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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-

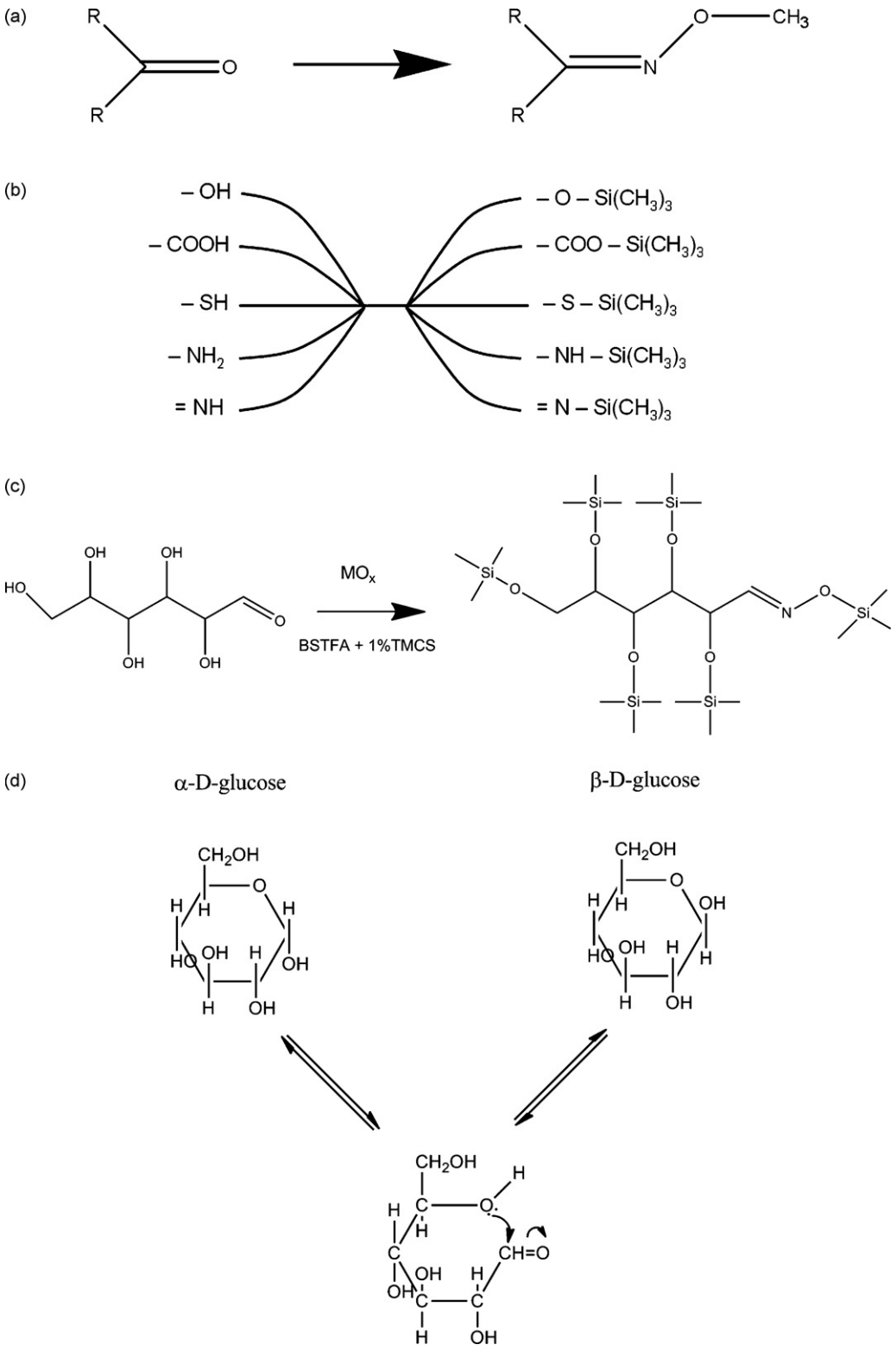

Fig. 1. Examples of derivatization processes. (a) Methoximation of a carbonyl functional group. (b) TMS groups formed for different functional groups through the addition of BSTFA + 1% TMCS. (c) Example of methoximation and trimethylsilylation of a metabolite (glucose and its glucose oxime hexa-TMS derivative). (d) Anomers formed from glucose through an open-chain intermediate.

posed a time-efficient method in which the derivatization time was decreased from 120 min to 6 min, without the loss of qualitative and quantitative information. This protocol was applied to a set of standard mixtures and to microbial-derived biological samples [23].

In the present study microwave derivatization is applied with a combined reagent to a series of chemical compounds (fatty acids, amino acids, organic acids, sugars and sugar alcohols) with subsequent analysis by GC/qMS and GC × GC/ToFMS, in order to produce a generally applicable method for the

Table 1
Specific compounds (in alphabetical order) present in the primary standard mixtures numbered as in Fig. 3b.

