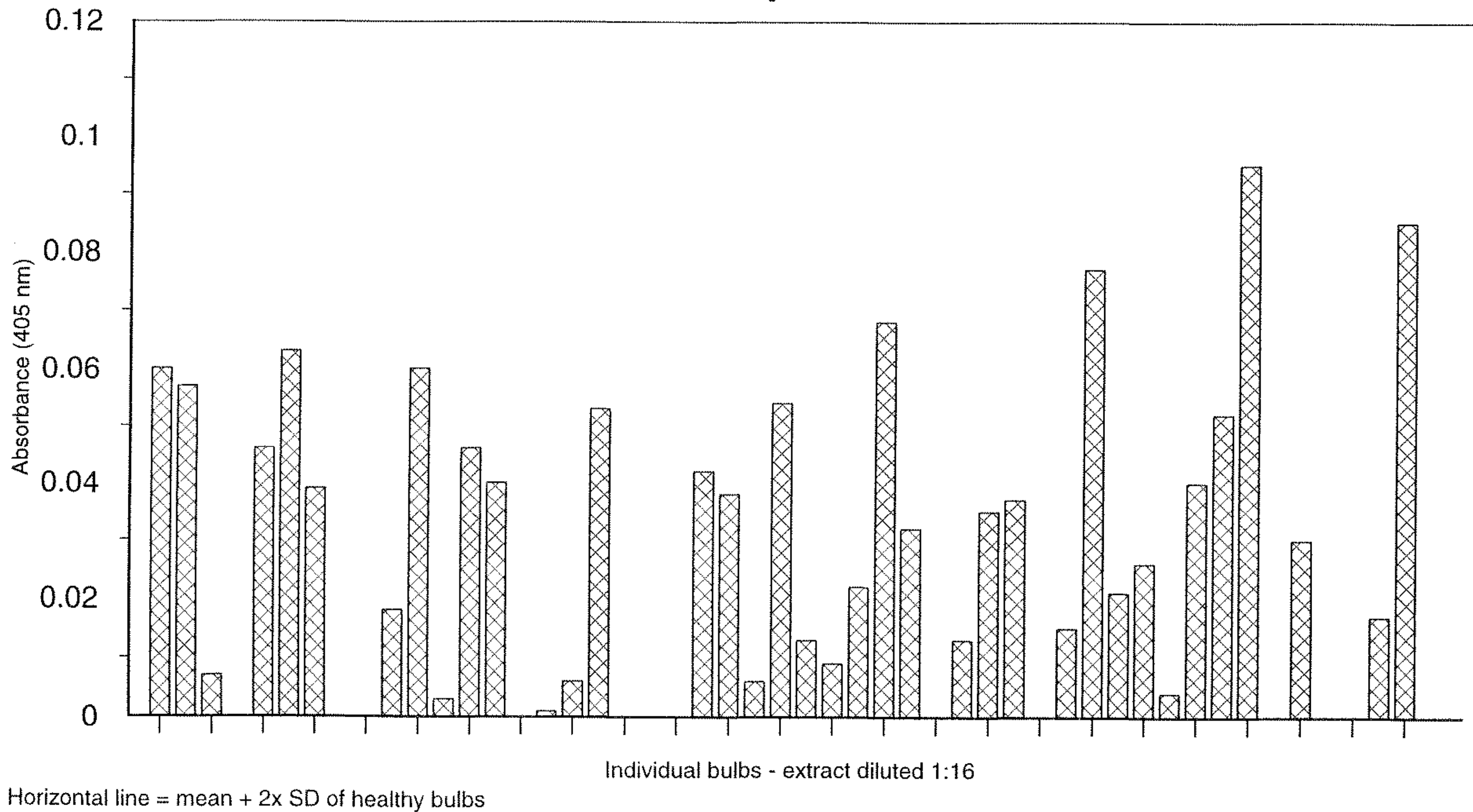

HDCFV134 Detection of latent Botrytis allii
Commercial sample H - 51-100
Absorbance (405 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

