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One-pot microwave derivatization of target compounds relevant to metabolomics with comprehensive two-dimensional gas chromatography

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ABSTRACT

Metabolomics has been defined as the quantitative measurement of all low molecular weight metabolites (sugars, amino acids, organic acids, fatty acids and others) in an organism's cells at a specified time under specific environmental/biological conditions. Currently, there is considerable interest in developing a single method of derivatization and separation that satisfies the needs for metabolite analysis while recognizing the many chemical classes that constitute the metabolome. Chemical derivatization considerably increases the sensitivity and specificity of gas chromatography–mass spectrometry for compounds that are polar and have derivatizable groups. Microwave-assisted derivatization (MAD) of a set of standards spanning a wide range of metabolites of interest demonstrates the potential of MAD for metabolic profiling. A final protocol of 150 W power for 90 s was selected as the derivatization condition, based upon the study of each chemical class. A study of the generation of partially derivatized components established the conditions where this could potentially be a problem; the use of greater volumes of reagent ensured this would not arise. All compounds analyzed by comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry in a standard mixture showed good area ratio reproducibility against a naphthalene internal standard (RSD < 10% in all but one case). Concentrations tested ranged from 1 μg/mL to 1000 μg/mL, and the calibration curves for the standard mixtures were satisfactory with regression coefficients generally better than 0.998. The application to gas chromatography–quadrupole mass spectrometry and comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry for a typical reference standard of relevance to metabolomics is demonstrated.

1. Introduction

1.1. Metabolic profiling

Metabolomics has received attention as an '-omics' technology, notwithstanding the specific interest in classes of compounds that can be construed to comprise the metabolome. Molecular and systems biology over the last few decades has shown that the flow of information from genes to function is linear and is translated through transcripts, proteins and finally metabolites [1]. There is considerable debate about the precise use of these terminologies (metabolomics, metabolic profiling, metabolic fingerprinting, metabolite target analysis), and it is generally advisable to exert caution in defining these terms [2]. Proposed minimum reporting standards for this type of chemical analysis have been promulgated by the Chemical Analysis Working Group (CAWG) which provide methods of reporting of information describing metabolomics [3].

Oliver et al. introduced the term 'metabolomics' in their systematic functional analysis of the yeast genome [4], proposing the challenge to discover what each of the gene products does, and how they interact in a living yeast cell (after the genome sequence of the yeast *Saccharomyces cerevisiae* had been completed). Sensitive analytical tools for the determination of flux-control coefficients are required, as it is not known which metabolites would have their concentrations altered due to a gene's deletion or overexpression [4]. Generally, the low molecular mass organic compounds of inter-

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est to metabolomics include, e.g. fatty acids, amino acids, carboxylic acids, carbohydrates, vitamins and lipids [5]. The composition of the metabolome can vary considerably, depending on the organism analyzed; *S. cerevisiae* has an estimated 600 metabolites [6] and the plant kingdom comprises up to 200,000 primary and secondary metabolites [7]. Therefore there is a great challenge involved in both the design of instrumentation and in the development of software for general metabolic profiling.

Currently, no single method can comprehensively (i.e. completely, at the level of detection) measure the metabolome, although there are a range of technologies that can generate quantitative metabolite profiles of several hundred metabolites. Applications have developed from primary work in the 1980s that used gas chromatography–mass spectrometry (GC/MS), chemical ionization mass spectrometry (CIMS) and nuclear magnetic resonance (NMR) spectroscopy [8]. Nonetheless, the analytical procedure is essentially constrained to the identification and quantification of a specifically chosen set of metabolites in a biological sample. Sample preparation usually focuses specifically on chemical properties of these chosen compounds, so as to reduce matrix effects through selective extraction or similar strategies, known as metabolic profiling (or metabolite profiling). It is an established and, at the limits of available methods, powerful technique applied in many facets of drug discovery. It allows characterization of pathological states and disorders of cells and organisms, in taxonomic and pathological studies and in metabolomics [2]. GC/MS has had a long history in metabolic profiling, e.g. through detailed study of inborn errors of metabolism (IEM) in humans, such that the technique is a necessity in identification of a large number of IEM. It continues to be widely applied to the analysis of plant extracts [9,10].

Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC/ToFMS) is an emerging technology that provides a two-dimensional separation and a full mass spectral profile based on retention time coordinates of compounds in the two-dimensional separation space. GC × GC has all the advantages of 'normal' GC techniques: sensitive analysis, readily automated detection of compounds and reliable methods. In addition, GC × GC separations offer additional information content and structured chromatograms where related compounds tend to cluster in the 2D plane in specific patterns [11]. In GC × GC a sample is separated firstly on a "conventional" high-resolution capillary GC column, then effluent is modulated into a short, fast elution second dimension (^{2}D) column according to the modulation ratio employed [12]. For a non-polar/polar (NP/P) column combination, co-eluting compounds of similar volatility at the time (temperature) of elution, may have different 'polarities'. The ^{2}D separation is achieved by the activity coefficient differences between solutes and the polar phase, to allow compounds of different polarities to be separated. Compounds belonging to the same chemical family, will have about the same activity coefficients, therefore show similar ^{2}D retention times, to form clusters along a retention axis in the 2D plane. The high peak capacity in 2D space accommodates resolution of highly complex mixtures associated with metabolite profiling.

Synovec and co-workers performed amino and organic acid analysis using GC × GC/ToFMS on a set of amino and organic acid standards and finally on rye grass samples [13]. Advantages of the GC × GC separation was demonstrated, and shown to be applicable to target analysis as well as pattern recognition and fingerprinting studies.

1.2. Chemical derivatization

The chemical diversity of metabolites can be most appropriately analyzed if at least two different physicochemical properties of the target analytes are used, for example GC and MS (volatility and mass analysis) or high-performance liquid chromatography with MS (hydrophobicity and mass analysis). Derivatization in GC chemically modifies a compound in order to increase its volatility, and/or improve its stability and separation performance and/or sensitivity [14]. The most popular method used for GC is silylation which reduces sample polarity and replaces active hydrogens with trimethylsilyl (TMS) groups. Pyridine is commonly used in this process, as an acid scavenger that drives the reaction forward. Methoximation (MO_x) is necessary for specific classes of compounds (e.g. keto acids, sugars) and must be performed before silylation (Fig. 1a) as it protects carbonyl moieties (converted to methoximes). This improves their GC properties by preventing multiple derivatization products, simplifying chromatograms [15]. Bis-trimethylsilyltrifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) is an appropriate derivatizing reagent, as it is sufficiently volatile to provide little interference with early eluting peaks, and it acts as its own solvent. TMCS is used as a catalyst to increase TMS donor potential [15]. BSTFA was chosen as the derivatization reagent as *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (and other reagents) are known to produce a derivatization by-product which can attack the initially formed ester to yield an artifact. This is not observed with BSTFA, as it and its by-products do not contain any active hydrogens and its by-product does not add as easily across active carbonyls [16]. Fig. 1b summarizes the derivatizable groups formed through adding BSTFA reagent to various functional groups.

Paik and Kim performed sequential ethoxycarbonylation, methoximation and tert-butyldimethylsilylation for the simultaneous determination of amino, carboxylic and keto acids [17] not only allowing simultaneous recovery of the different compound classes, but also linearity and accuracy were satisfactory for the accurate and precise quantification of the diverse amino, carboxylic and keto acids. However tert-butyldimethylsilyl (TBDMS) derivatives gave rise to incomplete derivatization for other classes of compounds such as polyols, and the derivatives elute at higher retention times which may be a problem for multiply derivatized compounds [15]. Recently, derivatization with alkylchloroformates has also been reported, and although derivatization to the alkylchloroformates is simple and rapid, it has only been applied to a limited number of biological samples [15]. Mayadunne et al. successfully reported the separation characteristics of alkylchloroformate-derivatized amino acids by GC × GC in a range of food and beverage products, including wine, beer and honey [18].

1.3. Microwave-assisted derivatization

Microwave-assisted derivatization (MAD) involves the effective heating of materials by the use of "microwave dielectric heating" effects [19]. This depends on the ability of a material (whether it be solvent or reagent) to absorb microwave energy, heating the material, and increasing the reactivity of the compounds. The amount of energy created by the above process is related to the ability of the matrix to align or 'couple' itself with the frequency of the applied field [19]. Deng et al. developed a MAD method for the analysis of amino acids in blood samples by GC/MS [20]. The optimization of the reaction solvent, microwave power, and the derivatization reaction process was completed; microwave irradiation improved the silylation of amino acids with BSTFA, and only 1 min was required for the derivatization to reach completion [20]. Silva and Ferraz reported a novel MAD method, in which sugars and organic acids were derivatized in a domestic microwave at 180 W for 5 min, and average analytical recoveries were above 97% [21]. Another study reported a rapid MAD method for the analysis of steroid estrogens by GC/MS, in which BSTFA + 1% TMCS was used as the derivatization reagent [22]. Recently, Liebeke et al. pro-