Amino acids	Sugars	Organic acids	Sugar alcohols	Fatty acids
[1] L-Alanine	[17] α-L-Arabinose	[7] Fumaric acid	[14] Erythritol	[40] Arachidic acid
[26] L-Asparagine	[19] β-L-Arabinose	[6] Maleic acid	[4] Glycerol	[11] Capric acid
[10] γ-Amino-*N*-butyric acid	[30] α-D-Galactose	[3] Malonic acid	[36] Inositol (meso)	[32] Myristic acid
[18] D-Glutamic acid	[31] β-D-Galactose	[12] Malic acid	[29] Mannitol	[39] Nonadecanoic acid
[2] Glycine	[33] α-D-Glucose	[15] Oxalic acid		[38] Oleic acid
[5] L-Leucine	[34] β-D-Glucose	[20] Succinic acid		[35] Palmitic acid
[13] Methionine	[41] α-Lactose	[16] L-Tartaric acid		[37] Stearic acid
[25] L-Phenylalanine	[44] β-Lactose			
[8] Proline	[42] α-Maltose			
[9] L-Serine	[43] β-Maltose			
	[27] α-D-Mannose			
	[28] β-D-Mannose			
	[21] α-D-Ribose			
	[22] β-D-Ribose			
	[23] α-D-Xylose			
	[24] β-D-Xylose			

analysis of a wide variety of chemical classes relevant to metabolomics.

2. Experimental

2.1. Experimental design

To develop a protocol to perform an experiment for metabolic profiling, a number of factors (e.g. time and power) can be identified which can potentially affect the responses (i.e. metabolite peak area). The goal for such analytical systems is to find the most suitable conditions that maximize response as well as reproducibility [24]. In the study undertaken the efficiency of MAD is investigated by varying two factors (power and time). This was achieved by varying one factor whilst keeping the other factor constant, i.e. derivatization efficiencies were studied using 30 s, 60 s, 90 s and 120 s while keeping the power constant at 150 W then 300 W, 450 W and 600 W. The two factors were performed at four levels providing $2^4 = 16$ experiments, and each experiment was run in triplicate (48 experiments were conducted for each separate standard mixture).

2.2. Derivatization procedure

The standard compounds (amino acids, organic acids, sugars, sugar alcohols and fatty acids; Sigma–Aldrich, Castle Hill, Australia) were prepared at an initial concentration of approximately 1000 μg/mL of each of compound listed in Table 1. From the separate standard solutions, both individual working standards, and various mixtures were prepared. Amino acids, sugars and sugar alcohols were prepared in H_2O and organic and fatty acids were prepared in MeOH (methanol). Primary standard mixtures, for each compound class, e.g. amino acids, sugars, etc. contained 50 μg/mL of each respective compound. In addition, a mixture comprising all components was prepared. Standard mixtures were generally diluted 5-fold (using H_2O or MeOH), providing a final concentration of 10 μg/mL. The diluted standard mixture (10 μL) was transferred to a GC vial that was dried at 60 °C under N_2. Once dry, 20 μL of 20,000 μg/mL methoxyamine hydrochloride in pyridine was added and immediately after 50 μL BSTFA + 1% TMCS was added. Naphthalene was added as an internal standard (10 μL of a 250 μg/mL solution in pyridine). The final step in the process was to use microwave irradiation (CEM Corporation MARS-5, Matthews, NC) to heat the mixture. Note that both sequential additions of derivatization reagents with microwave treatment after each addition, addition of both reagents prior to microwave treatment, and also conventional heating experiments were conducted.

A set of experiments was performed using different power and time conditions of the separate standard mixtures in order to study the variation in peak response whilst varying experimental factors. Internal standard (naphthalene) was added as an inert, but relatively volatile standard to check for losses arising from evaporation or similar solute loss and to compare recovery across the different standards. Naphthalene was chosen specifically as it is not reactive under the derivatization conditions. Peak responses for naphthalene remained fairly constant across all the different sets of experiments performed (generally <2% RSD – data not shown), indicating its suitability as an IS for the derivatization process, and the ability of the system to prevent volatile component losses.

2.3. GC/qMS analysis

The analysis of the different standard mixtures was performed using an Agilent model 6890 GC system (Agilent Technologies, Nunawading, Australia) equipped with an Agilent model 5973 quadrupole mass spectrometer (qMS). The column used was a conventional 30 m BPX5 (5% phenyl polysilphenylene-siloxane) capillary column (SGE International, Ringwood, Australia) with an internal diameter of 250 μm and a film thickness (d_f) of 0.18 μm. All injections were performed in splitless mode (1 min vent open time) with 1 μL injected volume, and an oven ramp beginning at 70 °C with a hold of 2 min, then increasing at 5 °C/min to 260 °C with a hold time of 5 min. Helium gas was used at a rate of 1.3 mL/min. The transfer line was held at 280 °C and mass spectra were collected from 45 to 650 *m*/*z*.

