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Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry detection: analysis of amino acid and organic acid trimethylsilyl derivatives, with application to the analysis of metabolites in rye grass samples

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Abstract

First, standard mixtures of trimethylsilyl (TMS) derivatives of amino acid and organic acid are analyzed by comprehensive two-dimensional (2D) gas chromatography (GC) coupled to time-of-flight mass spectrometry (GC × GC/TOFMS) in order to illustrate important issues regarding application of the technique. Specifically of interest is the extent to which the peak capacity of the 2D separation space has been utilized and the procedure by which the derivative standards are identified in the 2D separations using the mass spectral information. The resulting 2D separation is found to make extensive use of the GC × GC separation space provided by the complementary stationary phases employed.

Second, in order to demonstrate GC × GC/TOFMS on two real sample types, trimethylsilyl metabolite derivatives were analyzed from extracts of common lawn grass samples (i.e., perennial rye grass), as a means to provide insight into both the pre and post harvest physiology. Various chemical components in the two rye grass extract samples were found to either emerge or disappear in relation to the trauma response. For example, a significant difference in the peak for the TMS derivative of malic acid was found. The successful analysis of various components was readily facilitated by the 2D separation, while a 1D separation would have produced too much peak overlap, thus impeding the analysis. The importance of using a GC × GC separation approach for the analysis of complex samples, such as metabolite extracts, is therefore demonstrated. The real-time analysis capability of GC × GC/TOFMS for multidimensional metabolite analysis makes this technique well suited to the high-throughput analysis of metabolomic samples, especially compared to slower, stopped-flow type separation approaches.

Keywords: Two-dimensional gas chromatography; Time-of-flight mass spectrometry; Metabolites; GC × GC/TOFMS; Comprehensive two-dimensional separation; Amino acids; Organic acids; Sugars

1. Introduction

Metabolomics determines the physiological status of a biological system by analyzing the type and quantity of small-molecules in the sample. Genomics, transcriptomics, and proteomics play important roles in understanding complex biological systems [1–5]. Metabolomics plays a key role in the next step of the progression of biological information. By describing the effects that a stress or a disease state has on the activity of various biological pathways and by studying changes in the chemicals produced in and through those pathways, metabolomics promises insights into cellular physiology that will enable the development of new small-molecule and biotechnologically-developed drugs, reduce the cost of drug research and development, and enable new industrial uses of biotechnology [5]. However, metabolites are present

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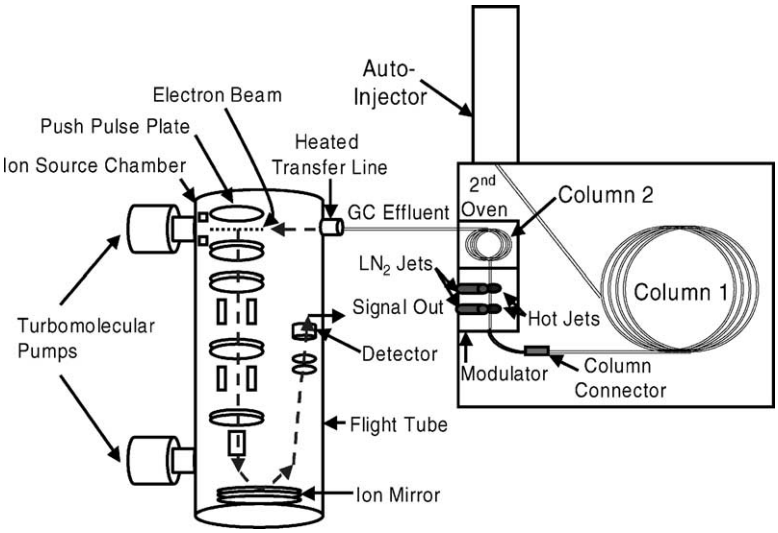

Fig. 1. GC × GC/TOFMS instrument schematic. Sample is injected by the auto injector into Column 1 (DB-5), 10 m with an 180 μm i.d. and a 0.18 μm film thickness. Delivery of Column 1 effluent onto Column 2 was performed using cryo-modulation. Effluent from Column 1 was concentrated at the head of Column 2 during each Column 2 separation. A hot pulse of nitrogen gas was used to begin each 2 s Column 2 run. Following each Column 2 separation the GC effluent was transferred to the TOFMS via a heated transfer line. Mass spectra were collected from 70–625 m/z at 100 spectra s^{-1}.

in a wide concentration range, and are produced by an extremely complex web of metabolic processes. Consequently, metabolomics presents a challenge both in designing suitable instruments, and in developing computational analysis methods.

