SHORTER COMMUNICATIONS

CHEMICAL ANALYSIS OF FIVE SPECIES OF *ASPERGILLUS* BY COMBINED SCANNING ELECTRON MICROSCOPY AND X-RAY SPECTROMETRY

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THIBAUT, M. & ANSEL, M. 1976. Chemical analysis of five species of *Aspergillus* by combined scanning electron microscopy and X-ray spectrometry. *Trans. Amer. Micros. Soc.*, 95: 210–214. A new apparatus, which combines an electronic probe micro-analyzer with an electron microscope, has been used in conducting a systematic elemental analysis of several pathogenic species of the fungus *Aspergillus*. All elements in the periodic table whose atomic numbers lie between 5 (boron) and 92 (uranium) can be detected. All chemical information can be obtained on the same sample. The analysis is made under visual check and all artifacts can be eliminated. The species examined were: *Aspergillus clavatus, A. chevalieri, A. glaucus, A. oryzae,* and *A. versicolor.*

Among physical methods of analysis, X-ray spectrometry combined with scanning electron microscopy offers biology many research possibilities. This method of elemental analysis allows detection of all elements of Mendeleef's periodic classification ranging from 5 (boron) to 92 (uranium). We deemed it interesting to apply this method to the study of fungi pathogenic for humans. The present paper reports our results for five species of *Aspergillus*. We have previously published a note on the approach (Thibaut & Ansel, 1973), using two other species (see Discussion, below).

MATERIALS AND METHODS

The species studied belonged to four groups; the number in parentheses refers to the stain designation used in the culture collection maintained in the parasitology laboratory of the Medical School of the University of Paris:

A. clavatus group: *A. clavatus* Desmazières (469)
A. glaucus group: *A. chevalieri* (Mangin) Thom & Church (629)
A. glaucus Link (392)
A. flavus group: *A. oryzae* (Ahlb) Cohn (393)
A. versicolor group: *A. versicolor* Tiraboschi (746)

We used in vitro cultures of the same age (12 days old) in Sabouraud's glucose infusion. They were washed with sterile demineralized water in order to eliminate any trace of nutrient medium, then dried. Then they were carried over to aluminium slide-cylinders and stuck on them with silver lacquer so as to secure the conductivity of the electrons. The samples were placed in a vacuum evaporator where they were plated with aluminium. For this investigation, we used the Camebax system, combining in the same assembly an electron microprobe with three wave-length X-ray dispersive spectrometers and a scanning electron microscope. The analysis was performed with the help of an inclined spectrometer that allowed working with samples whose relief was up to 1 mm. Ten batches were used and 20 analyses were carried out for each case.

TRANS. AMER. MICROS. SOC., 95(2): 210–214. 1976.