2.4. GC × GC/ToFMS analysis

The primary standard mix was analyzed using an Agilent model 6890 GC interfaced with a LECO Pegasus III (LECO Corporation, St Joseph, MI) ToFMS system, operated in GC × GC mode. The ^{1}D column was a 30 m BPX5 (5% phenyl polysilphenylene-siloxane) capillary column (SGE International) with an internal diameter of 250 μm and 0.25 μm d_f. The ^{2}D column was a 1 m BPX50 (50% phenyl, 50% dimethylpolysilphenylene-siloxane) capillary column (SGE International) with an internal diameter of 100 μm and 0.1 μm d_f. All injections were performed in splitless mode (1 min vent open) with 1 μL injected volume, and an oven ramp beginning at 70 °C with a hold of 2 min, increasing at 3 °C/min to 260 °C, with a final hold time of 5 min. Helium gas was used at a flow volume of 1 mL/min. The transfer line was held at 280 °C. Mass spectra were collected from 45 to 650 *m*/*z*. The modulator used was a longitudinal modulated cryogenic system (LMCS) (Chromatography Concepts, Doncaster, Australia), with the modulation temperature

kept at 0 °C and a modulation period of 4 s was applied throughout the duration of the analyses.

3. Results and discussion

3.1. Optimization of methoximation and silylation

The methoximation and silylation steps in the proposed method were optimized accordingly by varying specific parameters, such as the different amounts of methoximation and derivatization reagents. Firstly, methoximation was performed on the combined mixture by the addition of 5 μL, 10 μL, 15 μL, 20 μL and 25 μL of 20,000 μg/mL methoxyamine hydrochloride in pyridine. The methoximation reaction was found to be complete after the addition of 20 μL therefore this was used as the optimal volume for the amounts of analyte used. In addition to the methoximation reaction, 1 μL, 2 μL, 5 μL, 10 μL, 20 μL, 50 μL, 100 μL, 200 μL, 400 μL and 500 μL of BSTFA + 1% TMCS were added to the combined mixtures, with 50 μL chosen based on the observation that derivatization was maximized (according to peak area). It was also found that the addition of more than 50 μL of derivatization reagent resulted in an increase in the number of peaks detected (artifacts), due to excess reagent. These 'new' artifacts were present mainly at low masses and/or low retention times, as well as trace peaks now being more prominent.

The methoximation and silylation steps were carried out by:

1. Methoximation using MAD, followed by silylation using MAD.
2. Methoximation and silylation as a single step using MAD (Fig. 1c).

No structural differences in various analytes (i.e. multiple peaks or artifacts) were seen by performing the methoximation and silylation steps as a one-step process, specifically when analyzing the keto acids and sugars and monitoring the chromatograms for evidence of multiple product formation (experimental conditions were kept constant for, e.g. the amount of derivatization reagent added). Fig. 1d describes an equilibrium mixture of α- and β-glucose anomers in solution formed through an open-chain intermediate. This observation suggests a one-step process is equally effective as the two-step sequential process, but has the advantages of being less time consuming and having fewer opportunities for sample losses. In all cases, MAD is much faster than the traditional application of heat to accomplish the derivatization step.

3.2. Conventional derivatization vs. microwave-assisted derivatization

Conventional derivatization methods may require a long time (for example, more than 70 min) at a reaction temperature of up to 120 °C for the silylation reaction to take place to completion (particularly for amino acids) [25]. Conventional derivatization uses heat, which is transferred from the vessel wall to the reactants, whereas in microwave-assisted derivatization the energy is directly distributed evenly and directly to the solvent and the sample by microwave heating. A sample of amino acids was used to contrast conventional derivatization with the MAD method, chosen specifically as they are more difficult to derivatize than the other classes of compounds. A temperature of 120 °C for 2.5 h was required to achieve complete silylation using the conventional method (at 80 °C for 1 h incomplete derivatization of the compounds was obtained; data not shown), as compared to 150 W for 90 s for the microwave derivatization method. In the case where methoximation would need to be implemented before silylation (e.g. sugars), double the time would be needed to perform both derivatization steps.

3.3. Optimization of microwave derivatization

Two important parameters for the MAD reaction were considered: microwave power output (W) and irradiation time (s). The relative response ratios of the analyte peaks vs. the internal standard (naphthalene) were plotted against the irradiation time for all standard mixtures, in order to establish which power and time settings would be optimal across the different classes of compounds.

3.3.1. Amino acid mixture

The results of the optimization of the amino acid mixture are presented in Fig. 2a and b. From these it is evident that a power and time setting of 150 W and 90 s provided the largest relative response area ratios, and thus greater sensitivity and a more complete reaction at 150 W in 90 s. An increase in power above 150 W gave rise to decreasing relative peak areas, suggesting derivatized compound degradation begins to occur, as well as an increase in artifact formation. This is due to an increase observed in the abundance of artifacts due to the side reactions formed during the derivatization process. Fig. 2b shows an approximately constant response for amino acids as time is increased, for a power of 150 W and an increased time showed no improvement in amino acid response.

