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"AUTOMATED STATISTIC MEASUREMENT OF THE VOLUME OF SPORES RELEASED BY LARGER FUNGI "

P.BAUMGART * A. LAURENT ** JP MAURICE ***
* 9 Chemin des Echalandes 88160 LE THILLOT France
** 65 Bd. Valonnière 54600 VILLERS - LES - NANCY France
*** 45 rue de France 88300 NEUFCHATEAU France

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Summary : An experimental method is proposed to determine directly the volume of the spores released by mushrooms. The principle of the method is founded on the electrical resistivity of an aqueous slurry containing the dispersed spores. Its application and its statistical analysis are illustrated with some examples.

I - Introduction

In our quest to improve our knowledge of taxa (genus, specie, group of specie's) in mushrooms, we have investigated a new approach in the determination of spores.
Sporographs have already largely argued about sporal dimensions (Length, Width and Thickness)
All "contemporary" mycologists are deeply convinced about the high specificity of sporal dimensions (Heinemann and Rameloo, 1985). Nevertheless, they outlined how difficult it still be to develop a "***standardized methodology"*** with regards to the determination using optical microscopes which would be reproducible from one Mycologist to the other.
The determination of sporal volumes seems to be essential in the mushrooms taxonomy and the influence of its different parameters appears to be systematic.
Breitenbach and Kränzlin (1991) approached the notion "sporal volume" in a theoretical way, based on the studies from Gross (1973-1976), from Parmasto E. and Parmasto I.(1987) as well as from Möls (1987) and finally Krüger (1987). Both Swiss authors came to calculate the average sporal volume (called V_m) with the following relation: $V_m = 4/3\ \pi\ a^2\ b$, where "a" is the half minor axis and "b" is the half major axis of the revolution ellipsoidal spore. As a result, for each and every specie (450 in their study), one theoretical volume was calculated.
To our opinion, this only contributed to move the problem and to amplify the discrepancies observed when using an optical microscope and thus lead to errors.
This is the reason why it seemed preferable to us to apply a ***direct measurement*** of the volume of these micro particles, using the ***variation of resistivity*** in liquid.
Numerous automated methodologies based on this principle already exist in the fields of industry or biology. We have just adapted it to the measurement of spores released by mushrooms.

II - Methodology

21- Collection of the sample

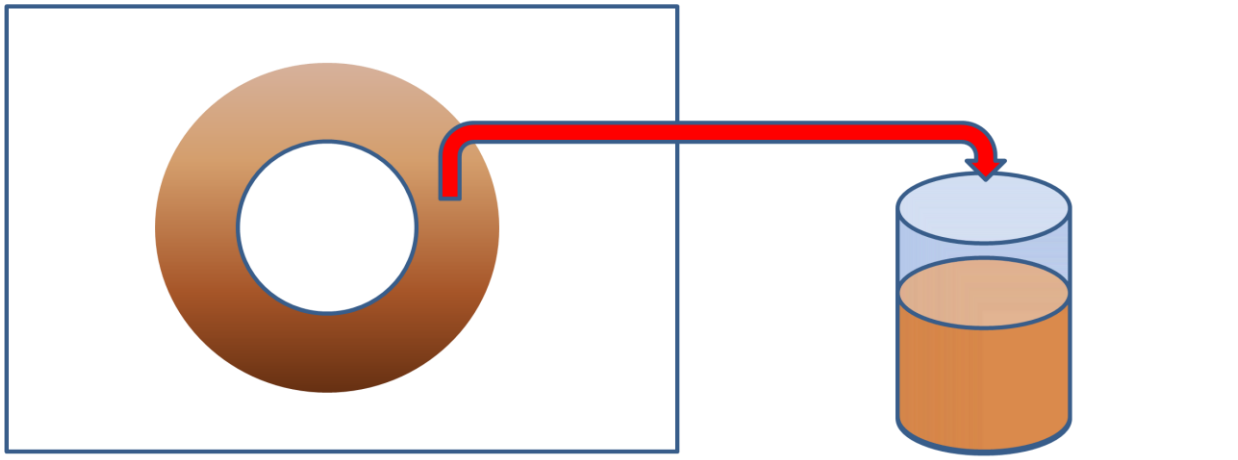
As it is described in the dedicated literature, this step is very crucial and delicate: ***only the spores which naturally fall off the "sporophore" have to be collected:*** it implies a few precautions:

- Only work on "fresh" and in a good state species. We'll see later how to keep these spores in a good condition.
- In order to ensure a heavy sporulation, work at the optimal temperature and at the optimal level of the hygrometry: there are numerous ways to reach these conditions.
- Cardboard layer with holes in order to let the base of the mushroom dipping into water.
- Separate "hat" and "leg" for small specimens.
- Special conditions limited, for our measurements, to the purity of the spore collection, i.e absence of other particles, dust, spore of other mushrooms, microscopic moulds, microorganisms etc…

NB : Today, it is possible to get rid of external pollutions in a close environment, but it remains difficult to evaluate the fall of "basides", cystides" or other cellular elements coming from the hymenium.

- Once the sporal collection has been placed on a glass slide for instance, it has to be stored away from dust and avoid an external contamination during measurements.
- It is essential to only use sterile disposable equipments.