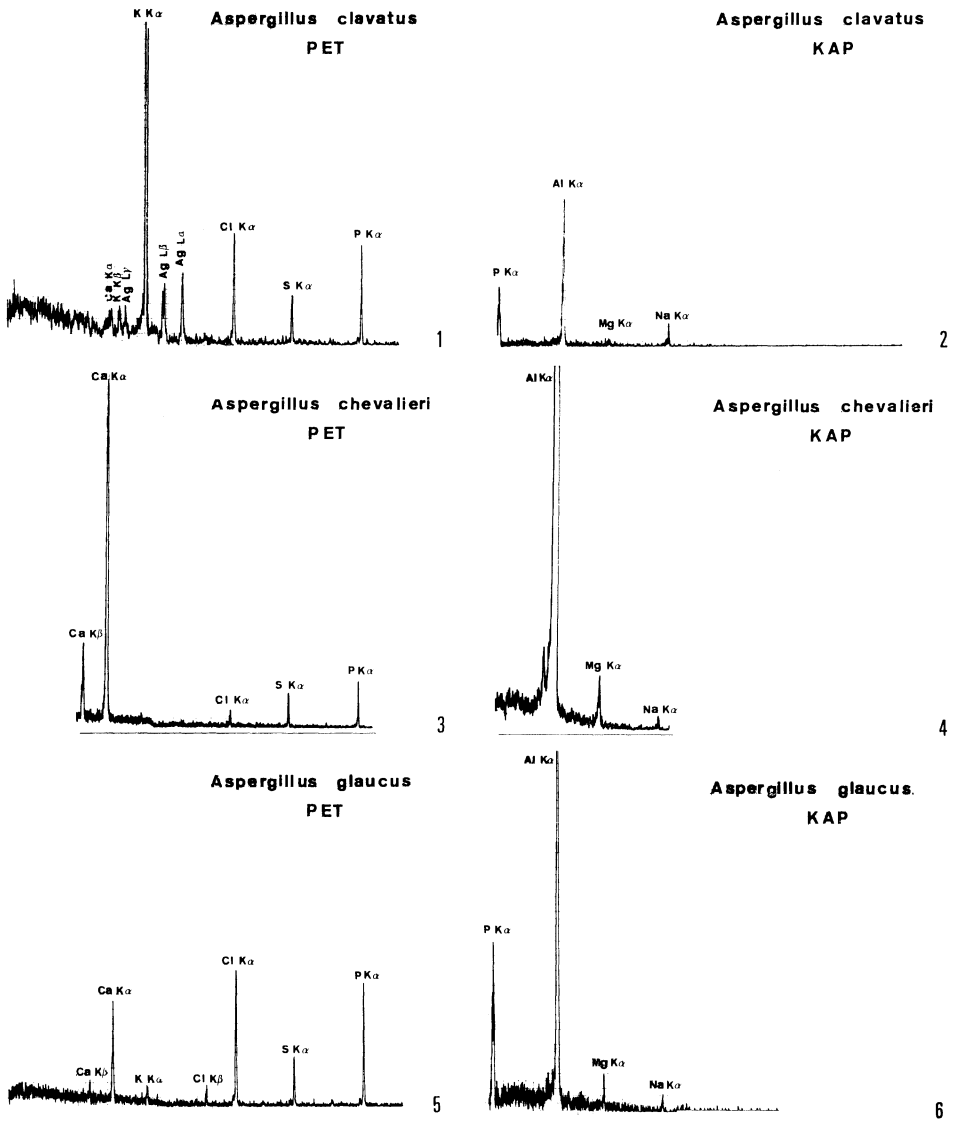

FIG. 1. Study of *Aspergillus clavatus* under wave-length dispersive spectroscopy (Camebax system). With the PET crystal detector are identified: calcium (traces, Kα line), potassium (Kβ and Kα lines), chlorine, sulphur, and phosphorus (Kα lines). The Ag Lγ, Lβ and Lα lines came from the silver lacquer used to stick the samples on the supports. Fig. 2. *A. clavatus*. Study with the KAP crystal detector. The aluminium Kα line comes from metallization. Presence of magnesium (traces) and sodium. Fig. 3. *A. chevalieri*. Characteristic X-ray spectra of various elements (PET crystal detector): calcium (Kβ and Kα lines), chlorine, sulphur, and phosphorus (Kα lines). Fig. 4. *A. chevalieri* (KAP crystal detector): aluminium Kα line coming from metallization, magnesium, and sodium Kα line. Fig. 5. *A. glaucus*. With the PET crystal detector are identified: calcium (Kβ and Kα lines), potassium (Kα line), chlorine (Kβ and Kα lines), sulphur, and phosphorus (Kα lines). Fig. 6. *A. glaucus*. With the KAP crystal detector: magnesium and sodium are identified. The aluminium Kα line comes from metallization.