3.3.2. Fatty acid mixture

The fatty acid mixture described produced similar results to the amino acid mixture, in that an optimal setting of 150 W and 90 s provided the largest relative response ratios. The only clear distinction observed was the large drop in the response ratios at 600 W and 120 s. This is proposed to be due to compound degradation, therefore greater power and time settings should be avoided. Fatty acid derivatization appears to be better at higher time settings, apart from the longest time (120 s), where a greatly reduced response is shown. For this and the next two sets of standard mixtures, separate figures are not provided, since they show the trends that can be adequately described in the text.

3.3.3. Organic acid mixture

The organic acid mixture was quite different to the previous compound classes, as there was only a small change to the response area ratios observed when varying the power and time, with a slightly improved response when higher power and/or time settings were used. Nevertheless, it may be concluded that a power and time setting of 450 W and 60 s is suitable for organic acid derivatization, but if other compound classes better suit lower or higher settings, there should still be adequate derivatization of organic acids.

3.3.4. Sugar and sugar alcohol mixture

The sugar and sugar alcohol mixtures showed similar characteristics in that a power setting of 450 W provided the optimum relative response ratio with a reduced response at both higher and lower power settings. A time setting of 90 s was found to be optimal. The response at 600 W was similar to that at 150 W, but with an increased time (from 30 s to 120 s), an improved response for all sugars is observed.

3.3.5. Primary standard mixture

The data suggest that the different chemical classes of compounds present in the mixture would ideally be derivatized with different power and time settings. Clearly, in a mixture of all metabolites a compromise must be found. A general setting can still be applied which takes into consideration the optimum relative response ratios across all the different classes of compounds present. Based upon our results, settings of 150 W and 90 s are suggested as the best compromise as these conditions result in the

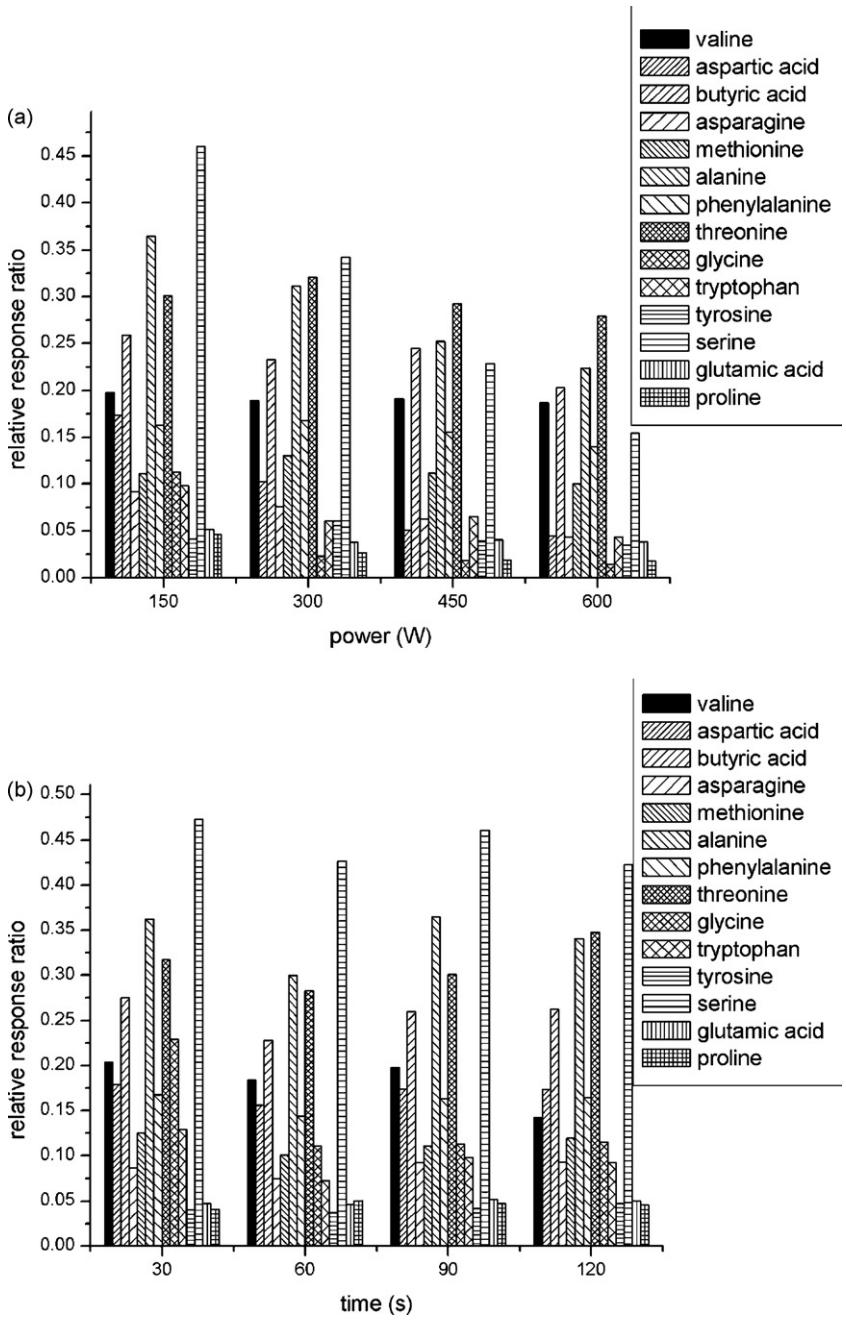

Fig. 2. Effect of power and time on MAD of an amino acid mixture. (a) Effect of power on mean response area ratios of amino acids at time 90 s. (b) Effect of time on mean response area ratios of amino acids at power 150 W. Area ratios *vs* naphthalene.

