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A new HPLC analytical method to study fungal caffeine metabolism

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A simple and rapid HPLC method has been developed to analyse all the methylxanthines that can be produced by *N*-demethylation of 1,3,7-trimethylxanthine (caffeine). This method is particularly suitable to study caffeine metabolism of a filamentous fungus (*Aspergillus* sp V12A25) cultivated in a synthetic liquid medium containing caffeine as the sole source of nitrogen.

Abbreviations

1MX, 1-methylxanthine; 3MX, 3-methylxanthine; 7MX, 7-methylxanthine (heteroxanthine); 13dMX, 1,3-dimethylxanthine (theophylline); 17dMX, 1,7-dimethylxanthine (paraxanthine); 37dMX, 3,7-dimethylxanthine (theobromine); 137tMX, 1,3,7-trimethylxanthine (caffeine).

Introduction

Caffeine (1,3,7-trimethylxanthine) is an alkaloid present in more than 60 plant species, the more common of which are Coffea, Camellia, Theobroma and Cola genera (Suzuki and Waller, 1988). The biological and pharmacological effects of caffeine are well known: stimulation of the central nervous system, toxicity when fed excessively (Arnaud, 1987), and mutagenicity on microorganisms (Haynes and Collins, 1984).

Coffee pulp is the principal by-product generated in large quantities during the wet method of coffee cherry processing (Zuluaga, 1989). It contains about 1% (dry wt) of caffeine which considerably limits its potential use as animal feed, in spite of its high protein and sugar content (Bressani, 1978). Nevertheless, a number of fungi can specifically degrade caffeine to detoxify coffee pulp (Perraud-Gaime and Roussos, 1997). With the aim of finding an alternative way to chemical decaffeination of coffee, investigations have also been made in the past to isolate fungi that were able to degrade caffeine (Kurtzman and Schwimmer, 1971). However, the metabolism of caffeine by such microorganisms has not been clearly explained.

The primary purpose of the current study was to develop a new analytical method of methylxanthine quantification by HPLC, suitable to identify the initial steps of fungal caffeine metabolism, using a caffeine-degrading filamentous fungus cultivated in a synthetic liquid medium.

Materials and methods

Strain

Aspergillus sp V12A25 was isolated in Mexico from agro-industrial coffee by-products. It has been shown to be particularly effective in degrading caffeine (Roussos *et al.*, 1995).

Culture media

Maintenance and conidia production medium

The coffee infusion/sucrose-medium (CIS-medium) was prepared as follows: 40 g commercial ground coffee (Grand'Mère "Familial", Café Grand'Mère S.A., Wattignies, France) was boiled in 1 l distilled water and filtered to remove coffee grounds. Then, sucrose 2.0 g; KH_2PO_4 1.3 g; $Na_2HPO_4.2H_2O$ 0.12 g; $MgSO_4.7H_2O$ 0.3 g and $CaCl_2.2H_2O$ 0.3 g were added, pH was adjusted to 5.6 with 1 M KOH, and the volume was brought to 1 liter. The medium was then supplemented with 20 g agar and sterilized at 121°C for 20 min.

Growth medium

The composition of the synthetic caffeine and sucrose medium (CS-medium) was (g/l): anhydrous caffeine 3.0; sucrose 28.4; KH_2PO_4 1.3; $Na_2HPO_4.2H_2O$ 0.12; $MgSO_4.7H_2O$ 0.3 and trace-element stock solution 10 ml. Initial pH was adjusted to 4.0 by addition of H_2SO_4 1 M. This medium (100 ml) was put into 500 ml-Erlenmeyer flasks and sterilized at 121°C for 15 min. The sterile trace-element stock solution contained (g/l): $FeSO_4.7H_2O$ 3.0 and $ZnSO_4.7H_2O$ 2.2. It was added after sterilization of the CS-medium.

Culture conditions and sampling

The CS-medium was inoculated with conidia suspension at 10^6 conidia/ml, and flasks were incubated at 30°C for 5 days with agitation 150 rpm. Conidia suspension used for inoculation, was obtained from cultures in 250 ml-Erlenmeyer flasks containing 25 ml CIS-medium, incubated at 30°C for 7 days. Conidia suspension was prepared as follows: 30 ml sterile distilled water containing Tween 80 (0.2% w/v) was added to the culture flasks and conidia were gently suspended with a magnetic stirrer (100 rpm).

Samples were removed regularly from the cultures. Biomass was discarded from the supernatant by filtration on a 0.45 μm filter. Filtrates were stored at −20°C before analysis.

HPLC assay of methylxanthines

Chemicals

Caffeine was purchased from Farmitalia Carlo Erba (Milano, Italia); other methylxanthines and β-hydroxyethyltheophylline, from Sigma. Acetonitrile and tetrahydrofurane were chromatography grade products from Merck.