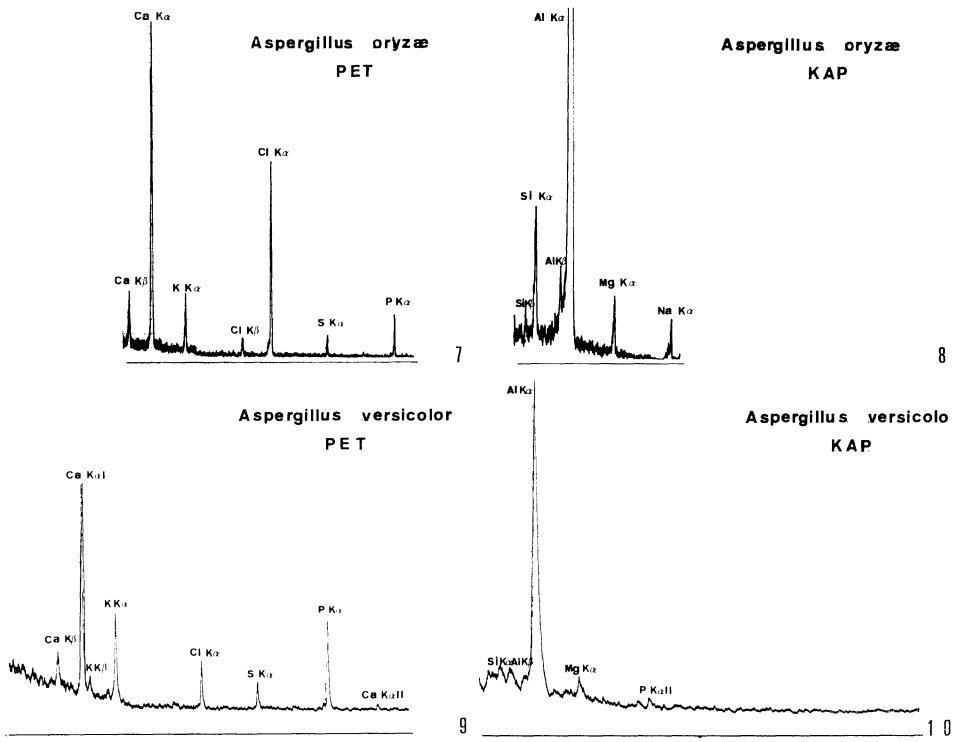

FIG. 7. *A. oryzae.* X-ray spectra of various elements: calcium (Kβ and Kα lines), potassium (Kα line), chlorine (Kβ and Kα lines), sulphur and phosphorus (Kα lines). PET crystal detector. Fig. 8. *A. oryzae.* Spectra of silicon (Kβ and Kα lines), magnesium (Kα line), and sodium (Kα line). The aluminium Kβ and Kα lines come from metallization. KAP crystal detector. Fig. 9. *A. versicolor.* Study with the PET crystal detector: calcium (Kβ, KαI, and KαII lines), potassium (Kβ and Kα peaks), chlorine, sulphur, and phosphorus (Kα lines). Fig. 10. *A. versicolor.* With the KAP crystal detector, the following elements are identified: silicon and magnesium (Kα lines). The aluminium Kβ and Kα peaks come from metallization.

The principle of the electron microprobe is as follows: the impact of a focalized electron beam on an anticathode gives rise to X-rays. The small bombarded area of the sample plays the part of the anticathode. In addition to a continuous spectrum of X-rays, each element emits several sharply defined lines of characteristic wave-length, which can be identified with a crystal spectrometer. Furthermore, the relative intensity of the lines from the different elements gives an accurate indication of the amounts of those elements present. The X-rays emitted were analyzed with crystal detectors: pentaerythritol (PET), acid potassium phthalate (KAP), and lithium fluoride (LIF). X-ray microanalysis was performed with a gas flow proportional counter (argon-methane) with low-pressure feeding in order that soft rays could be produced. The counter contained 10% methane and 90% argon. The conditions required for detection are: accelerating voltage 20 kv, current delivered 50 nA.

The instrument scans the surface of the sample with an electron probe and then analyzes the X-rays excited by the beam that are characteristic of the elements irradiated. Electron microanalysis thus makes possible the qualitative study of the elements within the tissues during the visualization of the sample by scanning.

Results

The analysis of *A. clavatus* in wave-length dispersive spectrometry allowed detection of seven elemental chemical components. With the PET crystal detector specific spectra of the following elements appeared: calcium (traces), potassium, chlorine, sulphur, phosphorus. With the KAP crystal detector: the Kα lines of magnesium (traces) and sodium.

The study of *A. chevalieri* allowed detection of six elemental chemical components. With the PET crystal detector spectra of the following elements: calcium, chlorine, sulphur, phosphorus; with the KAP crystal detector, magnesium and sodium.

