New archeometric method for wood based on an enzymatic biosensor

Luigi Campanella*, professor, Andrea Antonelli*, graduate in chemistry, Gabriele Favero*, PhD in chemical sciences and Mauro Tomassetti*, professor

Summary A new method is proposed for the determination of wooden artefacts based on the correlation between the number of carboxyl groups present in the wood sample and its age, on the number of carboxylic groups the immobilized enzymatic activity depends. A glucose oxidase biosensor in which a wooden disk on which the enzyme glucose oxidase is immobilized is used, with a gaseous diffusion oxygen amperometric transducer.

Key-words Archeometry, wood, enzyme biosensor.

The growing interest in the scientific dating of historical and artistic finds has stimulated the search for new, possibly simpler and cheaper techniques, to replace the consolidated conventional techniques.

In particular, with reference to the dating of non metal and non stone objects made of cellulose material, the current line of research is aimed at developing methods alternative to conventional procedures, such as dendrochronology [1], ^{14}C dating [2], or more recent methods such as that based on infrared spectroscopy [3]. These methods have many advantages but also several drawbacks that sometimes make them inapplicable. For example, using the ^{14}C method, it is not possible to date wood samples produced after about 1650 owing to the changes occurred in the Earth's atmosphere as a result of increased CO_2 concentration. On the other hand, when infrared spectroscopy is used, the presence of termites in the wood complicates the dating procedure as they modify the chemical composition of the wood, and above all consume some of its components. Lastly dendrochronology is affected by local climatic factors that prevent calibration curves of general validity being constructed.

The aim of the present investigation was thus to develop an innovative method of dating wooden artefacts as an alternative to the more complex and expensive conventional methods. This approach is based on an enzymatic biosensor consisting of a gaseous diffusion amperometric electrode for oxygen determination used as a transducer and a disk-shaped sample of the wood material to be tested on which the enzyme glucose oxidase was chemically immobilized.

Experiment

Reagents

D(+)glucose monohydrate; N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride were supplied by Fluka; anhydrous sodium acetate, potassium dihydrogenphosphate and potassium monohydrogenphosphate were supplied by Carlo Erba; glucose oxidase by Sigma.

Apparatus

The measurements were performed using an Orion mod. 970899 amperometric gaseous diffusion electrode for oxygen determination connected to an Orion mod. EA940 potentiometer and to an Amel mod. 868 analog recorder; a Casio quartz chronometer; a Compaq Presario personal computer.

For all the sample pretreatment and solution preparation operations the following were used: a Lab-Line mod. 1263-1 multiple magnetic stirrer; a Crison mod. 52-02 glass electrode; a Crison mod. GLP 22 pH-meter; a constant-humidity container.

Wood types analysed

The method above described was applied to five different types of wood (chestnut, pine, cherry, olive and walnut), for each one of which dated samples of different age were available.

The choice of these wood types was determined mainly by two needs: the obvious impossibility of performing tests on all the types of wood, led to a choice which falls on these as considered most typical of wooden object construction in the various fields of human activity (artistic, architectural, tool and object production, etc). Furthermore, in order to obtain a correlation between experimental data and ageing it was necessary to have available samples of known age.

It was of course no easy task to obtain these samples. In some cases (chestnut and olive), it was possible to obtain samples dating back to the late 16^{th} century, while only to the 18^{th} century for others (walnut and cherry). In addition, by courtesy

* Department of Chemistry, University of Rome « La Sapienza », P. le Aldo Moro 5, 00185 Roma, Italy. E-mail: mauro.tomassetti@uniroma1.it

of the University of Moscow it was possible to analyse pinewood samples of the period between 11th and 12th centuries. The other samples were obtained from antique dealers, string instrument makers and cabinet makers in the Rome area.