HDCFV134 Detection of latent Botrytis alliii
Commercial sample P - 1-50

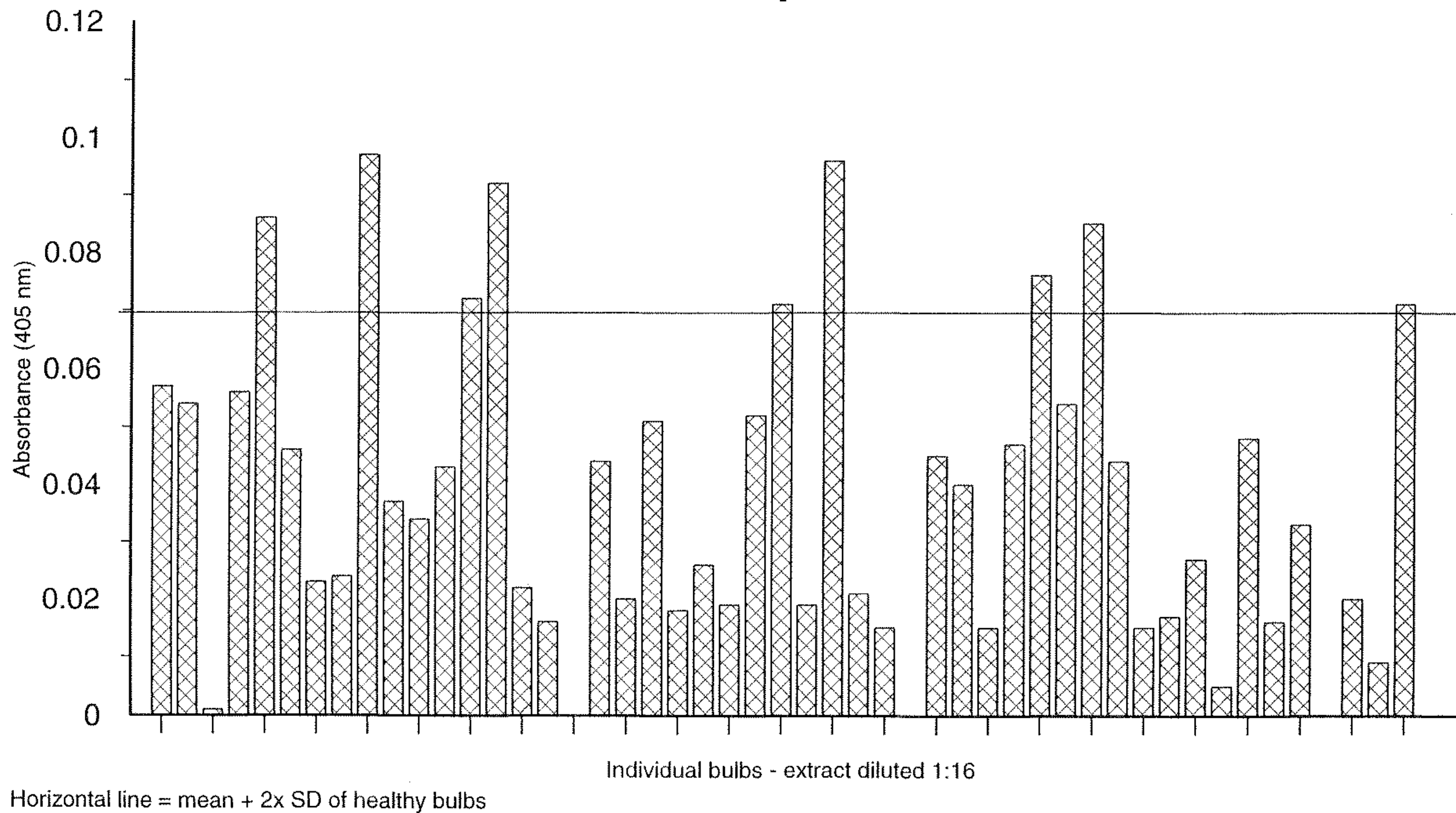
Horizontal line = mean + 2x SD of healthy bulbs