largest relative response ratios across all classes simultaneously while minimizing the formation of artifact peaks. Fig. 3 shows the GC/qMS (gas chromatography with quadrupole mass spectrometric detection) (Fig. 3a) and GC × GC/ToFMS (comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection) (Fig. 3b) of the primary standard mixture, illustrating its complexity. All the metabolites present in the standard mixture were clearly separated using GC × GC, but some metabolites were found to be co-eluting using one-dimensional GC/qMS (e.g. palmitic acid (peak 35) and meso-inositol (peak 36) or L-phenylalanine (peak 24) and β-D-xylose (peak 25) in Fig. 3). Consequently, quantification was performed on the basis of the GC × GC/ToFMS results.

A comparison of the relative response ratios of the metabolites present in the standard mixture using microwave and traditional methods is shown in Fig. 4. Three compounds were chosen from each compound class and generally demonstrated that across the different classes of compounds the relative response area ratios are higher when using microwave derivatization. Derivatization procedures were performed in triplicate (all processes), and RSDs obtained for response ratios across the different classes of compounds studied ranged from 1% to 5%. This confirms that the microwave derivatization method developed is a reliable method (reproducibility) which gives rise to larger mean peak area ratios (sensitivity) and less side reactions (artifacts).

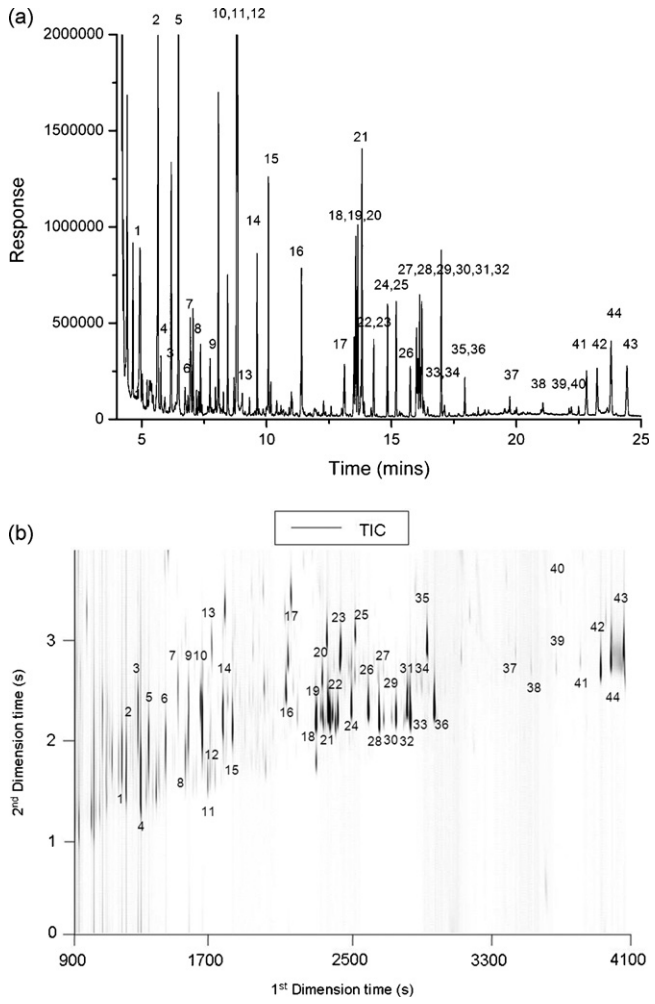

Fig. 3. (a) GC/qMS (BPX5) and (b) GC × GC/ToFMS (BPX5–BPX50) of the primary standard mixture of all components. Component numbering is given in Table 1.

3.4. Partial derivatization and artifact formation in trimethylsilyl derivatization reactions

3.4.1. Partial derivatization

Fig. 5 displays a chromatogram of a partially derivatized sample of the mixture used in Fig. 3b. The conditions that lead to partial derivatization were investigated, in order to establish the lowest amount of derivatization reagent required to ensure complete derivatization.

Partial derivatization was achieved by varying the amount of derivatization reagent added in the derivatization step. The optimal amount of derivatization reagent required was 50 μL (as mentioned previously) and this was used for all separate standard mixtures. The amino acid mixture was chosen on the basis that this mixture was the most difficult to derivatize, due to the fact that certain amino acids, e.g. arginine and lysine require longer derivatization times [26] (as they contain multiple amino/carboxyl groups).