Methods

Principle of the method

Each measurement is based on an enzymatic biosensor consisting of a Clark electrode (gaseous diffusion amperometric electrode) and a thin disk cut from the wooden artefact to be tested on which the glucose oxidase enzyme has been chemically immobilized. The biosensor is used to measure the activity of the immobilized enzyme, which can then be related to the amount of enzyme covalently bonded to the wooden test disk. The method of enzymatic immobilization adopted involves the formation of amide bonds between carboxyl groups in the wooden disk and amino groups in the protein component of the enzyme. Being an established fact that, with time, the constituents of the wood, particularly cellulose, are subject to complex degradation phenomena ultimately leading to the formation, as end products, of compounds having carboxylic groups [4], the abundance of the latter is related to the age of the wooden find; it thus follows that the activity of the immobilized enzyme correlates directly with the age of the wood sample used as a support for enzyme immobilization.

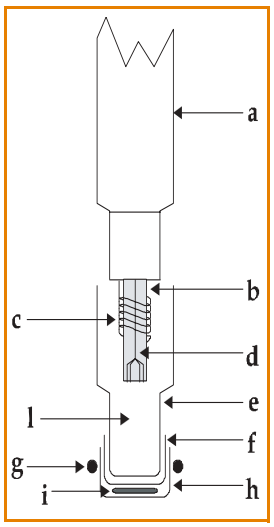

Figure 1 - Biosensor for dating wooden finds.
a: gaseous diffusion amperometric electrode; b: dielectric; c: Ag/AgCl anode; d: Pt cathode; e: electrode cap; f: gas-permeable membrane; g: rubber O-ring; h: nylon net; i: sample (wooden disk with immobilized enzyme); l: internal solution (phosphate buffer 0.067 mol.L^{-1}, KCl 0.1 mol·L^{-1}, pH 6.6).

Sampling and sample pre-treatment

A very thin strip of wood was sanded so as to thoroughly clean the surface. Wooden disks were then obtained from it using a common leather punch, 0.5 cm in diameter and 0.1 mm thick, so that they could easily fit the measuring surface of the electrode.
The disks obtained in this way were then placed inside a humidifier. The latter was in practice an essicator containing a saturated solution of KCl instead of silica gel. The samples were thus maintained in this airtight container at a constant humidity of 37 % for 48 hours. This operation was necessary to ensure uniform moisture content of the samples, which was initially highly variable.

Enzymatic immobilization and biosensor assembly

The enzyme used was glucose oxidase; this enzyme was immobilized on the wooden disks by means of a chemical bond using carbodiimmide [5-6], which acts as an actual bridge, bonding on one side with the protein chain of the enzyme and on the other with the carboxyl groups of the wood. For this purpose, a 0.1 mol.L^{-1} solution of carbodiimide was prepared by dissolving 191.7 mg in 10 mL of phosphate buffer 0.1 mol.L^{-1} at pH 4.8.
The sample was then placed in the solution thus obtained and constantly stirred for one hour. Meanwhile a glucose oxidase solution had been prepared by dissolving 4 000 units of enzyme in 1 mL of acetate buffer 0.1 mol.L^{-1} at pH 5.1.
The enzymatic solution was then stored at a temperature of 4-5 °C until the sample was removed from the carbodiimide solution after the prescribed time had elapsed and placed in a vial containing the glucose oxidase solution described above.
After 24 h the sample thus treated was washed repeatedly to remove any enzyme not chemically bonded to the wood disk, but only adsorbed or physically entrapped inside it. In all, three 20 minute washings were performed, each time using 10 mL of acetate buffer 0.1 mol.L^{-1} at pH 5.1.
After this treatment the sample was ready for measurement and was fixed on the external surface of the Teflon membrane at the bottom of the Clark electrode.
As shown in *figure 1*, the sample was trapped between the gas-permeable membrane of the electrode and a nylon net, itself fixed to the cap by means of an O-ring.
The biosensor thus assembled was hooked up to the measuring apparatus coupled to a recording device.