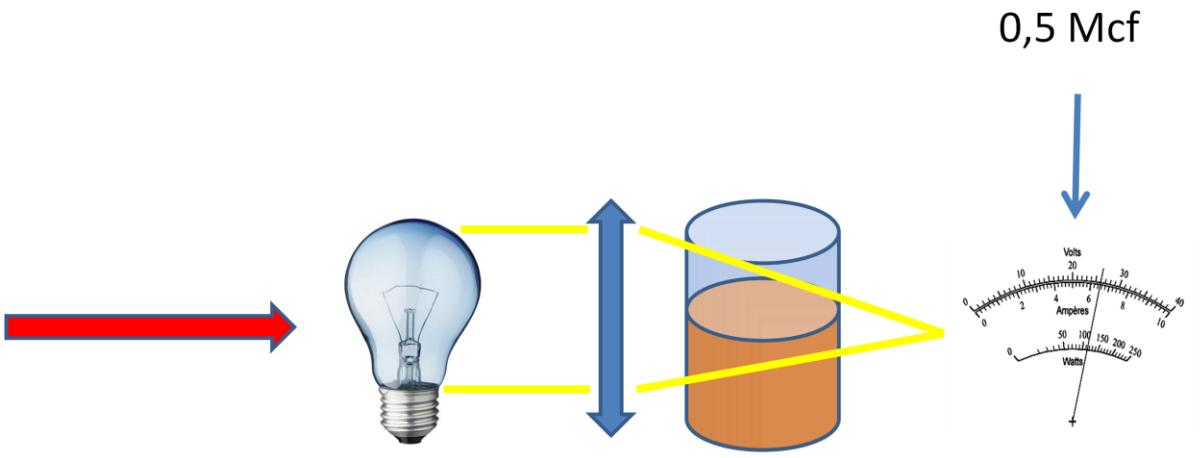
Description of the stages



Sporal collection

Spores put into suspension

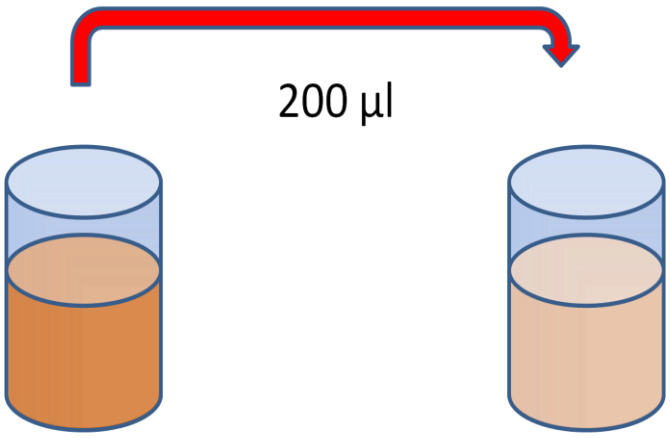
1



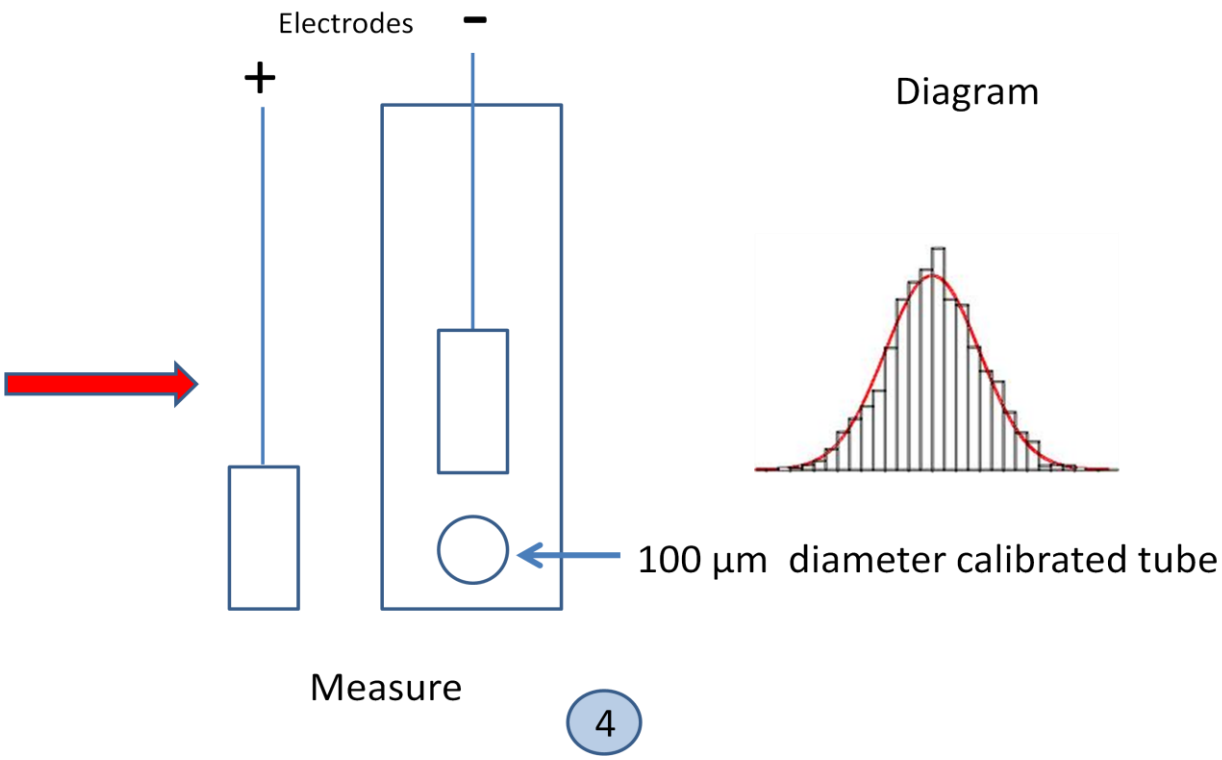
Calibrate this suspension
With a densitometer (Mc Farland)

2

dilution



3


Electrodes
+
−
Diagram
100 µm diameter calibrated tube
Measure
4

Densitometer allowing the measurement and the adjustment of spores suspensions, in order to standardize the quantity in the sampling probe.

22 - Standardization of the sporal collection

Once the collection has been put down on the slide, an homogeneous suspension in the measurement liquid should be prepared. This operation will be completed in 3 steps:

- put into suspension this primary solution
- calibrate this suspension with a densitometer (Mc Farland)
- dilute this primary solution for test set up.