Sample preparation

Before analysis, samples of *Aspergillus* sp V12A25 cultures were diluted to achieve a final concentration for each methylxanthine between 2 to 20 mg/l. In the same time, an internal standard (β-hydroxyethyltheophylline) was added to reach a final concentration of 10 mg/l in the samples.

Chromatographic conditions

Methylxanthine concentrations were measured using a gradient-HPLC system consisting of a model PU 4100 Philips gradient liquid chromatograph with a model Pye Unicam PU 4021 Philips multichannel detector. An injection loop of 100 μl was used. The column used was a Supelcosil LC-18 (5 μm particule size), 4.6 × 250 mm (Supelco, Inc., Bellefonte, PA, USA), equilibrated at 32°C. Methylxanthines were resolved by gradient elution with two solvents. Solvent A consisted of H_3PO_4 1.75mM/acetonitrile/tetrahydrofuran (98:1:1, by vol) and solvent B consisted of acetonitrile/tetrahydrofuran (99:1, v/v). A flow rate of 2 ml/min was maintained during the run. The HPLC analysis was started with 100% of solvent A. After 5 min, solvent B was added reaching 20% by 5 min. After 1 min with 20% of solvent B, initial conditions were finally recovered within 3 min. All methylxanthines contained in the samples were detected by UV absorbance at 273 nm and quantified using against standard curves established for each methylxanthine, and for the internal standard. Linear standard curves were obtained for each methylxanthine ranging from 0 to 20 mg/l.

Results and discussion

Improvements in the separation and identification of methylxanthines

The new HPLC method we developed for the separation and the analysis of methylxanthines is simple and rapid (Fig. 1A). It provides a good separation of all methylxanthines in 19 min between two injections. It is sensitive to low concentrations of metabolites since the detection limit for each compound assayed is ranged from 1 to 2 mg/l. This method also allows to clearly determine all the intermediate metabolites possibly produced by reactions of *N*-demethylation of caffeine.

As far as the time used to carry out this methodology, the method described here can be considered an intermediate one between very rapid methods developed to measure only caffeine and dimethylxanthines (Aramaki *et al.*, 1991; Tanimura, 1994), and those for all caffeine metabolites found in mammalians and humans, including methylxanthines and methyluric acids (Lelo *et al.*, 1989; Berthou *et al.*, 1989) which take two to three times longer to run than our new method.

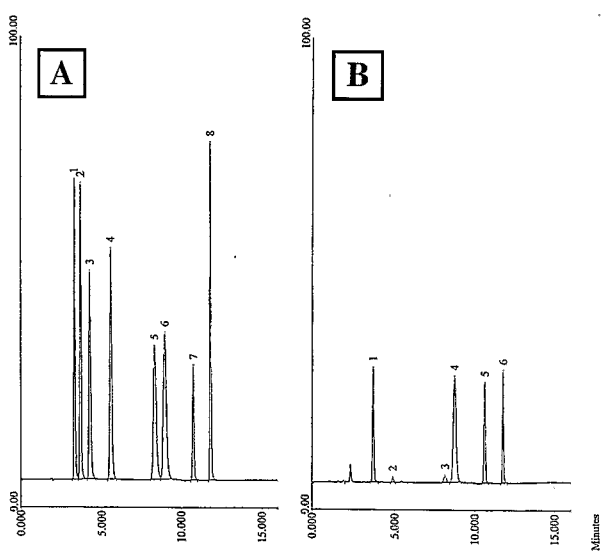

Figure 1 HPLC chomatograms of methylxanthines. **A.** Methylxanthine standards (1: 7-methylxanthine; 2: 3-methylxanthine; 3: 1-methylxanthine; 4: 3,7-dimethylxanthine; 5: 1,7-dimethylxanthine; 6: 1,3-dimethylxanthine; 7: Internal Standard; 8: 1,3,7-trimethylxanthine). **B.** Sample of *Aspergillus* sp V12A25 culture on CS-medium (1: 3-methylxanthine; 2: unknown; 3: 1,7-dimethylxanthine; 4: 1,3-dimethylxanthine; 5: Internal Standard; 6: 1,3,7-trimethylxanthine).

Method implementation towards the identification of fungal caffeine-demethylating metabolites

The described HPLC method has been successfully applied to the identification of caffeine-demethylating metabolites present in the supernatant of the *Aspergillus* sp V12A25 culture on CS-medium. Example of an HPLC chromatogram of a sample is shown in Figure 1B. No extraction was necessary in order to identify correctly the metabolites present in the supernatant. However, a high concentration of caffeine was used in the CS-medium (3 g/l) to optimize the detection of metabolites. HPLC-chromatograms of supernatant samples presented no interference between unidentified compounds and methylxanthines, and separation times of the metabolites did not change from those of the aqueous standard solution (Fig. 1B).