Measurement using the biosensor

After assembly, the biosensor was ready to perform measurements. It was then immersed in a beaker containing 10 mL of acetate buffer 0.1 mol.L^{-1} at pH 5.1, making sure that the end portion of the electrode was always at the same depth and that constant stirring conditions were rigorously maintained; in this way, it was ensured that also the exchange of oxygen between the solution and the atmosphere remained constant.
Under these conditions, after about 10 minutes, the signal flattened out to form a plateau. At this point 1.0 mL of a glucose solution 1.0 mol.L^{-1} was added as excess substrate.

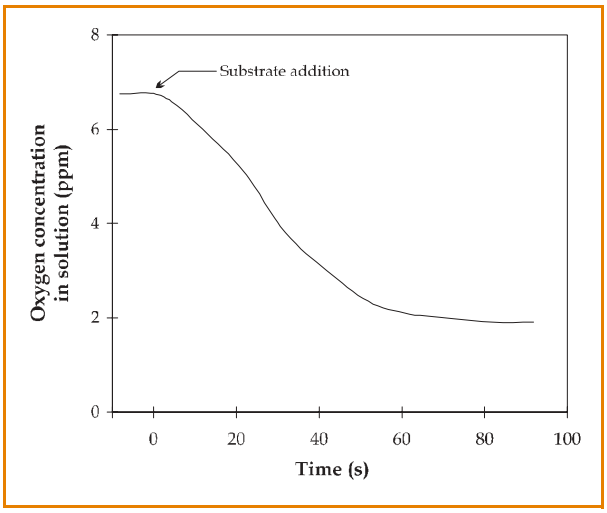

Figure 2 - Typical experimental curve relative to the response of the biosensor for dating wooden finds.

The addition of substrate led to a decrease of the dissolved oxygen concentration due to the enzymatic reaction.
Simultaneously with the addition of glucose a stop watch was started so that every 10 s the number of ppm of O_2 measured by the oxygen electrode could be recorded. The analog recorder connected to the measuring instrument allowed the corresponding curve to be recorded continuously, until the signal again became constant. A new plateau was normally reached after about 100 s (*figure 2*).

Results and Discussion

Figure 2 shows the experimental plot of a typical recorded curve referring to the consumption of dissolved oxygen due to the enzymatic reaction:

$$\beta - \text{glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{gluconic acid} + H_2O_2$$

The extent of the decrease of oxygen concentration recorded between zero time and 100 s since the beginning of the enzymatic reaction correlates with the activity of the immobilized enzyme and thus with the age of the wood under examination.
The method was set up using pinewood and later applied also to the other types of wood considered, using the same approach. Essentially, therefore, a determination was made of the total variation in oxygen concentration after 100 s referring to the pinewood supports of various ages available for testing.
The data were processed by computing the mean oxygen consumption rate, which was equal to the rate of consumption of the substrate (glucose), during the first 100 s of the enzymatic reaction. The immobilized enzyme activity per unit weight of sample, which was expected to correlate with wood age, was then computed.
Figure 3 contains a block diagram showing both the experimental values of oxygen consumption after 100 s and the specific immobilized enzyme activity