III - Presentation of the measurement system

9000 SERONO automated system

Capacity of measuring a volume range up to 400 μm^3

a) Introduction of the diluted sample into the system:

After an automated dilution (5ml), the solution will be transformed at the level of the sampling probe, and then automatically sucked up into the measurement chamber.

b) The Measurement "itself":

The principle of the measurement and of the electronic counting of the spores is based on a conductivity differential between the spores and the dilute solution in which they are in suspension.
It follows the principle of impedance. Spores will be counted and measured from the range of the electrical impulses. Every phase of the measurement can be followed up and controlled on a screen and the final result will be printed on thermal paper (cf. frequencies histogram). After the dilution step, the sample will be sucked up through calibrated tubes (100 µm of diameter) with a regular flow.

c) Decontamination:

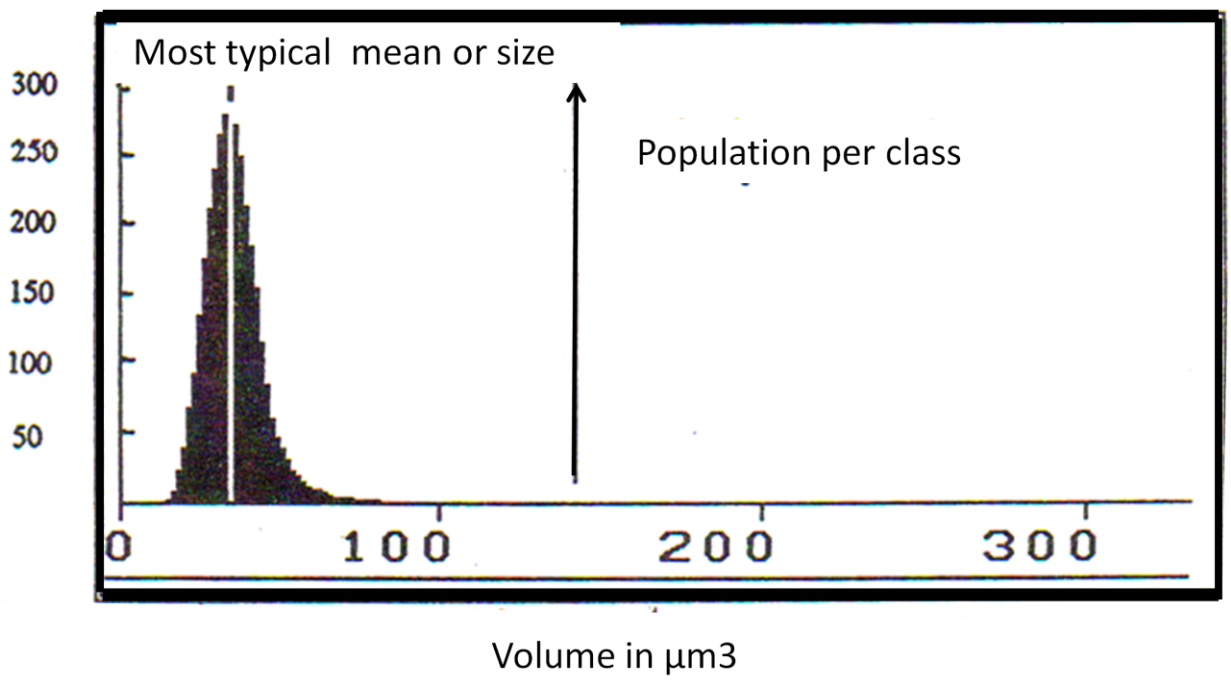
Once the measurement has been finished, the measurement and counting samples will be carefully cleaned up with a double rinsing. A rinsing solution is flowing through the priming pump, the counting chamber and the volumetric tube so that the waste will be rinsed away after each measurement.

d) Numerical interpretation of the results:

A predefined detection threshold algorithm with software allows the system to detect electronic pulses, to sort them out and to only keep the relevant data's, which are afterwards printed out and shown on the visualisation screen.
This will correspond to the results of enumeration and volume of the studied sporal collection. (cf frequency histogram)

IV - Interpretation and control of the results:

a) Example of histogram (Lycoperdon perlatum)



The number of the measured spores varies in the range from 3000 and 10000. This example shows the profile of the spore population. This model of presentation will be used for all measurements.

b) Control diagrams on the screen:

In addition to the printed result, a control diagram on the screen shows the variation of the flow during the counting and the measurement. Acceptable precision limits are constantly displayed for the operator.
If a problem is detected, the sample will be automatically re-processed by the machine and the operator will be informed by a message display.
The system has to be calibrated prior each series of tests with latex particles having a well known volume: during the calibration the acceptability ranges given by the supplier had to be respected.

NB: All measurements will be systematically done in combination with a microscopic examination of the sample in order to control its aspect and particularly the homogeneous repartition of the spores in the dilute solution.

V - Detailed study of one species

Lycoperdon perlatum

This species has been chosen according to the following parameters:

- facility to collect the spores
- "round" shape of the spores
- capability to produce numerous spores.

A - *Detailed statistical analysis of the frequencies' diagram:*

- conventional parameters have been used
- consolidation of the data in classes = volume in μm^3 = xi
- calculation of the median values.
- population per class = ni
- frequency = F
- calculation of the mean, the standard deviation and the variance ot the distribution
- complementary parameters, such as a dimensionless variable, a theoretical probability of the variance.