Current methods for the study of metabolomic samples include, nuclear magnetic resonance (NMR) [6], liquid chromatography (LC) [7], capillary electrophoresis (CE) [8,9], and gas chromatography (GC) [10–16], with GC often implemented using mass spectrometry (MS) detection. Because most metabolites are non-volatile in their native state, methods to derivatize the compounds have been developed, so they may be separated by GC. The volatility of metabolites is increased by substituting acidic protons of alcohols, amines, and acids with trimethylsilyl groups. Hence, GC has been demonstrated as an effective method for examining the metabolome [4]. However, the complexity of metabolomics samples is such that the separation power of single dimension GC, even with the added power of MS detection, may often not be sufficient to solve emerging chemical analysis challenges.

Comprehensive 2D GC with time-of-flight mass spectrometry (GC × GC/TOFMS) is a recently emerging technology [17–26] that provides considerably more chemical selectivity than GC/MS, and thus should be ideally suited to the analysis of metabolites. GC × GC/TOFMS provides 3D data, retention time on two columns, and a complete mass spectral scan at each retention time coordinate in the 2D separation space. The 2D separation of GC × GC/TOFMS is done in real-time, unlike many other 2D methods, which require stopped-flow analysis to adequately sample the first dimension separation in the second dimension. This real time analysis capability makes GC × GC/TOFMS well suited to the high-throughput analysis of metabolomic samples. It has been shown that separations of complex products using GC × GC benefit from the increased selectivity offered by the use of two columns with stationary phases that provide complementary selectivity [27–29]. Implementing two complementary separation dimensions provides optimal information orthogonality [30–33]. Thus, a well-designed GC × GC system for metabolomic studies should, and will, fully utilize the 2D separation space and provide significantly more analytical information per unit time than will a single dimension GC separation of equivalent run time.

Typical GC × GC instrumentation (Fig. 1) utilizes a longer length of a non-polar stationary phase in the first dimension column (Column 1) from which effluent is transferred onto a shorter section of polar second dimension column (Column 2) to provide a complementary separation in the second dimension. With currently available technology one can perform a comprehensive GC × GC separation followed by mass spectral analysis at each point in the chromatographic space. The 3D data that are reported herein are amenable to analysis using various chemometric analysis techniques. With such large and potentially informative data sets the need for third order chemometric data analysis procedures is critical [25,26,34].

In this manuscript, we demonstrate the GC × GC/TOFMS analysis of derivatized amino acid and organic acid standards, as a means to obtain ample peak capacities, sufficient for comprehensive metabolomics analyses. We also analyzed metabolite extracts of perennial rye grass; evaluating both the pre and post harvest physiology of the samples. Although, the application of GC × GC/TOFMS to complex samples has been reported, this is the first report of applying this technology to the analysis of metabolites in real samples. Thus, the rye grass sample analyses provide a proof-of-principle study

of the GC × GC/TOFMS approach to studying metabolites. The two types of perennial rye grass samples are found to exhibit different GC × GC/TOFMS metabolite separation patterns. We further demonstrate the importance of a GC × GC approach for the analysis of complex mixtures such as these, which fully use the separation space available in such a multidimensional approach.

2. Experimental

2.1. Derivatization Procedure—standard samples

The standard mixtures contained approximately 50 mg of each of the dry chemicals listed in Table 1 and Table 2 for amino and organic acids, respectively. Chemicals were purchased from Sigma Aldrich (St. Louis, MO). Both of the standards mixtures were dissolved in water and 10% (v/v) of each solution was transferred to a clean vial where it was dried at 50 °C for approximately 60 h. Fifty micro litre of pyridine (Fisher Scientific, Fairlawn, NJ 99.9%) and 100 μl of Sylon BTZ reagent mixture were added to the dried standard samples. This mixture was heated to 70 °C for 30 min. The reagent mixture, Sylon BTZ, is a mixture of N,*O*-bis(trimethylsilyl)acetamide (BSA), chlorotrimethylsilane (TMCS) and *N*-trimethylsilylimidazole (TMSI) in a ratio of 3:2:3 (Supelco, Bellefonte, PA). The samples were allowed to cool prior to analysis via GC × GC/TOFMS.

Table 1
List of amino acids included in the amino acids mixture

L-Alanine
L-Arginine
L-Asparagine
L-Aspartic acid
L-Cysteine
L-Glutamic acid
L-Glutamine
Glycine
L-Histidine
L-Isoleucine
L-Leucine
L-Lysine
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine
L-Valine
γ-Amino-*N*-butyric acid
β-Alanine
L-Cystine
D-Glutamic acid
L-Ornithine
DL-Pyroglutamic acid
L-Homoserine
Trans-4-hydroxy-L-proline

Table 2
List of organic acids included in the organic acids mixture

L-Ascorbic acid
Benzoic acid
Citric acid
Fumaric acid
D-Gluconic acid
D-Gluconic acid lactone
D-(+)-Glyceric acid
4-Hydroxy benzoic acid
6-Hydroxy benzoic acid
DL-isocitric acid
Maleic acid
Malic acid
Nicotinic acid
Oxalic acid
Pyruvic acid
(−)-Quinic acid
Succinic acid
L-(+)-Tartaric acid