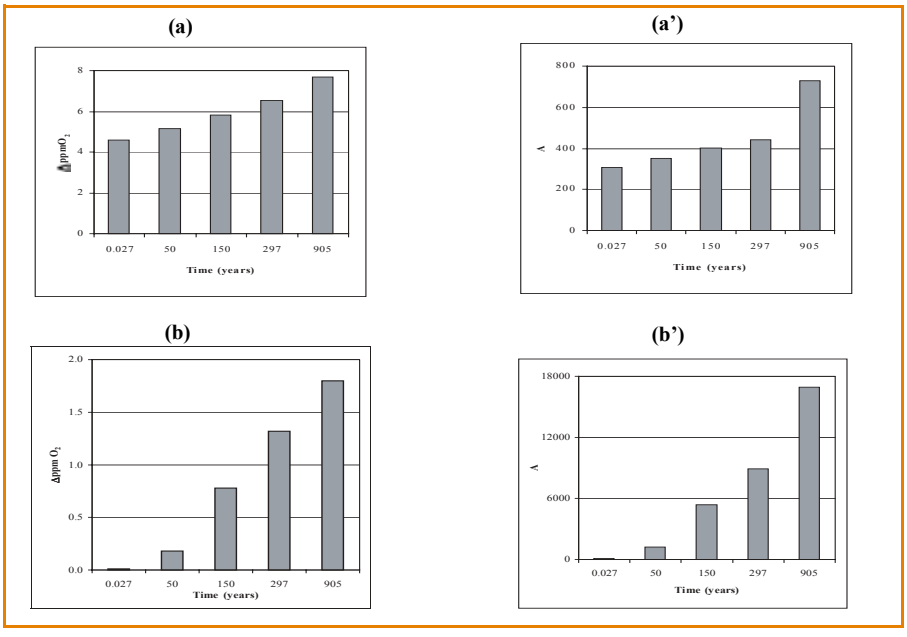

Figure 3 - Block diagrams **(a)** and **(a')**: comparison of trends in experimental data obtained ($\Delta ppmO_2$) or enzymatic activity values (A), computed (for t = 100 s), as a function of sample age. Block diagrams **(b)** and **(b')**: comparison of trends in experimental data obtained ($\Delta ppmO_2$) or enzymatic activity values (A), computed (for t = 10 s), as a function of sample age.

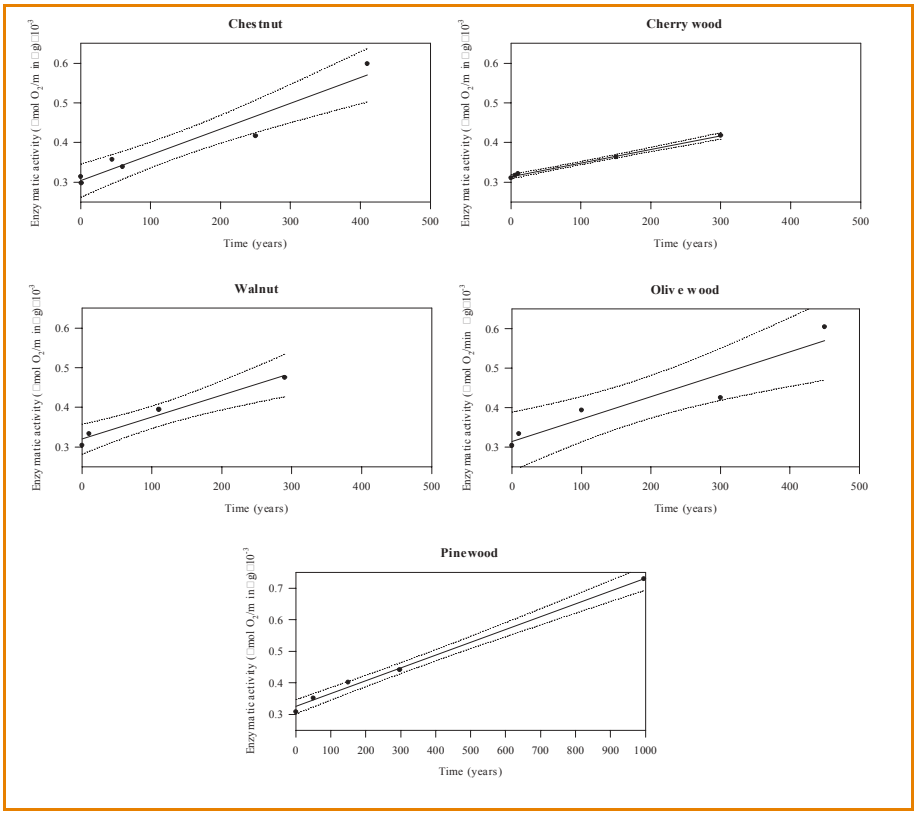

Figure 4 - Archeometric curves: specific immobilized enzymatic activity, per unit weight of sample, measured, for t = 100 s, as a function of wood sample age, obtained using the biosensor for wooden find dating and for different wood types.