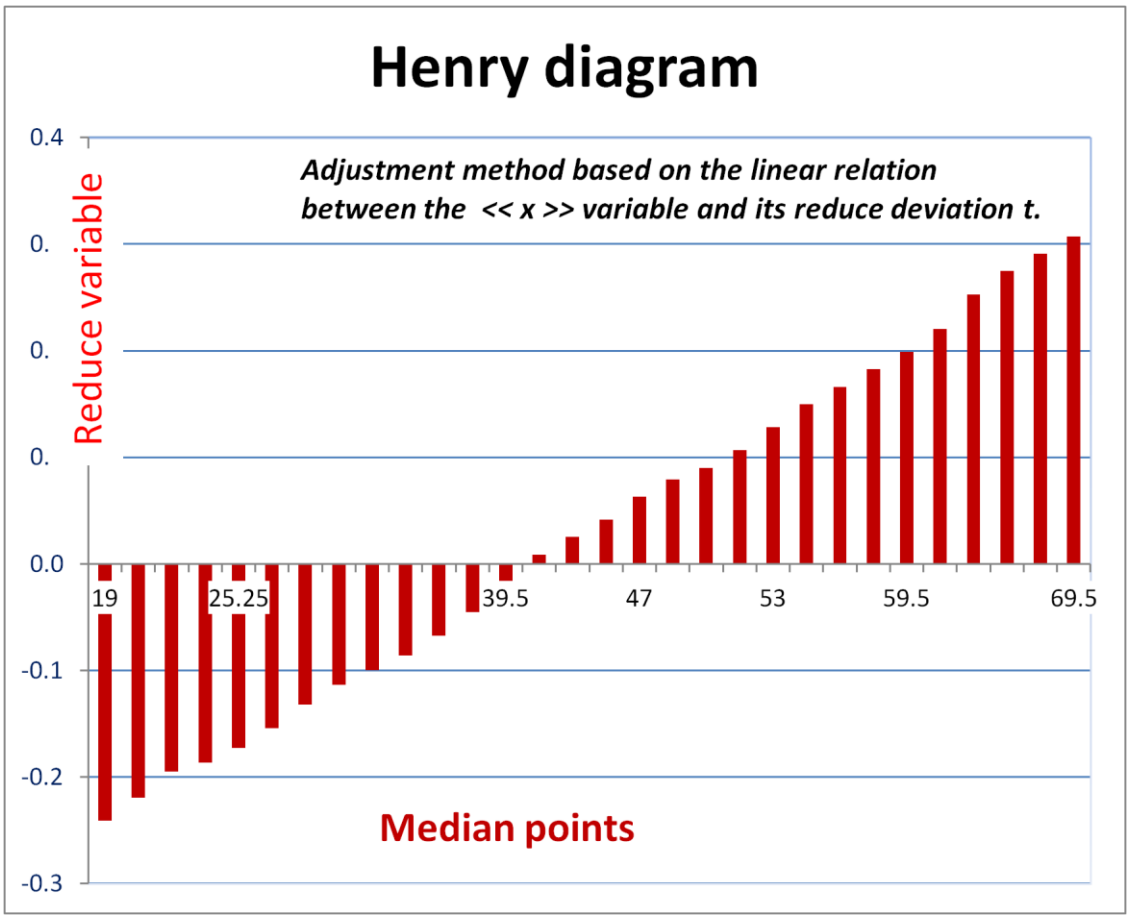
The observation of the 3291 measurements presented in the table (1) gives a good idea of the distribution of the population of the spores. For a better readability, darkened lines in this table represent the limits of the mean differential on both sides of the average: between 32 and 51 μm^3 for 1 X the value of the mean differential, and between 23.25 and 59.5 μm^3 for 2x the value of the mean differential.
According to this, we are able to conclude that the volume distribution of the spores of *Lycoperdon perlatum* follows a "Gaussian" type of repartition : 1 x the mean differential representing 70.6 % and 2 x the mean differential 95.8 % of the population.
(Refer to clarkered lines on table 1)

Table (1) STATISTIC MEASUREMENT OF Lycoperdon perlatum

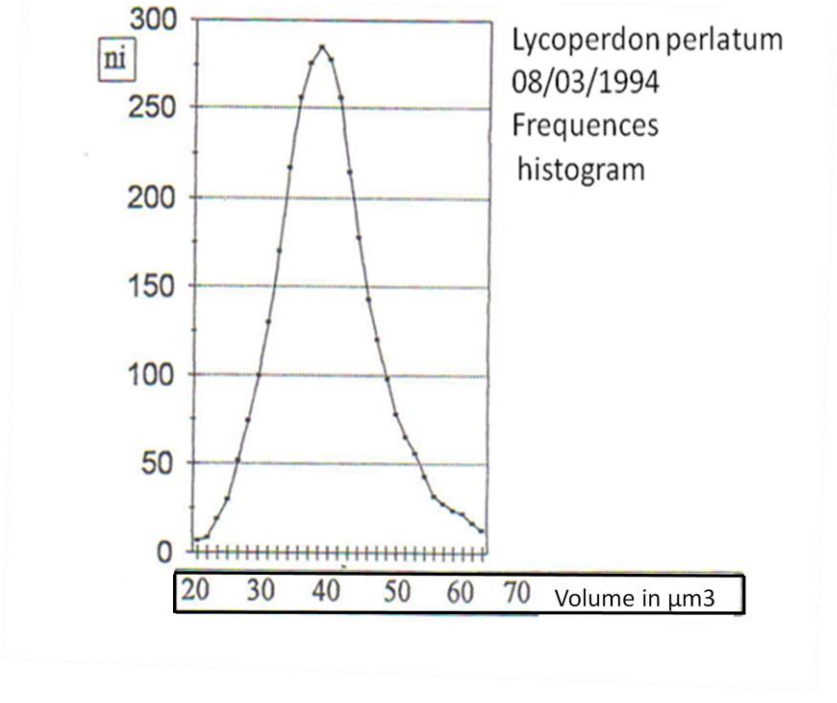
	Class interval volum µm³	Median points	number	Frequence	Mean			Cumulative reduce variable frequences		
		xi	ni	f	xini	$(x-X)^2$	$ni(x-X)^2$	t=x-X/s	Fc	Q(t)
1	18-20	19	7	0.0021	133.00	492.54	3447.78	-2.4087	0.0021	0.00
2	20-22	21	9	0.0027	189.00	407.77	3669.91	-2.1916	0.0048	0.00
3	23-23.5	23.25	19	0.0058	441.75	321.96	6117.25	-1.9474	0.0106	0.00
4	23.5-24.5	24	30	0.0091	720.00	295.61	8868.24	-1.8660	0.0197	0.00
5	24.5-26	25.25	52	0.0158	1313.00	254.19	13217.74	-1.7304	0.0355	0.00
6	26-28	27	74	0.0225	1998.00	201.45	14907.19	-1.5404	0.0580	0.00
7	28-30	29	100	0.0304	2900.00	148.68	14867.55	-1.3234	0.0884	0.00
8	30-31.5	30.75	130	0.0395	3997.50	109.06	14178.00	-1.1334	0.1279	0.00
9	31.5-32.5	32	170	0.0517	5440.00	84.52	14367.71	-0.9978	0.1796	0.00
10	32.5-34	33.25	217	0.0659	7215.25	63.10	13691.68	-0.8621	0.2455	0.00
11	34-36	35	256	0.0778	8960.00	38.36	9819.24	-0.6722	0.3233	0.00
12	36-38	37	276	0.0839	10212.00	17.58	4853.01	-0.4551	0.4071	0.00
13	38-41	39.5	285	0.0866	11257.50	2.87	817.13	-0.1838	0.4937	0.00
14	41-43	42	278	0.0845	11676.00	0.65	180.93	0.0876	0.5782	0.20
15	43-44	43.5	256	0.0778	11136.00	5.32	1362.20	0.2504	0.6560	0.40
16	44-46	45	215	0.0653	9675.00	14.49	3115.63	0.4132	0.7213	0.59
17	46-48	47	178	0.0541	8366.00	33.72	6001.86	0.6302	0.7754	0.76
18	48-49	48.5	143	0.0435	6935.50	53.39	7634.56	0.7930	0.8189	0.91
19	49-50	49.5	120	0.0365	5940.00	69.00	8280.24	0.9016	0.8553	1.06
20	50-52	51	98	0.0298	4998.00	96.17	9424.88	1.0644	0.8851	1.20
21	52-54	53	78	0.0237	4134.00	139.40	10873.14	1.2814	0.9088	1.33
22	54-56	55	65	0.0198	3575.00	190.63	12390.70	1.4985	0.9286	1.46
23	56-57	56.5	56	0.0170	3164.00	234.30	13120.60	1.6613	0.9456	1.60
24	57-59	58	43	0.0131	2494.00	282.47	12146.07	1.8241	0.9586	1.73
25	59-60	59.5	32	0.0097	1904.00	335.14	10724.38	1.9869	0.9684	1.85
26	60-63	61.5	28	0.0085	1722.00	412.36	11546.19	2.2040	0.9769	1.97
27	63-66	64.5	24	0.0073	1548.00	543.20	13036.91	2.5296	0.9842	2.15
28	66-67	66.5	22	0.0067	1463.00	640.43	14089.49	2.7466	0.9909	2.34
29	67-69	68	17	0.0052	1156.00	718.60	12216.23	2.9094	0.9960	0.00
30	69-70	69.5	13	0.0040	903.50	801.27	10416.53	3.0722	1.0000	0.00
		1314.5	3291		135567.00	7008.21	279382.97			
	TOTAL MESURE 3291									

