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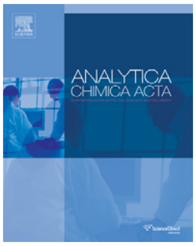

Rapid analysis of fungal cultures and dried figs for secondary metabolites by LC/TOF-MS

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ABSTRACT

A liquid chromatography–time-of-flight mass spectrometry (LC/TOF-MS) method has been developed for profiling fungal metabolites. The performance of the procedure in terms of mass accuracy, selectivity (specificity) and repeatability was established by spiking aflatoxins, ochratoxins, trichothecenes and other metabolites into blank growth media. After extracting, and carrying out LC/TOF-MS analysis, the standards were correctly identified by searching a specially constructed database of 465 secondary metabolites. To demonstrate the viability of this approach 11 toxigenic and four non-toxigenic fungi from reference collections were grown on various media, for 7–14 days. The method was also applied to two toxigenic fungi, *A. flavus* (200–138) and *A. parasiticus* (2999–465) grown on gamma radiation sterilised dried figs, for 7–14 days. The fungal hyphae plus a portion of growth media or portions of dried figs were solvent extracted and analysed by LC/TOF-MS using a rapid resolution microbore LC column. Data processing based on cluster analysis, showed that electrospray ionization (ESI)–TOF-MS could be used to unequivocally identify metabolites in crude extracts. Using the elemental metabolite database, it was demonstrated that from culture collection isolates, anticipated metabolites. The speed and simplicity of the method has meant that levels of these metabolites could be monitored daily in sterilised figs. Over a 14-day period, levels of aflatoxins and kojic acid maximised at 5–6 days, whilst levels of 5-methoxysterigmatocystin remained relatively constant. In addition to the known metabolites expected to be produced by these fungi, roquefortine A, fumagillin, fumigaclavine B, malformins (peptides), aspergillic acid, nigragillin, terrein, terrestric acid and penicillic acid were also identified.

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1. Introduction

In recent years, liquid chromatography–time-of-flight mass spectrometry (LC/TOF-MS) has become accessible for use as a routine analytical tool. TOF-MS uniquely offers the possibility of providing accurate mass data at high sensitivity and can function across a wide mass range without loss in sensitivity. The possibilities of exploiting applications of LC/TOF-MS are really only just becoming apparent, with publications appearing in the fields of emerging environmental contaminants [1], estrogens in river sediments [2], pesticides in fruit and vegetables [3], herbicides in olive oil [4], fungal metabolites and mycotoxins [5,6]. In these examples, the mass accuracy of the TOF-MS has provided discrimination of the target compounds from other interferences frequently present in complex matrices like olive oil. This was clearly demonstrated for example in a validation study when seven laboratories reported the herbicide diuron present from 0.03 to 0.05 mg kg^{-1} in an olive oil

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sample which subsequently by LC/TOF-MS was shown to be a co-eluting peak with the same nominal mass as diuron, but had a mass difference of 0.1 Da [4]. Nielsen and Smedsgaard [6] showed that positive ESI–TOF-MS could be used to provide confirmatory data to support tentative characterization of fungal metabolites by UV and LC retention time parameters, but did not take this approach any further in terms of developing a searchable database. The ease of obtaining accurate mass data for a number of analytes in an LC run, lends itself to subsequent searching an accurate mass database for target compounds. This approach has been used for monitoring pesticide residues, and searching of an elemental database has been applied to 350 pesticides in apples and oranges [7]. Others have similarly evaluated this approach for multi-residue screening of more than 100 analytes belonging to different veterinary drug classes together with their metabolites in urine [8], and for pharmacologically active compounds in traditional Chinese medicines [9].