2.2. Extraction Procedure—rye grass samples

The extraction of grass samples (perennial rye grass lawn samples, University of Washington, Seattle, WA) was performed over the course of 2–3 h. Two representative grass samples were obtained to produce a fresh sample "Sample 1" and a partially decomposed grass sample "Sample 2", representing pre and post harvest physiology, respectively. Immediately after cutting the grass samples, Sample 1 was placed in a sterile, capped 15 ml polystyrene conical tube (Becton Dickerson, Franklin Lakes, NJ) and held in a methanol bath at −78 °C using dry ice, i.e., frozen prior to extraction. The grass sample used to prepare Sample 2 was also immediately placed in a sterile, capped 15 ml polystyrene conical tube, but then left unfrozen for approximately 1 h between the time it was cut and the time that the extraction began. Thus, the grass sample used to prepare Sample 2 was allowed to "decompose" for 1 h prior to extraction. The grass samples were returned to the laboratory where they were transferred into unsilanized glass test tubes, weighed and crushed. Approximately 0.3 g of plant matter were used for each derivatization sample preparation. 50 μl of deionized water and 1.4 ml of methanol (Fisher Scientific 99.9%) were added, and the test tubes were heated to 70 °C for 25 min. After the samples had cooled to room temperature they were centrifuged (International Equipment Co. Boston, MA) for 3 min and the liquid was then decanted to 5 ml silanized glass vials with caps. 1.4 ml of water and 0.75 ml of chloroform (Fisher Scientific 99.9%) were added prior to the vials being vortexed for 1 min. Samples were allowed to sit for 30 min as the aqueous and organic layers separated. The aqueous layer was transferred to a clean vial and was centrifuged until a visible separation of layers was again apparent. The aqueous layer was collected and placed in an oven at 50 °C to dry for approximately 60 h. Once dried, derivatization was performed as described below.

2.3. Derivatization Procedure—rye grass samples

The derivatization of grass samples following their extraction as described above, was performed using a mixture of 25 μl of pyridine (Fisher Scientific 99.9%), 25 μl of acetonitrile (Baker 99%) and 100 μl of Sylon BTZ reagent mixture. The samples were stirred until the solid extract matter appeared to dissolve (5 min), then the contents of the test tubes were transferred to silanized glass inserts in GC autosampler vials which were then capped and held at 50 °C for 100 min. Once the samples had cooled to room temperature they were analyzed by GC × GC/TOFMS.

2.4. GC × GC/TOFMS analysis

The analyses of the standard mixtures and the extracted grass samples were performed using a Leco Pegasus III instrument with the 4D upgrade (Leco Corp., St. Joseph, MI), shown schematically in Fig. 1. The first column (i.e., Column 1) of the GC × GC/TOFMS instrument was a 10 m DB-5 capillary column with an internal diameter of 180 μm and a film thickness of 0.18 μm (J & W Scientific, Alltech, Deerfield, Il), a relatively standard separation phase for metabolite analysis studies [10,13,14]. The second column (Column 2), was a 2 m length of DB-17 with a 100 μm internal diameter and a 0.1 μm film thickness. These columns were joined using a mini union (Scientific Glass Engineering SGE, Austin, TX). Modulation, or delivery of Column 1 effluent onto Column 2 was performed using cryomodulation. Effluent from Column 1 was concentrated at the head of the Column 2 during each Column 2 separation. A hot pulse of nitrogen gas was used to begin each new Column 2 separation. The hot pulse was 0.40 s in duration, and the total Column 2 run time was 2 s. Ultra high purity helium (0.8 ml min^{-1}) was used as the carrier gas for both the standard mixture analysis as well as for the grass sample analyses. The injection of standard mixtures was done in the splitless mode with a 1 μl injected volume and a Column 1 oven ramp beginning at 70 °C with a hold time of 5 min then increasing at 5° min^{-1} to 250 °C with a hold time at 250 °C of 5 min. Column 2 was held in a separate oven (see Fig. 1), which was 40 °C higher than the Column 1 temperature. The first 5 min of each run was considered a solvent delay and no mass spectra were taken during that time. However, all GC × GC/TOFMS separations were plotted with the 5 min included on the Column 1 time axis so the data reflect the correct retention time. The analysis of the grass sample extracts differed only slightly from the above procedure. For the grass sample analyses, 1 μl of derivatization solution was injected using a 25:1 split. All studies utilized an Agilent 7683 autoinjector and an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA). The transfer line was held at 260 °C and the ion source was held at 200 °C. The detector voltage was 1600 V and the filament bias was −70 V. Mass spectra were collected from 70–625 m/z at 100 spectra s^{-1}. Mass spectra from the GC × GC separation were searched against the NIST MS database to provide the peak matches listed (NIST MS Search 2.0, NIST/EPA/NIH Mass Spectral Library; NIST 98). NIST matches to reference spectra are based on a weighted dot product of the two spectra with higher *m*/*z* spectral peaks having more weight than lower *m*/*z* spectral peaks. A similarity value, in a range from 0 to 999 with 999 being a perfect match, is produced as a measure of the quality of the match.