3291

mean **(X)**	41.19
variance	84.89
standard deviation **(s)**	9.21



Curve drawn with experimental results



These results have been also confirmed by a calculation methodology based on the computerized analysis of a curve, or by setting up the "Henry" straight line with the same methodology.
We will observe that in most of the cases, we obtained a "normal" distribution of the volume of the spores of the studied mushroom, except for certain species where we're likely to obtain other types of distribution..

B - *Results explanations.*

The population profile in our example gives an average of 41 µm^3 per spore: this volume corresponds to the most typical one. Limits will be given as "v" for the smaller (corresponding to 2 x the value of the mean differential) and as "V" for the greater volume (corresponding to 2x the value of the mean differential). The most marginal values (2 extremes) will be put between brackets ( ). The % of population corresponds to a "normal" distribution. For each example, the quantity of measured spores will be indicated, as well as the date and the site of the collection. Additional volumes indicated as the bottom of each test result have been calculated from the values "L = 2 b" and "l = 2a" coming from the literature. The applied calculation formula is the one from Breitenbach and Kranzen (1991):

$$V_m = 4/3\ \pi\ a^2\ b$$

This formula can only be applied if the shape of the spores are considered as a "revolution ellipsoid" and thus cannot take into account the influence of the thickness "e" of the spores (in case of compressed or flattened spores): it just considers it as a circle.

VI - Comments on the theoretical measurements of sporal volumes

Our methodology, based on an automated statistical measurement of the volume of spores is rigorously standardized and provide highly reproducible results with a pretty good precision in comparison with a theoretical estimation of this volume, calculated until now from more or less manually determined spore's dimensions. This has been a very motivating factor to pursue the study of our methodology on further species.
We very well know that the spores we come to observe never have the perfect shape of an ellipsoid: indeed, spores harbour various geometrical shapes, which leads us to make two comments:

- Based on the microscopic examination to determine the dimension of spores and with the application of the "theoretical formula of the "revolution ellipsoid", the calculation methodology gives quite good results in case on "round" shaped spores only. As soon as the shape of the spores gets longer or more complicated, this methodology becomes unsuitable.
- The smaller the spores, the more important the impact of measurement errors of the dimensions of the spore on the variations regarding the calculation of the volume.

We are going to illustrate the interest of the methodology of the direct measurement of the volume of spores with the help of the precision rate of both methodologies. Let's suppose the following spore's dimensions:

- L = Length = 7 µm
- l = Width = 6 µm
- e = Thickness = 5.6 µm

Let's consider the precision rate of the measurements at +/- 0.5 µm.
The spore's dimensions would then be the following ones:

- 6.5 < L < 7.5 µm
- 5.5 < l < 6.5 µm
- 5.1 < e < 6.1 µm

When we apply the formula of the "revolution ellipsoid":

$$V = 4/3\ \pi\ x\ L/2\ x\ l/2\ x\ e/2 = \pi/6\ x\ L\ x\ l\ x\ e$$

We obtain the following minimal, mean and maximal values of the spore's volume:

$$95.46 < 123.15 < 155.7\ \mu m^3$$

This leads us to note that a precision rate of +/- 0.5 µm respectively on the length, width and thickness of the spores gives a gap of 27.7 μm^3 between the smallest value and the average and a gap of 32.5 μm^3 for the greatest one.
According to the supplier, the precision rate of the direct measurement of the spore's volume by resistivity is less than 1 μm^3.
To have a rigorous approach of our approach, let's consider a maximal precision rate of 3 μm^3 for the measured volume of 123.15 μm^3. The minimal, mean and maximal spore's volumes will then be:

$$120.15 < 123.15 < 126.15\ \mu m^3$$

The incidence of the precision rate, which has been purposively amplified, on the estimation of the maximal and minimal values of the volume of the spores will be 10 times lower on the values L, l and e. Indeed the recalculated precision rate on the dimensions of the spores will only reach +/- 0.05 µm for the most atypical spores, whereas the usually admitted rate is +/- 0.5 µm.