Initially 1×, 2×, 4×, and 8× molar ratios of metabolite vs. derivatization reagent were added to the primary standard mixture, but partial derivatization was not observed for most compounds with only minor partial derivatization product in the 1× and 2× molar ratios. After the addition of 0.5× molar ratio there were no compounds derivatized (only baseline response was observed).

It was observed that between 0.5× and 2× molar ratios of metabolite vs. derivatization reagent were needed to obtain par-

Table 2
Repeatability (RSD), R^2 and relative responses vs. internal standard at four different concentration levels of relative responses of some selected metabolites ($n = 3$) EIC mode m/z 73 for TMS derivatives and m/z 128 for naphthalene) in GC × GC.

Compound	Equation and R^2	Relative response vs. internal standard[a]	RSD (%)[a]
Amino acids			
L-Alanine	$y = 0.019x - 0.318$ $R^2 = 0.998$	18.87	6.4
		1.359	3.9
		0.104	13.0
		0.007	7.4
L-Leucine	$y = 0.012x - 0.087$ $R^2 = 0.999$	12.78	2.5
		1.136	3.2
		0.135	6.2
		0.011	4.8
L-Serine	$y = 0.005x - 0.004$ $R^2 = 1$	5.37	6.5
		0.549	4.4
		0.048	2.4
		0.004	8.2
Sugars			
D-Glucose	$y = 0.011x - 0.055$ $R^2 = 0.999$	11.84	0.9
		1.264	4.8
		0.13	2.3
		0.010	3.4
Maltose	$y = 0.004x + 0.007$ $R^2 = 1$	4.82	2.4
		0.586	5.3
		0.051	1.7
		0.006	3.6
D-Ribose	$y = 0.009x + 0.031$ $R^2 = 0.999$	9.21	0.8
		1.164	2.9
		0.106	1.7
		0.012	3.7
Organic acids			
Oxalic acid	$y = 0.009x + 0.031$ $R^2 = 0.999$	9.24	0.9
		1.065	4.1
		0.105	3.3
		0.011	5.8
Benzoic acid	$y = 0.017x + 0.078$ $R^2 = 0.999$	17.83	1.6
		1.945	2.8
		0.204	1.2
		0.019	3.9
Sugar alcohols			
Glycerol	$y = 0.004x + 0.029$ $R^2 = 0.999$	4.10	1.4
		0.459	4.6
		0.042	1.0
		0.004	2.5
Inositol (meso)	$y = 0.009x + 0.013$ $R^2 = 1$	9.72	1.1
		1.052	3.2
		0.11	2.7
		0.010	5.3
Fatty acids			
Pentadecanoic acid	$y = 0.013x - 0.113$ $R^2 = 0.999$	13.22	4.6
		1.192	2.6
		0.107	7.1
		0.011	4.2
Heptadecanoic acid	$y = 0.021x - 0.029$ $R^2 = 0.999$	21.54	3.8
		2.261	5.1
		0.269	1.1
		0.025	7.8

[a] Each set of values is given for 1000 μg/mL, 100 μg/mL, 10 μg/mL and 1 μg/mL, respectively.