3. Results and discussion

A standard mixture of amino acids, listed in Table 1, was prepared and analyzed as described in the Section 2. The total ion current (TIC) GC × GC chromatogram for the amino acid standard is shown in Fig. 2A. In this separation, nearly the entire GC × GC separation space is being used, and the 2D separation exhibits a significant level of information orthogonality [30–33]. The 2D chromatogram shows the importance of using a 2D chromatographic separation technique employing complementary separation on both axes for the analysis of metabolite samples. The combination of both non-polar and polar columns provides a larger peak capacity and more selectivity than either column on its own. Further studies, not shown for brevity, indicate a high level of instrumental reproducibility for this technique. With replicate injections displaying a relative standard deviation in peak volume of 5%, where the peak volume is the total integrated signal. Reproducibility can be further improved using a set of internal standards to account for extraction, derivatization, and injection variations.

The combination of GC × GC separation with TOFMS detection is a powerful technique. In this study, full mass spectral scans are collected at 100 spectra s^{-1} through the entire separation space. TOFMS can be readily used to identify resolved peaks in the GC × GC separation, or alternatively, third order chemometric methods can be employed to deconvolute the mass spectra of peaks that are overlapped in the GC × GC separation space [23,26]. The deconvoluted mass spectra can then be used, with GC × GC retention time information for identification purposes [17–26,35]. The TIC contour plot of the same amino acid standard mixture and GC × GC separation shown in Fig. 2A is shown in Fig. 2B for a different perspective on visualization. In the figure, representative peaks are labeled and the mass spectral matches are listed. Representative peaks are labeled for the following amino acid derivatives: (a) serine; (b) and (c) L-threonine; (d) L-methionine; (e) alanine; (f) proline; (g) phenylalanine and (h) homoserine. The presence of more than one threonine peak is to be expected as a result of the degree of derivitization of the samples. The silanization process used herein replaces active hydrogen, hydrogen bound to oxygen, nitrogen, etc., with trimethylsilyl (TMS) groups. The degree of silanization of a sample is dependant on the strength and steric interactions of the reagents used as well as the parameters under which the derivitization takes place. Under the conditions used in this work, there is some selectivity in the degree of

silanization of amine groups. Carboxylic acid groups, which are generally more easily derivitized, appear to have reacted to completion. Matches have been made with NIST similarity scores of 800 or greater, to peaks representing zero, one and two TMS substitutions to a primary amine site. NIST similarity scores can range from 0 to 999, and scores greater than 750 are considered reasonable. This is shown with the peaks for (b) threonine, (d) methionine, and (e) alanine in Fig. 2B. The threonine peak labeled (b) has no amine TMS substitutions, the methionine peak (d) has one of two amine hydrogen atoms substituted with a TMS group, and the alanine peak (e) has both amine hydrogen atoms substituted with TMS groups.