VII – Drawbacks and advantages on the methodology

A - Drawbacks

- Cost of the system / of the consumables (disposable and sterile containers, thermal paper for the printer, ultra filtered measurement solutions ….) which make it difficult to be a routine methodology.
- Applicable only on fresh collected spore's samples, only coming from carpophores in a very good state.
- Difficulty to obtain a profuse spore's sample without any contaminant.
- Collection of the sample with dedicated specific saline solutions.

B - Advantages

- Rapidity of the measurement: approxamatively 1 min to measure 1 million spores just after the collection of the spore's sample.
- Rapid detection of heterogeneous populations or abnormalities. Determination of the heterosporie.
- Rapid checking (future studies) between the mean size of spores coming from different carpophores or sites.
- Greater precision compared to conventional methodologies.
- Rigorously standardized methodology, with no "human" intervention during the reading.
- All the spores will be measured = no subjectivity with regards to the choice of the measured spores.
- Rapid delivery of an arithmetic average: important in the determination of a species, less in case of a routine examination.
- Possibility to proceed to correlation studies with other methodologies.
- Automatic correction in case of "flattening".
- Easiness to proceed to studies related to the age of the carpophore, environmental conditions of growth etc ….

VIII - Conclusion

From the studied species, we are already able to perceive the advantages of this methodology.
Of course, this is only a preliminary study. The very convincing results, already obtained from the study of a few species, encourage us to confirm them with the analysis of further species, in order to have a proper validation of the methodology.

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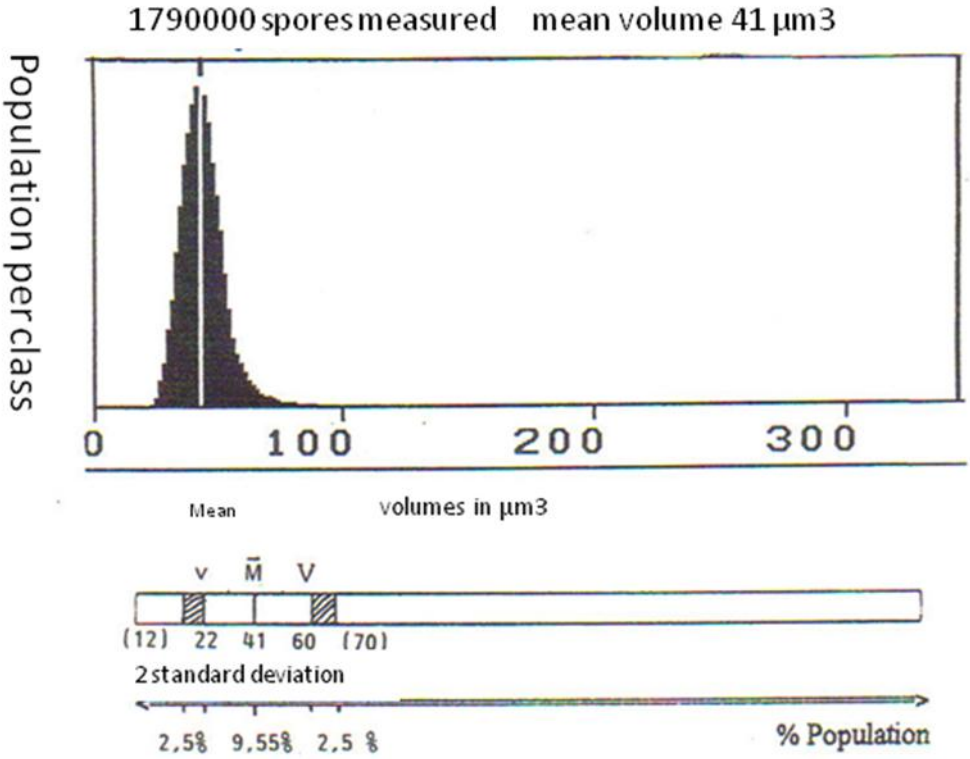
DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

Date 04 30 1994
Location/site : Le Thillot 88 France

Lycoperdon perlatum Pers.:Pers.



Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	22 µm³
Romagnesi H.	22 à 48 µm³
Jülich W.	13 à 45 µm³
Breitenbach J./Kranzlin F.	22 à 48 µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

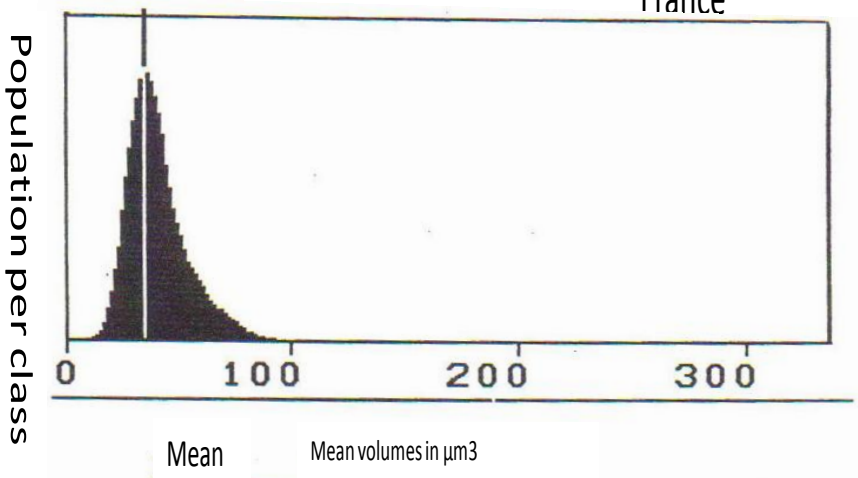
Gymnopus peronatus(Bolton:Fr.) Antonin,Halling & Noordel.
S yn. Marasmius peronatus(Bolton)Fr.