In *A. glaucus*, the elements calcium, potassium, chlorine, sulphur, phosphorus were detected with the PET crystal detector; and magnesium and sodium, with the KAP crystal detector.

The chemical analysis of *A. oryzae* showed the presence of eight elements. With the PET crystal detector, potassium, chlorine, sulphur, phosphorus; with the KAP crystal detector, silicon, magnesium, and sodium.

The analysis of *A. versicolor* with the PET crystal detector showed calcium, potassium, chlorine, sulphur, phosphorus; with the KAP crystal detector, the Kα lines of silicon and magnesium.

In the five species, the LIF crystal permitted detection of no element. Besides, the other elements of Mendeleef's periodic classification were not detected. But carbon, hydrogen, oxygen, and nitrogen, components of living matter in general, were not sought for. As far as the negative results are concerned, the method may possibly not be sensitive enough to detect some infinitesimal amounts.

Discussion

The characterization of an element by its X-ray spectrum has been known since the pioneering work by Moseley (1913). The application of X-ray spectrography was developed by Castaing (1951). It soon became an attractive qualitative chemical analysis method due to the simplicity of spectra and to the reliability and rapidity of identification. In the biological area, it was used notably by Galle (1965). In mycology, this method has been used only by Gay (1972), for the study of *Saprolegnia* oospores, and by Thibaut & Ansel (1973), who analyzed two species of *Aspergillus*.

The analysis of our results shows differences in the elemental chemical composition of the five species studied as well as in that of other species, notably *Aspergillus fumigatus* and *A. niger* (Thibaut & Ansel, 1973; and unpublished results of ours). These chemical differences are not superimposable on the morphological differences that form the basis of the classification in various groups. Only *A. chevalieri* contains no potassium. *A. oryzae* and *A. versicolor* are the only species containing silicon.

The constant presence of chlorine, sulphur, phosphorus, and magnesium is also worth noting. Besides, the common denominator of the species of *Aspergillus* is their richness in calcium, except for *A. clavatus*, that is extremely poor in this element. The presence of calcium, therefore, is very well defined and found consistently. The Kα lines of calcium and phosphorus are found at the same topographic level. The presence of calcium phosphate is thus proven.

Histochemical methods (in optical microscopy) are inaccurate and not very specific, particularly with respect to the detection of calcium. Indeed, Lison (1960) showed that these methods did not allow distinguishing between calcium and its salts or to detect crystallic salts at tissue level. Only X-ray spectrometry is able to solve these problems. Previous attempts to localize

sodium or potassium in biological tissue have usually depended on electron histochemical techniques. These methods are unsatisfactory. First, the sodium and potassium ions would be washed out during the tissue preparation process. Secondly, there are doubts about the specificity of the histochemical reaction.

The chemical identification of the substances present in tissues is a problem ever more frequently encountered in biology. Usually this problem cannot be solved by the conventional methods of cytochemistry, for the number of usable and truly specific chemical reactions is rather restricted. Furthermore, even in favorable cases, these techniques require some presumptions as to the nature of the substances present before selecting the proper histochemical reaction. When no hint allows one to suspect beforehand the chemical nature of the material under study, a systematic elemental chemical analysis must be carried out. Electron probe microanalysis thus supplies a means of learning the chemical nature of a given substance, yielding particularly interesting indications, some of which can be obtained with no other method.

X-ray spectrometric analysis has demonstrated the richness in calcium of four species of *Aspergillus*. Differences were found in the elemental chemical composition of the species analyzed. These chemical differences are not superimposable on the morphological differences that form the basis of the classification of the genus *Aspergillus* (Raper & Fennell, 1965).

Microanalysis offers many advantages over histochemical and cytochemical methods. The detection of all elements in Mendeleef's classification can be carried out on the same sample. Since the analysis is performed under visual check, at the magnifications made possible by electron microscope, artifacts can be eliminated with certainty.

It is apparent that the actual range of mycological problems that can be investigated by the combination of scanning electron microscopy and X-ray probe microanalysis is quite immense. Of particular interest is that the technique, as exemplified by the use of the Camebax system, is sufficiently sensitive to detect many elements in the periodic table. Study in wave-length dispersive spectrometry becomes truly exploratory and should lead to new advances in mycological concepts.

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