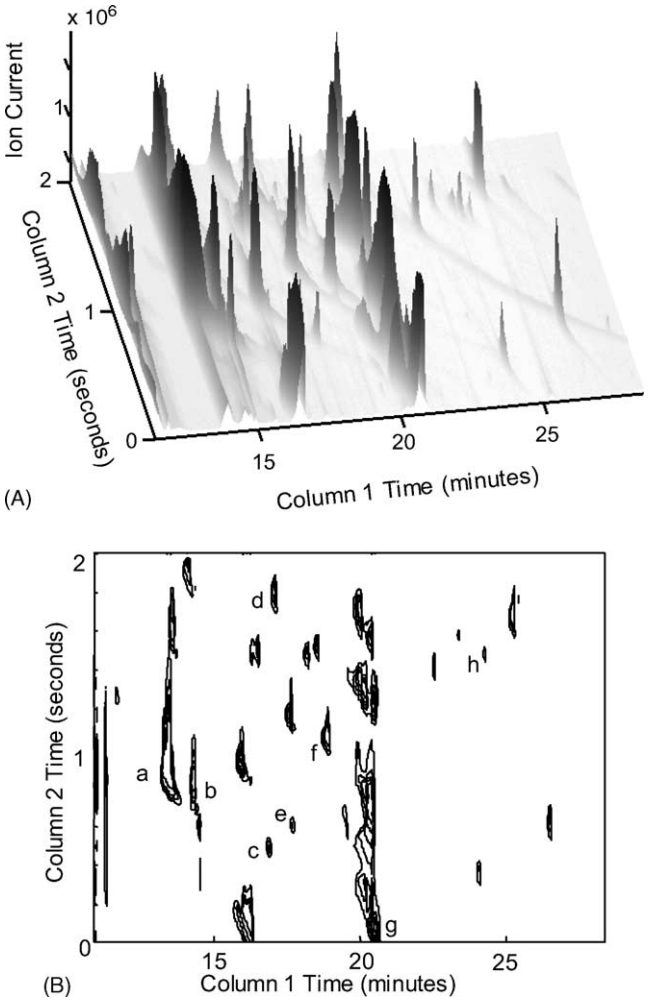

Fig. 2. (A) Total ion current (TIC) GC × GC chromatogram of the amino acid standards mixture (listed in Table 1), prepared and injected as TMS derivatives. (B) TIC contour plot of the separation in (A), with representative peaks identified with a NIST mass spectral database search. Peaks are labeled as follows: (a) serine (two TMS substitutions [NHTMS and COOTMS]); (b) L-threonine (two TMS substitutions [OTMS and COOTMS] no amine TMS substitutions); (c) L-threonine (three TMS substitutions [OTMS, NHTMS and COOTMS] one amine substitution); (d) L-methionine (two TMS substitutions [NHTMS and COOTMS]); (e) alanine (three TMS substitutions [NTMSTMS and COOTMS]); (f) proline (two TMS substitutions [OTMS and COOTMS]); (g) phenylalanine (one TMS substitution [COOTMS]) and (h) homoserine (three TMS substitutions [OTMS, NHTMS and COOTMS]).

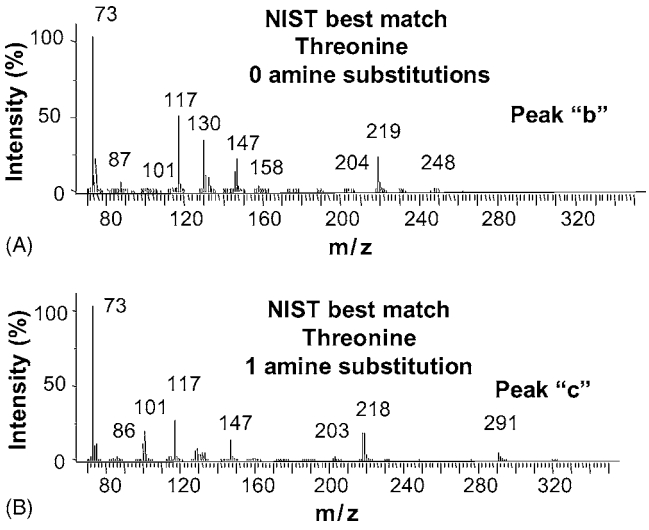

Fig. 3. Mass spectra for two separated and detected forms of threonine derivatives, labeled (b) and (c) from Fig. 2B. (A) Mass spectrum of the threonine derivative with no amine TMS substitutions. (B) Mass spectra of the threonine derivative with one amine TMS substitution.

Matches have also been made to multiple peaks of the same compound representing different levels of amine derivitization, such as in the case of threonine in Fig. 2B. Peaks (b) and (c) are threonine peaks, (b) having neither of the amine hydrogen atoms substituted, and (c) having one amine hydrogen substituted. The mass spectrum of a threonine derivative (b) is shown in Fig. 3A. The mass spectrum of the other threonine derivative (c) is shown in Fig. 3B. The two threonine derivatives match very well with their respective NIST reference spectra, with similarity scores of 930 and 920, respectively. The added spectral complexity of having multiple peaks for a given compound is a potential benefit for pattern recognition studies where a change in one compound can be tracked by corresponding changes in various parts of the separation space. This benefit can be described as adding "multivariate selectivity" to the data structure [25,26,33,34,36].

An organic acid standard mixture, which contained the compounds listed in Table 2, was prepared and analyzed as described in the Section 2. Fig. 4A is a TIC plot of the GC × GC separation of the organic acid standards mixture, again providing considerable use of the GC × GC separation space. Fig. 4B shows the same separation space in the form of a contour plot with representative peaks labeled for the following organic acid derivatives: (a) oxalic acid, (b) benzoic acid, (c) nicotinic acid, (d) maleic acid [(Z)-butenedioic acid], (e) succinic acid [butanedioic acid], (f) fumaric acid [(E)-butenedioic acid], (g) glyceric acid [propanoic acid], (h) malic acid, (i) 6-hydroxy nicotinic acid, (j) 4-hydroxy benzoic acid, (k) tartaric acid, (l) isocitric acid lactone, (m) citric acid, (n) gluconic acid lactone. The separation of maleic acid (d), succinic acid (e) and fumaric acid (f) is particularly noteworthy and the mass spectra of the three compounds are shown in Fig. 5A–C, respectively. While the separation of the two isomeric forms of butenedioic acid, maleic (d) and