per unit weight of sample, computed as described above. As expected, the two trends are clearly related and each is an increasing function of wood age. Archeometric curves were constructed to represent the activity as a function of wood sample age for all the different wood types investigated (*figure 4*). For the curves thus obtained the straight line regression equations and the correlation coefficient were computed for all the different wood types investigated. Examination of the coefficients of correlation shown in *table I* indicates that only in the case of pine and cherry wood there is a sufficiently linear correlation between enzymatic activity and wood age. In the other cases, the curve trend tends to be of the non linear type. However, only when a larger number of experimental data are available, that is, in practice when a larger number of wood samples of known date are available, will a more rigorous decision in this connection be possible.

It is also observed that the curves do not pass through the origin of the axes. In order to account for this in physical terms, the method was applied

Table I - Straight line equation and coefficient of correlation for immobilized specific enzymatic activity per unit weight of sample as a function of age, computed for samples of different wood species. Time span considered: 100 s.

Wood species	**Time span considered**	**Straight line equation** (y = µmol O_2/min × g; x = years)	**r^2**
Chestnut	XX – XVI century	$y = 0.649\,x + 304$	0.9359
Cherry wood	XX – XVIII century	$y = 0.342\,x + 314$	0.9960
Walnut	XX – XVIII century	$y = 0.555\,x + 320$	0.9698
Olive wood	XX – XVI century	$y = 0.567\,x + 315$	0.8987
Pinewood	XX – XII century	$y = 0.407\,x + 325$	0.9946

Table II - Experimental values of immobilized specific enzymatic activity, per unit weight of sample, found for modern pinewood samples obtained from trees cut down 5 to 50 years ago.

Pinewood age	A (µmol O_2/min×g)	RSD %
5 year old pinewood	309	5.66
50 year old pinewood	308	5.60

also to samples taken from living uncut pines of different biological ages ranging from 5 to 50 years. This type of measurement was performed to detect the presence of carboxyl groups also on wood samples taken from living trees. The results, shown in *table II*, show that, in this case, the value obtained for the activity is very close to that obtained as intercept on the y axis of the equation shown in *table I*. It is also interesting to note that, comparing the activity values obtained for two pinewood samples taken from living trees of different ages (in this specific case the trees were 5 and 50 years old, respectively) and growing in the same area, the activity data obtained were practically identical. This would seem to indicate that certain carboxyl groups are already present in the fresh wood, or else that the oxidation process giving rise to the carboxyl groups begins as soon as the tree is cut.

In any case this shows that whenever this method is used to determine the archeometric age of a wooden object, the result is independent on the tree's life span. If this was not the case it could sometimes be the non negligible cause of failure to obtain a precise determination. Indeed it must be borne in mind that pinewood, like other wood types, may be centuries old.

This type of investigative method gave the same result when applied to the other wood types investigated.

Examination of the curve in *figure 2* shows that measurement of the rate of variation of O_2 concentration, which correlates with the variation in glucose (substrate) concentration, could actually be performed more correctly if it was carried out only in the initial section of the curve, which displays a decreasing monotonic trend, rather than over the whole curve. A mean value of the reaction rate would thus be computed for the first 100 s. Taking this into account, also the oxygen consumption rate, equal to the rate of disappearance of substrate (glucose) was computed only for the first 10 s of the enzymatic reaction. Therefore, also in this case, the value of the specific immobilized enzyme activity per unit weight of sample, which was expected to be an increasing function of the sample's age, was computed.

Also using this procedure archeometric curves were thus constructed in which the activity is represented as a function of wood sample age for all the wood types considered (*figure 5*). The respective straight line regression equations and the coefficient of correlation are shown in *table III*.

Analysing several of these curves in greater detail the trend of the correlation between activity and wood age is found to be apparently logarithmic rather than linear in nature. Also in this case, however, this point will be clarified only when a larger number of experimental data will become available.

Lastly, the block diagram in *figure 6* shows the respective percentage relative standard deviations (RSD %) of the activity values (A) obtained for pinewood, using each one of the measurement methods described above, respectively, (i.e. for t = 10 s and t = 100 s).