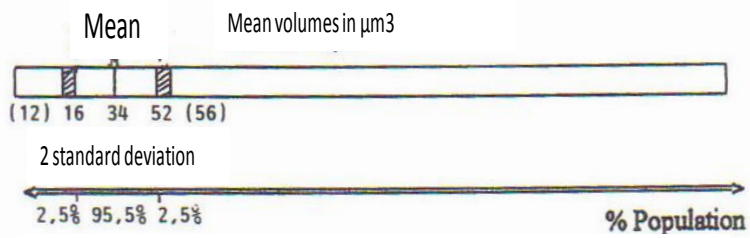
Spores elliptic to fisiform elliptic

Date 08 11 1994

Location/site : Le Thillot ,LePrey 88 France

2000 spores measured mean volume 34 µm3





Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	67 µm³
Romagnesi H.	28 à 75 µm³
Moser M.	28 à 67 µm³
Marchand A.	µm³
Breitenbach J./Kranzlin F.	83 µm³

DIAGRAMS OF THE SPECEES STUDIED

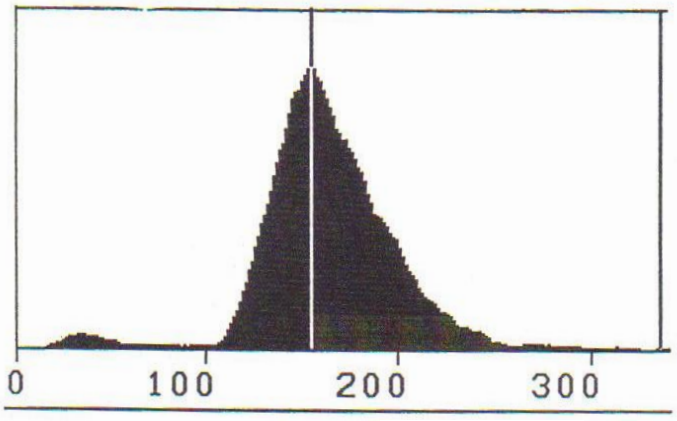
Automated statistic measurement of the volume of spores realised by larger fungi

Russula nigricans Fr.

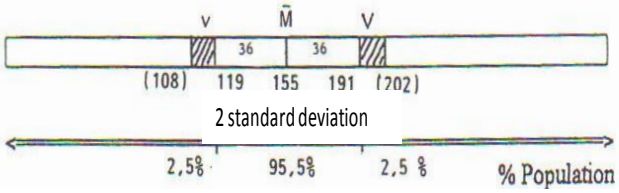
Date 08 23 1994

Location/site : Le Thillot 88 France

4600 spores measured mean volume 155 µm3



Mean volumes in µm3



Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	176 µm³
Romagnesi H.	132 µm³
Moser M.	154 µm³
Marchand A.	µm³
Breitenbach J./Kranzlin F.	µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

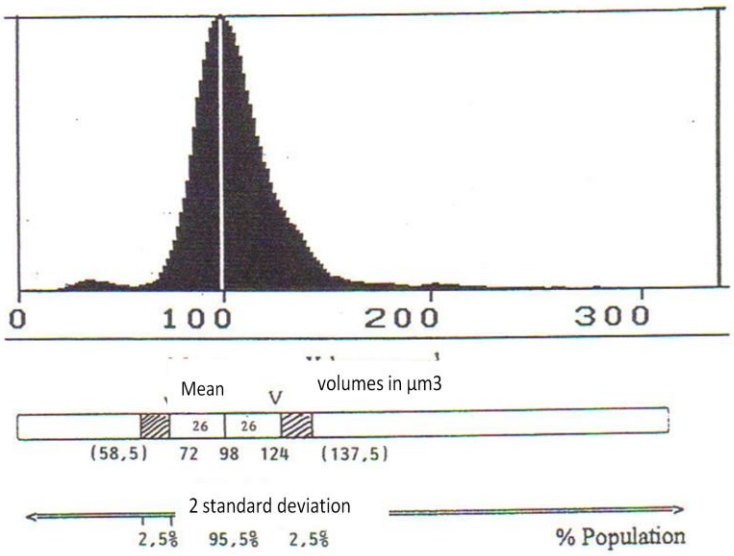
Russula vesca Fr.

Date 08 26 1994

Location/site : Le Thillot 88 France

Subglobular spore

3800 spores measured mean volume 98 µm3



Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	104 µm³
Romagnesi H.	91µm³
Jülich W.	µm³
Marchand A.	78 à 131 µm³
Moser M.	78 à 187µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

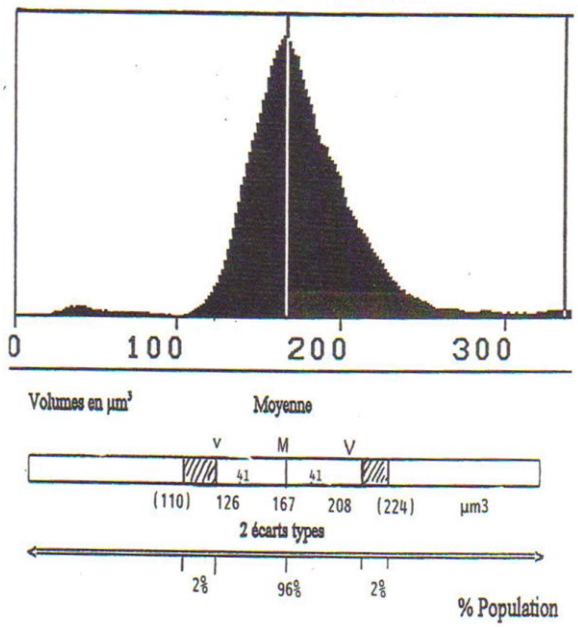
Russula foetens Pers.:Fr.

Date 07 23 1994

Location/site : Le Thillot France

Spores subglobose to ovoid

5200 spores measured . Mean volume 167 µm3



Quelques valeurs calculées du volume des spores à partir des tailles L et l trouvées dans la littérature. (sans correction de l'épaisseur).