Fungi play an important role in food safety due to their production of toxic secondary metabolites (known as mycotoxins) which occur naturally in agricultural commodities and ultimately can contaminate foodstuffs. Single fungal species can produce a wide range of secondary metabolites, frequently of differing molecular weight and functionality, meaning that in order to monitor all possibilities, a number of different analytical approaches have of necessity to be applied to the same sample. For this reason, there have been a spate of recent publications advocating the use of LC/MS/MS for multi-mycotoxin analysis covering as many as 39 mycotoxins in a single analytical run [10,11]. This approach is particularly useful for example for multi-*Fusarium* toxin analysis, it is nevertheless still targeted and by definition pre-supposes a knowledge of what mycotoxins might be anticipated. In contrast, LC/TOF-MS offers the possibility of screening for a much wider group of metabolites and even being able to include compounds for which only molecular formulae are known, but for which reference standards are unavailable. The high specificity offered by accurate mass measurement of molecular ions additionally means that potentially ‘crude’ sample extracts can be analysed directly, without elaborate and time-consuming sample clean-up. This makes the approach additionally attractive, offering speed and simplicity. Tanaka et al. [5] established an LC/TOF-MS method for simultaneous screening of nine Fusarium mycotoxins including ZEN and four *Aspergillus* mycotoxins in corn, wheat, cornflakes and biscuits. This method involved conventional sample clean-up but it was not established whether LC/TOF-MS had adequate specificity to be used on crude sample extracts without experiencing interference or ion suppression from the background.

In this paper, we have examined fungal secondary metabolite production in various growth media. This approach is of interest in two respects. Firstly, LC/TOF-MS enables a much wider range of possible metabolites to be screened than has ever previously been possible giving insights into possible mycotoxins that should be sought in targeted analysis of foods. Secondly, this approach potentially offers an objective tool for classification of fungal types based on their metabolic profiles. Traditional identification of fungi, based on morphology and behavior on different growth media, is inevitably slow and rather subjective. The traditional taxonomic approach provides no real insight into mycotoxin production as a diversity of toxins can be produced from the same species and not all strains are necessarily toxigenic. Smedsgaard [12] showed all metabolites profiles known to be produced by *Penicillium* species by comparing retention times and UV spectra. However, LC/TOF-MS with high specificity enables for the first time, rapid analysis of crude extracts from fungi providing immediate insights into their metabolic profiles.

This paper examines the feasibility of using LC/TOF-MS to profile fungal metabolites when fungi are grown under various conditions. A simple extraction has been developed and the procedure has been tested by spiking mycotoxin standards into growth media and demonstrating their unequivocal identification. This new method was applied to a number of well-characterised fungi from culture collections and identified metabolites of sterilised figs were compared with those predicted from the literature.

2. Experimental

2.1. *Reagents*

Malt extract agar (MEA, Merck, KGaA, Germany), potato dextrose agar (PDA, Merck, KGaA, Germany), and yeast extract sucrose agar (YES, Merck, KGaA, Germany) were used as fungal growth media. Ethyl acetate (J.T. Baker, Holland) with 1% formic acid (Merck, KgaA, Germany) and then acetonitrile (Merck, KgaA, Germany) were used for initial extraction of metabolites. Ethyl acetate (Merck, KgaA, Germany) with 1% formic acid and acetonitrile (Merck, KgaA, Germany) was used for a second extraction. Gradient grade acetonitrile and ammonium acetate in an aqueous solution of 1% formic acid (MEA, Merck, KGaA, Germany) was used as the HPLC mobile phase. Aflatoxins B_1, B_2, G_1, and G_2; aflatoxin M_1; ochratoxin A, zearalenone, 4-deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, fusarenone X, neosolaniol, fumonisins B_1, B_2, and B_3, nivalenol, HT-2 toxin, T2 toxin, kojic acid were obtained from Biopure (Tulin, Austria) and internal standard (benzophenone) was from Merck (Germany). The stock solutions were prepared in methanol and stored at −20 °C.

Toxigenic isolates of *A. paraciticus* (NRRL 2999), were obtained from the USDA culture collection (Peoria, Illinois, USA) and toxigenic isolates of *A. flavus* [(200198), (979), (993), (1303), (1004)] *A. ochraceus* (200700), *A. oryzae* (200828), *A. niger* (200807), *A. fumigatus* (200418), *P. citrinum* (501862) and non-toxigenic isolates *A. flavus* [(200141), (200916), (200120), (200333)] were obtained from the TÜBİTAK Marmara Research Center (Gebze, Kocaeli, Turkey) culture collection in slant agar.

2.2. *Apparatus*

An incubator (Binder, USA) was used for incubation of plates. All fungal confirmation was carried out with a microscope (Micros, MC300A, Austria). Vortex extraction used an Autovortex SA2 (Stuart Scientific, UK) and ultrasonication using LC 60/H ultrasonic device (Elma, Germany). Extracts were filtered through Whatman No. 4 filter paper. (Maidstone, England) and concentrated under a nitrogen stream (BOS, Gebze, Kocaeli,

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