It should be noted that, in both cases, RSD % values decrease with increasing wood age, that is, the precision of the method increases with increasing wood age. However, it is also significant that the RSD % values vary differently with increasing sample age according to whether the activity is

Table III - Straight line equation and coefficient of correlation for immobilized specific enzymatic activity per unit weight of sample as a function of age, computed for different wood species. Time span considered: 10 s.

Wood species	Time span considered	Straight line equation (y = µmol O_2/min × g; x = years)	r^2
Chestnut	XX – XVI century	$y = 30.04\,x + 237$	0.9918
Cherry wood	XX – XVIII century	$y = 24.35\,x + 353$	0.9326
Walnut	XX – XVIII century	$y = 32.61\,x + 301$	0.9895
Olive wood	XX – XVI century	$y = 24.87\,x + 887$	0.9452
Pinewood	XX – XII century	$y = 16.15\,x + 1698$	0.9330

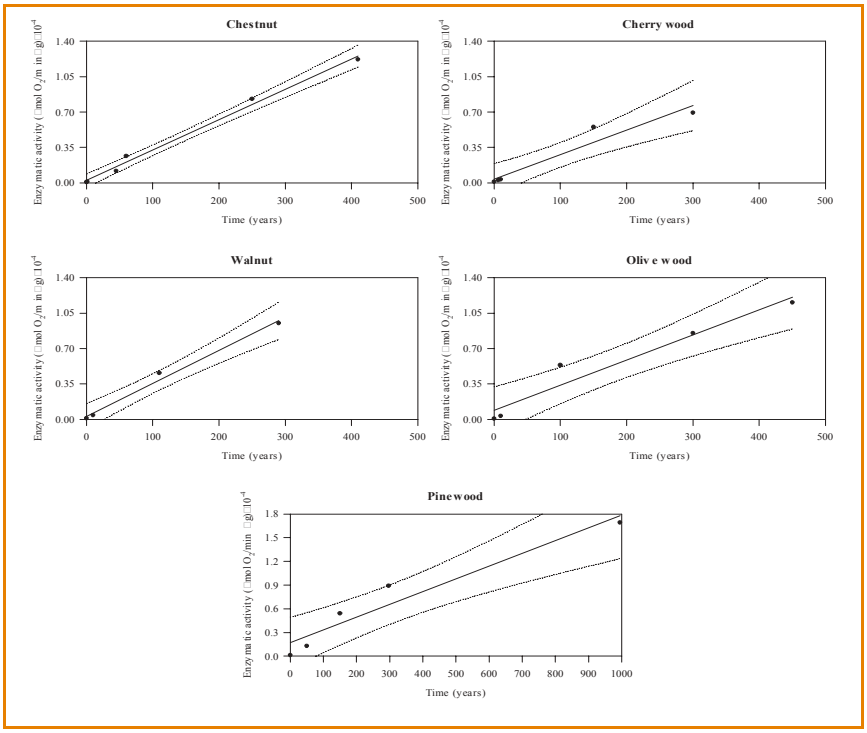

Figure 5 - Archeometric curves: specific immobilized enzymatic activity, per unit weight of sample, measured, for t = 10 s, as a function of wood sample age, obtained using the biosensor for wooden find dating and for different wood types.