Bon M.	334 à 500 µm³
Romagnesi H.	µm³
Jülich W.	µm³
Marchand A.	153 à 334 µm³
Moser M.	µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

Heterobasidion annosum (Fr.:Fr) Bref.

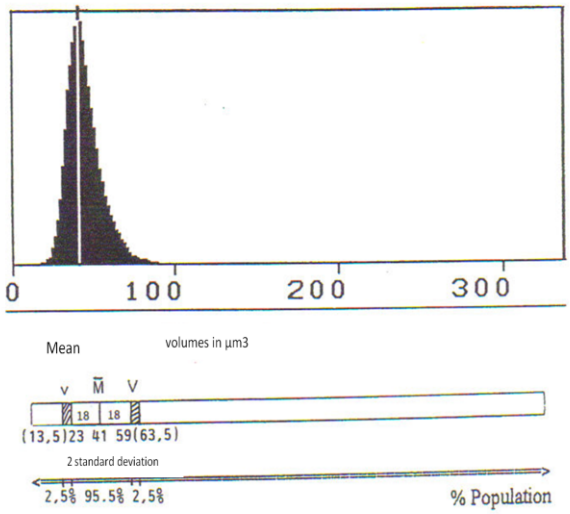
Date 08 10 1994

Location/site : Le thillot Flaconnière

70 France

Mainly ellipsoid spore with flattered dorsal profile

3900 spores measured mean volume 41 µm3



Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	41,8 µm³
Romagnesi H.	µm³
Jülich W.	28 à 63 µm³
Marchand A.	62 à 76 µm³
Breitenbach J./Kranzlin F.	37,6 à 63 µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

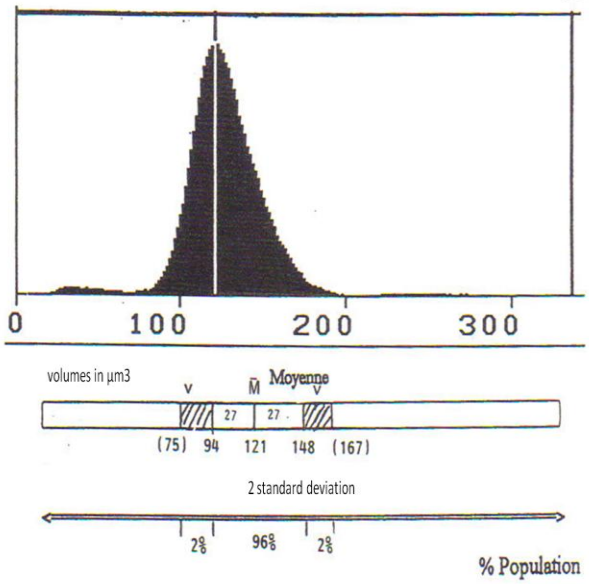
Russula antracina Romagn.

Date 07 23 1994

Location/site : Gray 70 France

Ellipsoid spore

7300 spores measured mean volume 121 µm3



Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	169 µm³
Romagnesi H.	µm³
Jülich W.	µm³
Marchand A.	178 µm³
Moser M.	µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

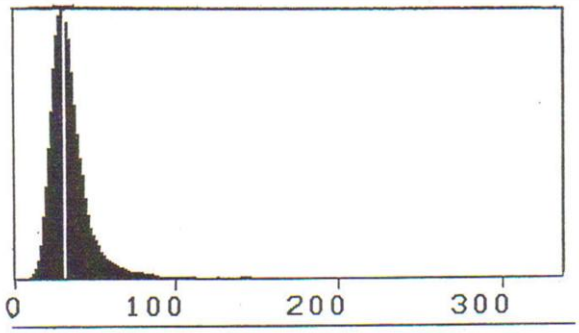
Mycena pura (Pers.:Fr.) Kumm.

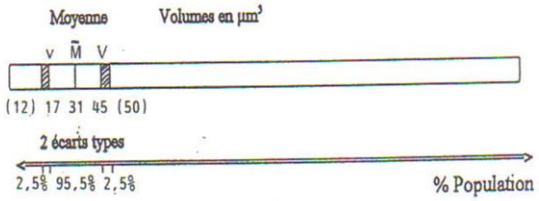
Date 09 04 1994

Location/site : Beulotte 70 France

Elliptic spore

1900 spores measured mean volume 31 µm3





Quelques valeurs calculées du volume des spores à partir des tailles L et l trouvées dans la littérature. (sans correction de l'épaisseur).

Bon M.	58 µm³
Romagnesi H.	35 à 55 µm³
Moser M.	32 à 71 µm³
Marchand A.	µm³
Breitenbach J./Kranzlin F.	55 µm³

DIAGRAMS OF THE SPECEES STUDIED

Automated statistic measurement of the volume of spores realised by larger fungi

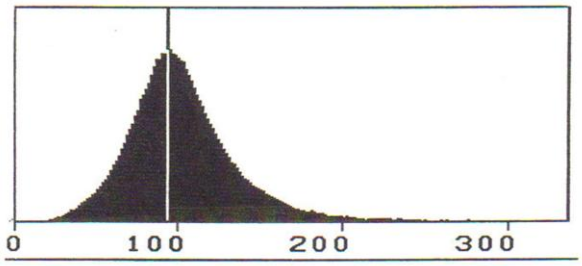
Gyroporus cyanescens (Bull.:Fr.) Quel.

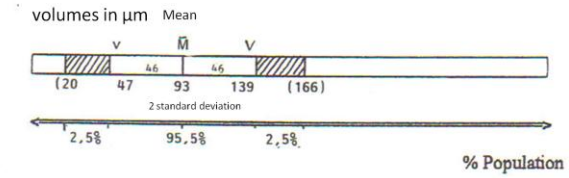
Date 10 03 1994

Location/site : Le thillot 88 France

Mainly ellipsoid spore

6900 spores measured mean volume 93 µm3





Some values of the volume of spores measured by using the length and the width found in the literature (no thickness correction)

Bon M.	307 à 535 µm ³
Romagnesi H.	104 à 188 µm ³
Jülich W.	µm ³
Marchand A.	142 à 158 µm ³
Moser M.	67 à 535 µm ³