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Automated SPME–GC–MS monitoring of headspace metabolomic responses of *E. coli* to biologically active components extracted by the coating☆

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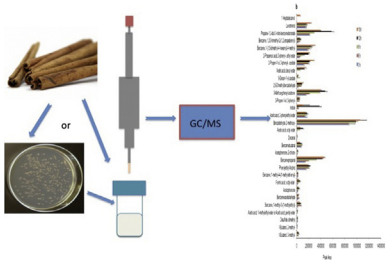
HIGHLIGHTS

- In vivo HS-SPME was used for monitoring of *Escherichia coli* metabolic profile changes.
- For the first time SPME fiber coating was used for simultaneous delivery of the antibacterial agent.
- Feasibility of automation of this process was demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

Monitoring extracellular metabolites of bacteria is very useful for not only metabolomics research but also for assessment of the effects of various chemicals, including antimicrobial agents and drugs. Herein, we describe the automated headspace solid-phase microextraction (HS-SPME) method coupled with gas chromatography–mass spectrometry (GC–MS) for the qualitative as well as semi-quantitative determination of metabolic responses of *Escherichia coli* to an antimicrobial agent, cinnamaldehyde. The minimum inhibitory concentration of cinnamaldehyde was calculated to be 2 g L^{-1}. We found that cinnamaldehyde was an important factor influencing the metabolic profile and growth process. A higher number of metabolites were observed during the mid-logarithmic growth phase. The metabolite variations (types and concentrations) induced by cinnamaldehyde were dependent on both cell density and the dose of cinnamaldehyde. Simultaneously, 25 different metabolites were separated and detected (e.g., indole, alkane, alcohol, organic acids, esters, etc.) in headspace of complex biological samples due to intermittent addition of high dose of cinnamaldehyde. The study was done using an automated system, thereby minimizing manual workup and indicating the potential of the method for high-throughput analysis. These findings enhanced the understanding of the metabolic responses of *E. coli* to cinnamaldehyde shock effect and demonstrated the effectiveness of the SPME–GC–MS based metabolomics approach to study such a complex biological system.

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1. Introduction

Essential oils (EOs) including cinnamon, clove, basil and oregano have significant antimicrobial activities [1–4]. Among these, cinnamon oil is one of the best antimicrobial agents, which contains cinnamaldehyde and has been proven to have the strongest antimicrobial activity compared to the other constituents of the cinnamon

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oil [2,5–8]. It is expected that the presence of cinnamon oil as well as cinnamaldehyde in the cell culture media would change the metabolite pattern of the bacteria. Some of the metabolites could be attributed to the diverse interactions and/or biotransformation routes and provided a possible basis to elucidate the mechanisms involved in the antimicrobial activity – either the preservation of food or the disinfection of highly resistant microorganism. Since cinnamon oil is a mixture of several components including cinnamaldehyde, there is a high possibility of co-elution of oil components with many cellular metabolites and, therefore, it is not easy to separate them well. It could be reasonable to use only cinnamaldehyde instead of cinnamon oil in order to demonstrate the potential utility of the technique as a tool for cellular metabolic analysis.

Cellular metabolomics (intracellular and extracellular metabolomics) have some fundamental advantages on providing important information for functional genomics, strain characterization, cell communication mechanism, metabolic engineering, biopharmaceuticals (e.g., antibodies, growth regulatory factors, immunomodulators), and industrial biotechnological processes [9–18]. However, measuring intracellular metabolites or metabolic fingerprinting is time-consuming and subject to technical difficulties, which are caused by the rapid turnover of intracellular metabolites and the need to quench the metabolism [19,20]. On the other hand, analysis of metabolic footprinting or extracellular metabolites is rapid, and does not need to quench the metabolism. These metabolites could be changed depending upon the environmental conditions. Recently several powerful standard analytical approaches (e.g., NMR, Microarray, GC–MS, LC-MS) for analyzing metabolomics of *Escherichia coli* to environmental perturbation (e.g., cold, heat, osmotic, oxidative, genetic alteration) have been reported [21–25]. The importance and utility of these methods are widely acknowledged and extensive research has been conducted in the laboratory for the characterization of phenotypes and the distinction of specific metabolic states due to environmental or genetic alteration. However, there are still no studies that have examined the effect of antimicrobial agents/drugs including cinnamaldehyde on cellular metabolic footprint analysis. Thus, at present, considerable attention has been focused on the development of a simple, rapid, sensitive and reliable metabolic footprinting approach due to cinnamaldehyde shock responses.