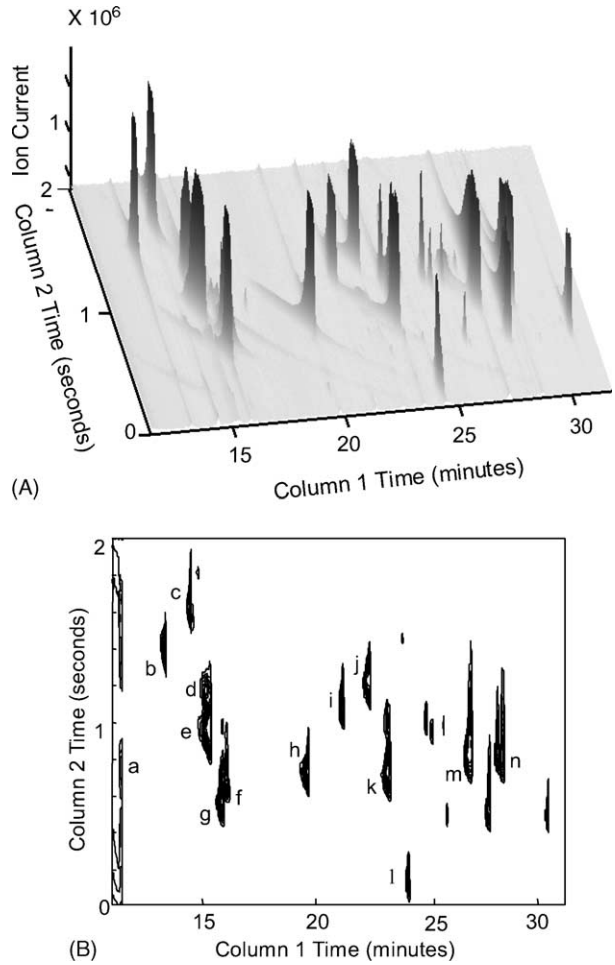

Fig. 4. (A) TIC GC × GC chromatogram of the organic acid standards mixture (listed in Table 2) prepared and injected as TMS derivatives. (B) TIC contour plot of the separation in (A), with representative peaks identified with a NIST mass spectral database search. Peaks are labeled as follows: (a) oxalic acid (two TMS substitutions [COOTMS]); (b) benzoic acid (one TMS substitution [COOTMS]); (c) nicotinic acid (one TMS substitution [COOTMS]); (d) maleic acid [(Z)butenedioic acid] (two TMS substitutions [COOTMS]); (e) succinic acid [butanedioic acid] (two TMS substitutions [COOTMS]); (f) fumaric acid [(E)butenedioic acid] (two TMS substitutions [COOTMS]); (g) glyceric acid [propanoic acid] (three TMS substitutions [OTMS and –COOTMS]); (h) malic acid (three TMS substitutions [OTMS and COOTMS]); (i) 6-hydroxy nicotinic acid (two TMS substitutions [OTMS and –COOTMS]); (j) 4-hydroxy benzoic acid (two TMS substitutions [OTMS and COOTMS]); (k) tartaric acid (four TMS substitutions [OTMS and COOTMS]); (l) isocitric acid lactone (two TMS substitutions [COOTMS]); (m) citric acid (four TMS substitutions [OTMS and COOTMS]) and (n) gluconic acid lactone (four TMS substitutions [OTMS]).

fumaric acid (f), was performed on Column 1, neither peak would have separated from the co-eluting succinic acid (e) or glyceric (g) acid without the Column 2 separation. The problem of co-elution on the Column 1 axis was more pronounced in the case of maleic acid (d) and succinic acid (e), which would have been completely overlapped without the additional separation power offered by Column 2. The NIST matches for these four peaks were all quite strong. Similarity scores were 934 for maleic acid, 924 for succinic acid, 952