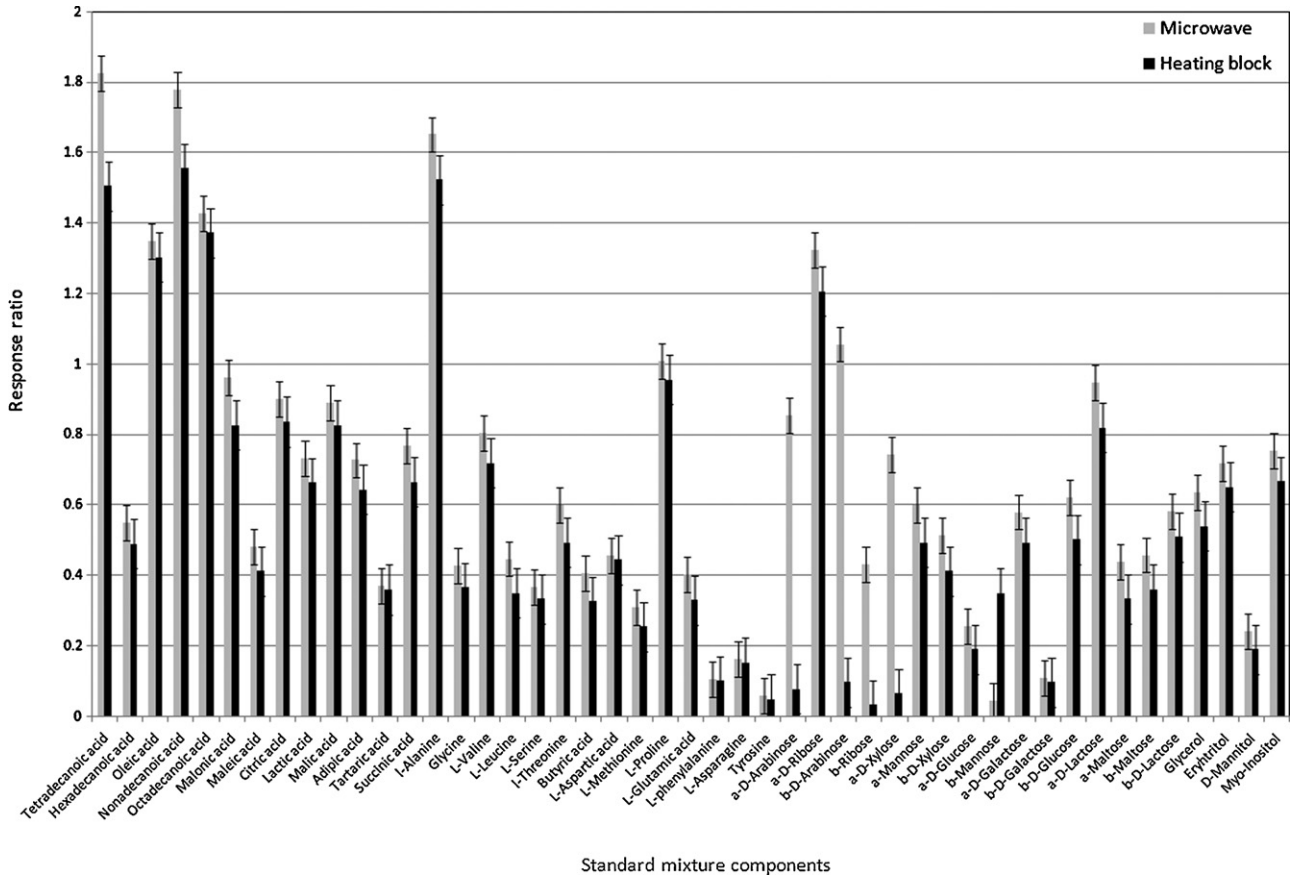

Fig. 4. Comparison of relative response ratios of components present in the standard mixture using microwave and traditional derivatization methods ($n = 6$).

tial derivatization for most compounds. Therefore 0.5×, 0.75×, 1×, 1.25× and 1.5× molar ratios were studied, with 1× being the most obvious case of partial derivatization. Fig. 5 presents an example of the partial derivatization observed under this case. Complete derivatization depends on there being sufficient derivatization reagent to meet the demands of all the analytes present in a mixture. Thus the analyst must ensure (by the use of mass spectrometry) that the compounds of interest are completely derivatized before proceeding with data analysis.

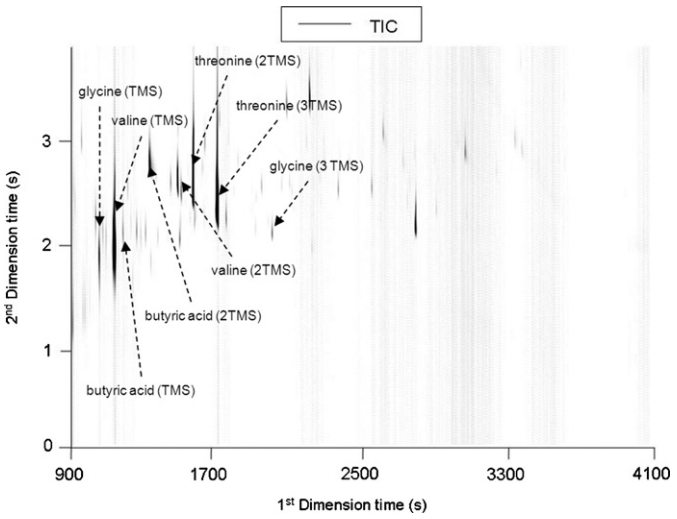

Fig. 5. GC × GC/ToFMS analysis showing partial derivatization of the amino acid mixture.

3.4.2. Artifact formation

Artifacts present in trimethylsilyl derivatization reactions are a common problem encountered with certain functional groups such as aldehydes, amides, carboxylic acids, esters, ketones and phenols. Artifact formation can lead to multiple peaks for the same compound, which in turn causes uncertainty in the quantification of the compound. Optimization of solvents, derivatization reagents, catalysts, reaction times and temperatures are necessary in order to minimize artifact formation.

Carboxylic acids tend to form silylation artifacts less than either ketones or aldehydes. It has been noted though, that carboxylic acids with at least one α-hydrogen occasionally form artifacts. Malonic acid, α-hydroxymalonic acid and α-methylmalonic acid also form artifacts from the silylation enol-form of their ester groups (e.g. α-hydroxymalonic acid forms the tris-TMS derivative as well as the unexpected tetrakis-TMS artifact). The tris-TMS derivatives of methylmalonic and hydroxymalonic acids have been known to form additional products by reacting with oxygen [15]. In our analysis, we only observed artifacts from the amino acid derivatization process, e.g. (L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester) was the expected derivative formed and (L-Proline, ethyl ester), (L-Proline, 1-acetyl-, methyl ester) and (L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester) were also formed as artifacts.