It is well known that solid-phase microextraction (SPME) is a non-exhaustive, environmentally friendly sample preparation technique that combines sampling, analyte extraction, and sample introduction in a single step while minimizing or completely eliminating the use of solvents [26–31]. In addition, SPME is portable and can be used with a direct extraction mode in combination with GC–MS. Moreover, recently it was shown that the SPME has the ability to capture variety of metabolites such as polar, nonpolar, short-lived and unstable metabolites. The practical utility of SPME is considered suitable for global metabolomics studies [32–35].

In this study, we have demonstrated, for the first time, an automated SPME–GC–MS-based extracellular metabolomics strategy. The assay protocols for partially and fully automated strategies are illustrated schematically in Fig. 1a and b *E. coli* BL21 is currently one of the most studied microorganisms and thus at first the growth pattern of this organism in super media and the minimum inhibitory concentration of cinnamaldehyde were obtained. The *E. coli* BL21 was then exposed to cinnamaldehyde stress and the temporal changes in the extracellular metabolic profile were compared to untreated controls to investigate (a) the overall and specific changes in the *E. coli* metabolome following cinnamaldehyde stress, (b) the trend of the time dependent metabolomics profiles due to both low and high doses of cinnamaldehyde, and (c) the metabolic variations due to the effects of both initial additions of cinnamaldehyde (cinnamaldehyde was added into the cell culture at zero incubation time and allowed to grow at 37 °C) and intermittent additions of cinnamaldehyde (cell culture was collected at different incubation times and then cinnamaldehyde was added to the culture). The findings from the SPME–GC–MS-based metabolomics investigation are useful to gain novel insights into cell metabolism. In addition, the system is very effective in separating different compounds of a complex biological sample rapidly and sensitively in headspace and has the potential of high-throughput due to the lower requirement of manual workup.

2. Experimental

2.1. Materials and solutions

All chemicals from commercial sources were of analytical grade and used without further purification. Peptone, yeast extract, NaCl, cinnamaldehyde (leaf), and SPME fibers (e.g., 100 μm PDMS, 65 μm DVB/PDMS, 75 μm CAR/PDMS, and 50/30 μm CAR/PDMS/DVB) were all purchased from Sigma–Aldrich. The 20 mL glass vials with polytetrafluoroethylene-lined screw caps were purchased from VWR. The *E. coli* BL21 was obtained from Professor John Brennan's Lab, McMaster University (ON, Canada) as a gift. Nano-pure water was obtained from a Milli-Q Synthesis A10 water purification system.

Stock solution of cinnamaldehyde was prepared with an organic solvent, ethanol (Sigma–Aldrich), and the final concentration of this organic solvent was less than 3%. Note that this level of organic solvent has been shown not to affect normal cell growth or headspace metabolomics.

2.2. Organisms and plate counting

Non-pathogenic bacteria (e.g., *E. coli* BL21) were used in this study. Standard Luria Bertani (LB) agar media (composition in 1 L: 10 g trypton; 5 g yeast extract; 5 g NaCl; 15 g agar) was used to count the number of colony forming units per mL (CFU mL^{-1}) in bacterial suspensions. For this, cultures are serially diluted with sterile water or media, and 10–20 μL of selected dilution is spread evenly over the surface of the warm agar plates. Plates were incubated at 37 °C for 24 h and then the colonies were counted. Dilutions showing between 30 and 100 colonies were used for calculation of CFU mL^{-1}. [CAUTION: Though *E. coli* BL21 is non-pathogenic, it should be handled following Level 1 biosafety procedures].

2.3. Determination of minimal inhibitory concentration (MIC) of cinnamaldehyde

Different concentrations of cinnamaldehyde (final concentration, 0.1–5 g L^{-1}) were added into cell suspension vials (5 mL each, initial cell concentration, 10^3–10^4 CFU mL^{-1}) separately. After incubation for 1 h at 37 °C with and without (control) cinnamaldehyde treatment in liquid phase, 20 μL of sample from each vial was plated into agar plates that contain LB agar media. Plates were incubated at 37 °C for 24 h and then the colonies were counted. Note that cinnamaldehyde is a highly antimicrobial agent and, therefore, it causes apparent bacterial inhibition by comparison with control. Three different sets of parallel experiments for each concentration of cinnamaldehyde were conducted.

2.4. Preparation of test solution

Super broth consisting of 32 g of trypton, 20 g of yeast extract, 5 g of NaCl, and 5 mL of NaOH (1 N) per liter was used as a basal medium. *E. coli* BL21 was inoculated at the initial concentration (~10^3 CFU mL^{-1}) into the super broth, and 5 mL of the super broth

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