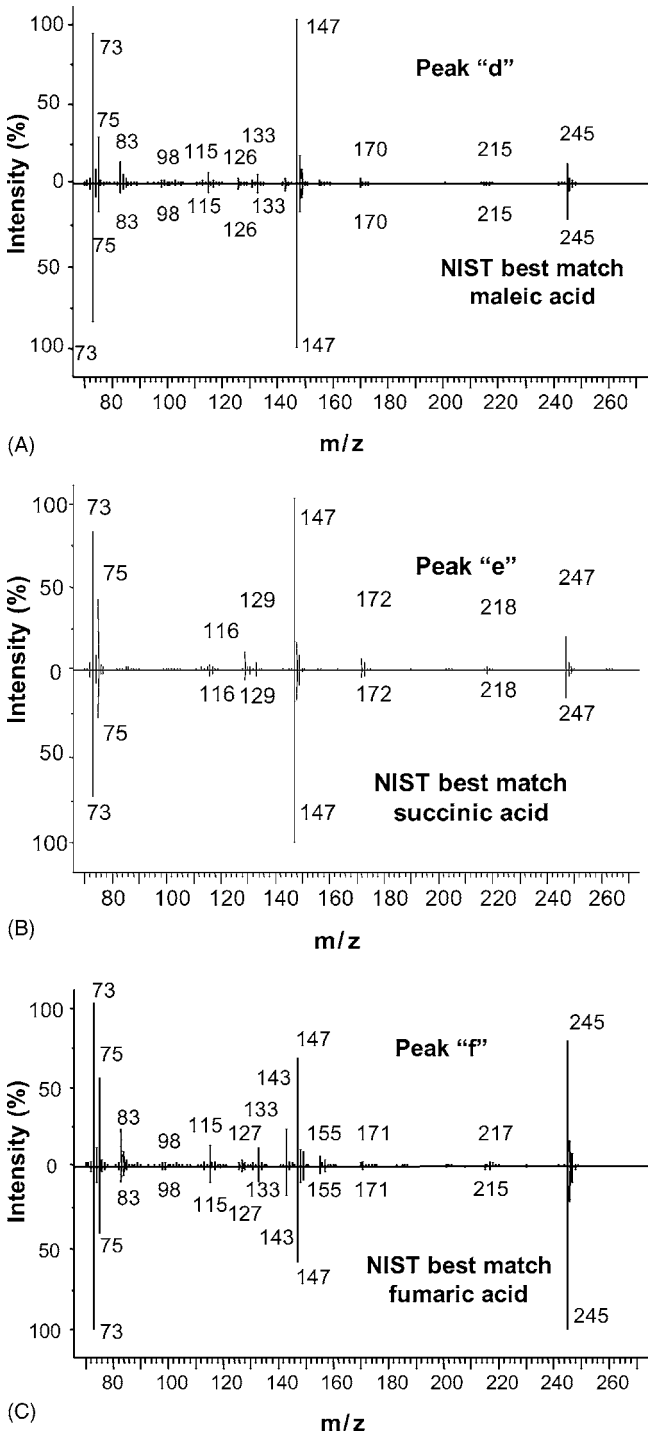

Fig. 5. (A) Mass spectrum of the maleic acid derivative with two carboxyl TMS substitutions, labeled peak (d) in Fig. 4B, which matches well with the NIST reference spectrum. (B) Mass spectrum of the succinic acid derivative with two carboxyl TMS substitutions, labeled peak (e) in Fig. 4B, which matches well with the NIST reference spectrum. (C) Mass spectrum of the fumaric acid derivative with two carboxyl TMS substitutions, labeled peak (f) in Fig. 4B which matches well with the NIST reference spectrum.

for fumaric acid and 926 for glyceric acid with a score of 999 being a perfect match.

As an initial evaluation of the benefit of using GC × GC/TOFMS for the analysis of metabolites, we evaluated the pre and post harvest physiology of perennial rye grass using extracted metabolite profiles. Informative sections of the GC × GC chromatograms from rye grass sample extracts are shown in Fig. 6A and B. In both cases grass samples were collected and extracted. The extract described as "Sample 1", shown in Fig. 6A, was frozen immediately upon being cut, as opposed to the extract labeled as "Sample 2", shown in Fig. 6B, which was left unfrozen for approximately 1 h between the time it was cut and the time that the extraction began. The most notable difference between these two extract samples occurs in the region between 16 and 24 min, i.e., near 19 min, after injection on Column 1. In the Sample 1 extract this region is rather devoid of peaks. There is a small peak, labeled (a), which matches to malic acid with a similarity score of 777. In Sample 2 (Fig. 6B), however, there are a series of peaks in this region with NIST matches as follows: (c) malic acid with a NIST match similarity score of 950, and (d), (e) and (f) which are other various metabolites with lower NIST similarity scores making their positive identification less certain. The largest, most prominent peak