Salts of organic and inorganic acids are not usually derivatized when reacting with BSTFA, however under specific conditions they will be detected as TMS derivatives. The presence of salts was not observed in our analysis.

Silylation reactions generally form the required derivative with minimal complications, although in some cases different artifacts can be observed. Useful references [14,27,28] discuss factors which

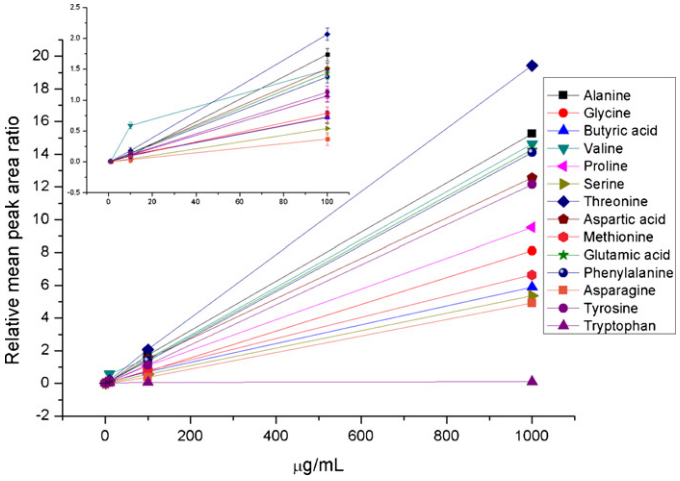

Fig. 6. Amino acid quantification by using GC × GC/ToFMS over the range 1–1000 μg/mL (inset 1–100 μg/mL).

should be noted when optimization of silylation reactions is performed, in order to minimize artifact formation.

3.5. Quantification of derivatized compounds using GC × GC/ToFMS

To effectively quantify the derivatized compounds in the standard mixtures, a measure of the effectiveness of the derivatization (i.e. the mass fraction of a compound that is transferred into its derivatized form) needs to be addressed. The derivatized compounds were quantified in a semi-quantitative manner using GC × GC/ToFMS, by assuming that the response of a compound in EIC mode (the selected ion m/z 73 for TMS was chosen in this case) was proportional to the concentration of compound injected. This can be achieved by comparing the response of the derivatized compounds with the reference compound (in this case naphthalene) of a known concentration.

All the 44 compounds (including sugar α and β anomers) that could be completely derivatized were derivatized reproducibly (RSD < 10% for all but one). Table 2 lists the repeatability (RSD), R^2 and relative responses vs. internal standard at varying concentration levels of the 44 compounds across the different chemical classes. Relative response ratios were compared for the microwave and conventional derivatization methods, and it was observed that the microwave method produced larger relative response ratios and fewer detectable artifacts across the different chemical classes of metabolites (Fig. 4). Concentrations ranged from 1 μg/mL to 1000 μg/mL, and the calibration curves (Fig. 6 – amino acid quantitation) for the standard mixtures (for all but one case) were satisfactory with regression coefficients better than 0.998 in most cases (Table 2). Reporting of regression coefficients up to 1000 μg/mL increases the R^2 significantly, and this may be slightly misleading as from 1 μg/mL to 100 μg/mL the regression coefficient obtained was generally lower, in the vicinity of 0.965–0.995 (see inset Fig. 6). Naphthalene at a concentration of 50 μg/mL was used as the internal standard in order to calculate the relative response of each compound. A linear trend is observed for the relative responses across the three levels analyzed, as the expected ratio between the 1000 μg/mL and the 100 μg/mL standard is 100:1 and 10:1 for the 100 μg/mL compared to the 10 μg/mL. For all compounds, an amount between 10 ng and 15 ng was finally injected into the GC × GC/ToFMS.

4. Conclusion

An optimized analytical method has been presented for the semi-quantitative analysis of a range of metabolites of interest to metabolomics. The analytical method proposed is quick, reliable (reproducible) and efficient (sensitive) as it takes advantage of the unique features of microwave-assisted derivatization as well as the separation power of GC × GC with ToFMS detection. A power setting of 150 W and an irradiation time of 90 s were found to be suitable for most of the different classes of metabolites studied. Methoximation and silylation reactions were also optimized to obtain maximum responses (sensitivity) across the different groups of metabolites, and partial derivatization of these compounds was also investigated in respect to artifacts formed during the derivatization process. Quantitation was performed using GC × GC/ToFMS (as all 44 compounds could be separated using GC × GC/ToFMS) and the linearity and reproducibility observed was sufficient (RSD less than 10%, R^2 greater than 0.998) in all cases but one. GC × GC was demonstrated as a useful tool to provide separation of metabolomic-type mixtures and the potential for resolution of target and matrix compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.04.036.

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