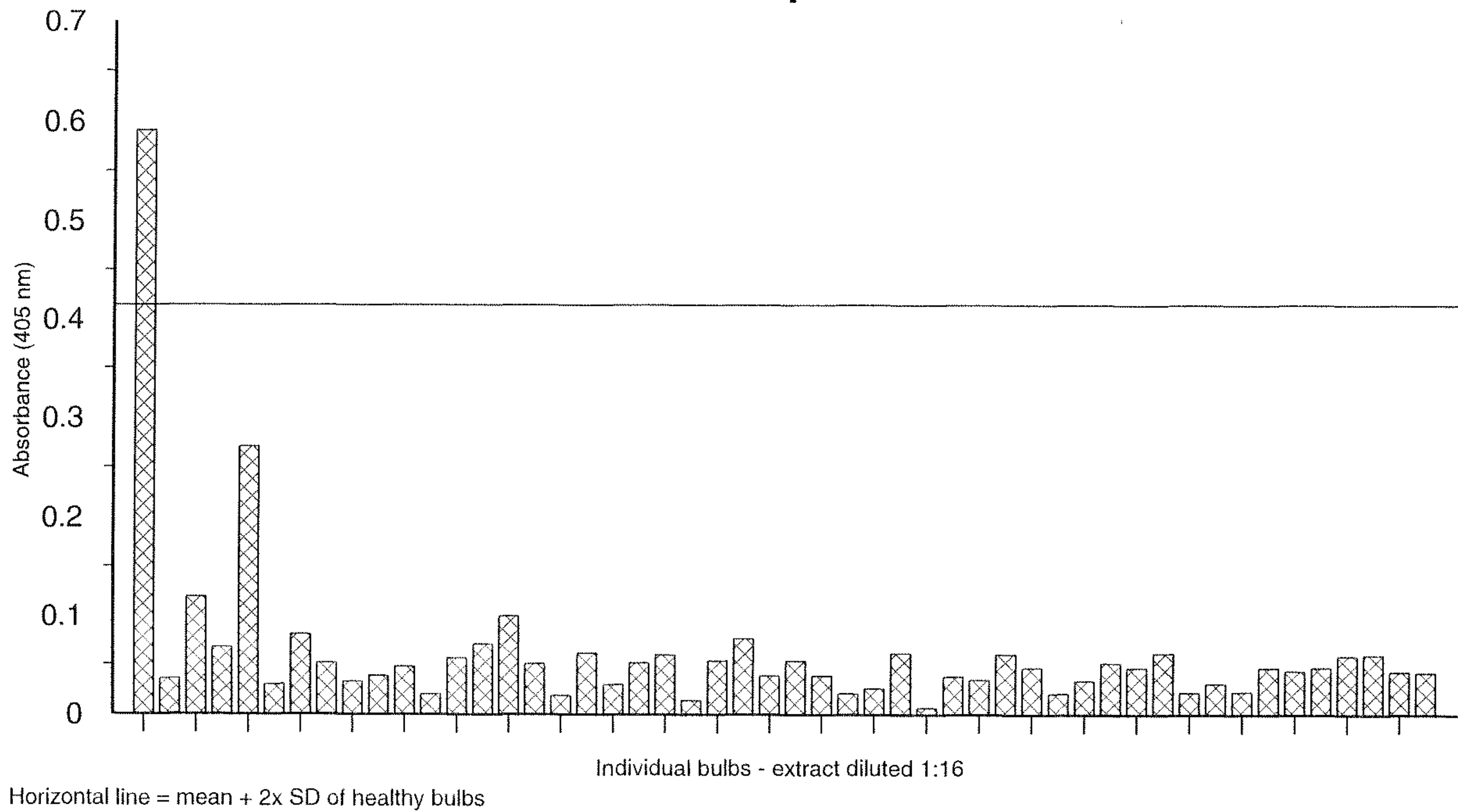

HDCFV134 Detection of latent Botrytis allii
Commercial sample P - 51-100
Absorbance (405 nm)
0.12
0.1
0.08
0.06
0.04
0.02
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample Q - 1-50
Absorbance (405 nm)
0.12
0.1
0.08
0.06
0.04
0.02
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs


HDCFV134 Detection of latent Botrytis allii
Commercial sample Q - 51-100
Absorbance (405 nm)
0.12
0.1
0.08
0.06
0.04
0.02
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs

HDCFV134 Detection of latent Botrytis allii
Commercial sample R - 51-99




HDCFV134 Detection of latent Botrytis allii
Commercial sample R - 1-50
Absorbance (405 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Individual bulbs - extract diluted 1:16
Horizontal line = mean + 2x SD of healthy bulbs