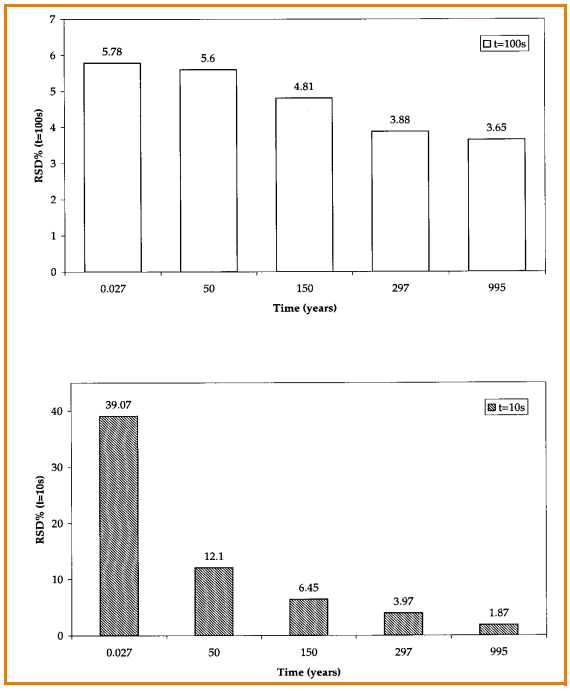

Figure 6 - Precision trends (computed as RSD %) in the measurement of specific immobilized enzymatic activity per unit weight, with increasing sample age (time expressed in years) for the case in which the value of the activity is measured as a function of the mean rate observed within the first 100 s (light coloured block diagrams), or on the basis the rate observed during the first 10 s (dark block diagrams).

determined during the first 100 s or during the first 10 s of the enzymatic reaction. The examination of these trends shows that the activity value computed for t = 100 s is apparently more suitable for evaluating wooden objects up to an age of about 300 years; while the activity value for t = 10 s is more suitable for older samples. It may be concluded that the results show that an activity value computed using one method or the other may be used for the purpose of obtaining a lower RSD %, depending on the age of the wood to be tested.

Lastly, the immobilized specific enzymatic activity may be determined per unit of sample area, as is often reported in the literature [7], rather than per unit weight. However, in the present research, as both the weights and the areas of all the samples tested were almost constant (in fact the weight of heaviest sample is about 1.5 the weight of the lightest one), the trends of the calibration curves found would practically be the same in any case.

Conclusions

In conclusion, the results obtained in the present research allowed a method to be developed which may be considered as a support for or an alternative to the existing techniques mentioned above.

Although it cannot yet be considered definitive, as more tests are required for its validation, it may nevertheless be claimed that the main experimental conditions for the method have been established.
The method makes it possible to analyse small quantities of sample, which is an indubitable advantage in the case of real samples. This justifies its inclusion among the « comparatively non destructive » methods. Furthermore, the limited sample pretreatment operations required are simple and easy to be performed. However, it must be admitted that even the simple sample preparation operations described are important as they ensure a good reproducibility of results. Indeed the use of the humidifier described in the experimental section is essential for good measurement repeatability.
As far as enzymatic immobilization is concerned, on the basis of precise literature indications [5-6], it was possible to optimize the method in such a way as to avoid any particular problems related to the formation of the chemical bond between the enzyme and the wood samples tested in the present investigation. It was thus possible to use this immobilization technique successfully in the development of the analytical method.
Moreover this seems quite reasonable for the following reasons: when a measurement is performed for t = 100 s, a variation of the signal is measured (see *figure 3*(a) and (a')), and thus of the mean rate and thus of the activity, the value of which does not vary appreciably with increasing wood age; consequently, also the measurement error remains practically constant (in this case the RSD % actually varies little with varying wood age); the opposite is true if the measurement is made over t = 10 s. The value of the signal and thus of the measured activity becomes much greater with increasing wood age (*figure 3*(b) and (b')); the value of RSD % thus becomes increasingly small, that is, a measurement is obtained that increases in precision with increasing wood age.
It is thus possible to construct archeometric curves that can give the approximate age of wooden samples of unknown age provided they belong to one of the species listed above.
The main features of the method are: it is simple and easy to apply, very cheap, and with no need of costly or sophisticated equipment.

Acknowledgement

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Le Pr Campanella est un spécialiste de la chimie analytique, s'intéressant en particulier aux biocapteurs et à leurs applications. Il a été également l'un des cinq rédacteurs-associés étrangers d'*Analusis* au cours des années 1990-2000. A ce titre, il a rédigé de nombreux articles, agi comme relecteur impartial, et aiguillé de nombreux auteurs européens à publier leurs résultats dans ce journal.