Evolution of the methylxanthines quantified to more than 10 mg/l in the supernatant of an *Aspergillus* sp V12A25 culture in CS-medium, is shown in Figure 2. The principal methylxanthines identified in the supernatant were the 13dMX and the 3MX. However, all other methylxanthines were also detected using the new HPLC method: the highest concentration measured for 17dMX was 46 mg/l, and concentrations less than 8.5 mg/l were found for 37dMX, 7MX and 1MX.

The time-lag observed between 13dMX and 3MX production during *Aspergillus* sp V12A25 growth shows that caffeine degradation is achieved by successive demethylation steps (Fig. 2). The first one is a demethylation in the 7-position of the purine ring, leading to an accumulation of 13dMX in the supernatant up to 850 mg/l. Then a demethylation at position 1 of 13dMX, leads to an accumulation of 3MX up to 340 mg/l. These results have been summarized in Figure 3, where the first steps of the main caffeine metabolic pathway of *Aspergillus* sp V12A25 is proposed. Nevertheless, the identification of other di- and mono-methylxanthines suggests that secondary pathways of caffeine degradation could exist, or that the fungal

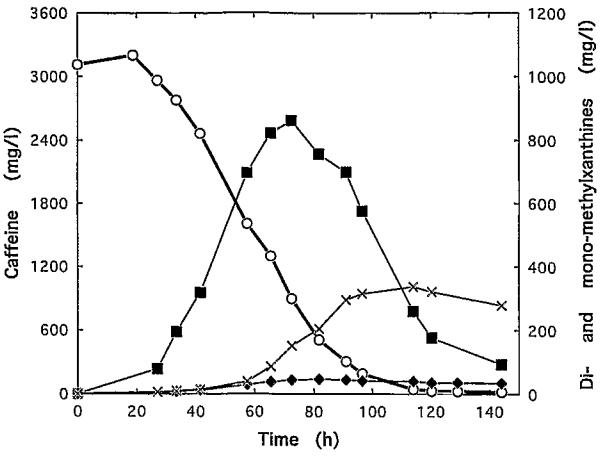

Figure 2 Consumption of caffeine and evolution of the major methylxanthines accumulated in the supernatant during growth of *Aspergillus* sp V12A25 on CS-medium. (○: 1,3,7-trimethylxanthine; ■: 1,3-dimethylxanthine; ◆: 1,7-dimethylxanthine; ×: 3-methylxanthine).

enzymes involved in the mechanism of *N*-demethylation are not specific.

Our preliminary results are identical to those of Ina (1971) who showed that an *Aspergillus niger* strain isolated from tea garden soil degraded caffeine to xanthine via 13dMX and 3MX. We can stress here that fungal metabolism differs from that of bacteria (mainly *Pseudomonas putida* stains) in which initially the 1-methyl group is primarily removed, followed by the 3-methyl group, leading to theobromine and 7-methylxanthine respectively (Blecher and Lingens, 1977; Hohnloser *et al.*, 1980; Asano *et al.*, 1993). It also differs from observations made in human caffeine metabolism where the 3-methyl group is first removed (Arnaud, 1987).

Differences in metabolic pathways of caffeine suggest that different enzyme systems might be involved in fungal, bacterial and mammalian catabolism of this alkaloïd. If the

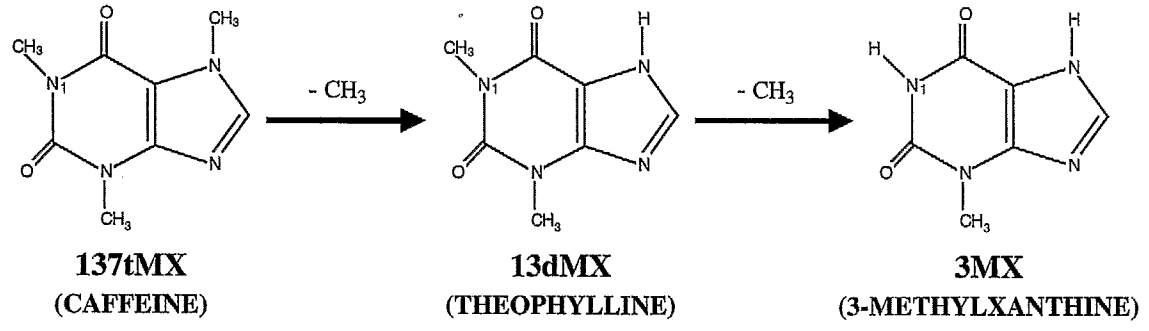

Figure 3 First steps of the caffeine degradation pathway by *Aspergillus* sp V12A25.

latter is well known and extensively studied, enzyme systems involving fungal degradation of caffeine have never been investigated. The new HPLC technique developed here for the determination of caffeine and its metabolites will allow a precise physiological study of caffeine-degrading fungi and the biochemical mechanisms involved.

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