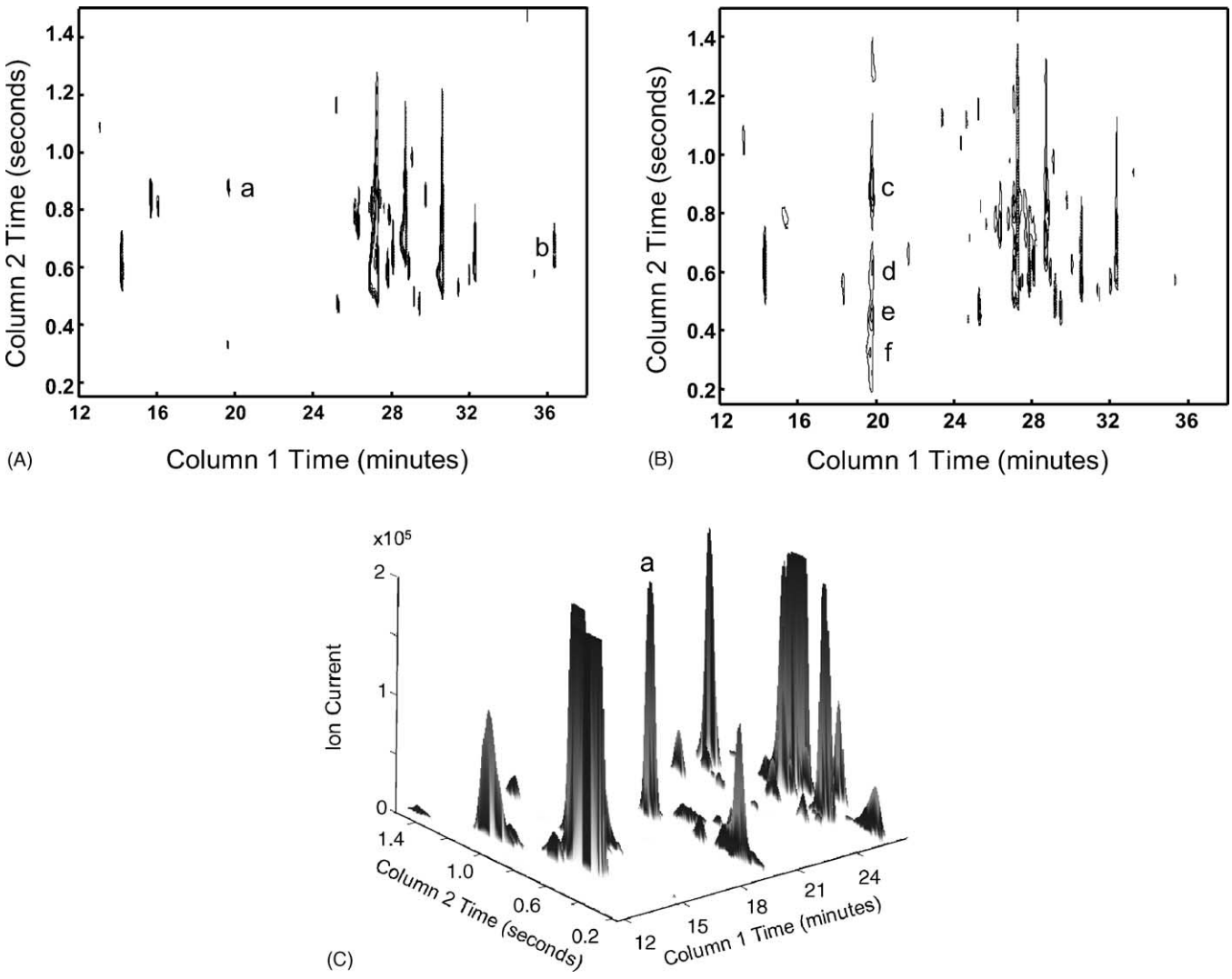

Fig. 6. (A) TIC contour plot of selected region of the Sample 1 lawn grass extract GC × GC chromatogram. The area in between 16 and 24 min on Column 1 is rather devoid of large peaks especially in comparison to the corresponding region in the Sample 2 lawn grass extract (in Fig. 6B). The small peak labeled (a) matches to malic acid (three TMS substitutions [OTMS and COOTMS]) with a similarity score of 777. Also in this figure is peak (b)), which matches to 2-*O*-glycerol-α-D-galactopyranoside (six TMS substitutions [OTMS]) with a NIST similarity score of 831. (B) TIC contour plot of selected region of Sample 2 grass extract GC × GC chromatogram. There are a series of notably large peaks that eluted at approximately 19 min after injection on Column 1, which are only separated from one another on Column 2. NIST matches for these peaks are as follows: (c) malic acid (three TMS substitutions [OTMS and COOTMS]) with a NIST match similarity score of 950, and (d), (e) and (f) which are other various metabolites with lower NIST similarity scores making their positive identification less certain, but would overlap with malic acid if only Column 1 were used alone. (C) Zoomed-in region of the lawn grass Sample 1 TIC surface plot, emphasizes the dynamic range of the instrument as well as the dynamic range of the analytes in the samples studied, as well as the potential limitation of visualization tools to study the data.

of the group is that of malic acid. The biological significance of the increase in malic acid in the extract of perennial rye grass that has been allowed time to respond to the harvesting process is unknown, however, it may correspond to a change in the activity of the citric acid cycle due to the prominent role that malic acid plays within that biological process. In addition to the appearance of prominent peaks in the region around a 19 min retention time, there is also the disappearance of the peak labeled (b) in Fig. 6A. This peak matches to 2-*O*-glycerol-α-D-galactopyranoside with a NIST similarity score of 831. This is one of many peaks in the grass samples whose mass spectrum tentatively matches to that of a sugar, as might be expected in an extract of plant matter such as this.

Evaluations of these samples using single dimensional GC were not undertaken in the present study. However, for the optimized single dimension separations used, peak regions such as those analyzed in this report would have been unresolved without the aid of the second dimension column. Peak regions of potential first column overlap were noted in many types of plant extracts performed in this study, including samples of various species of sage, as well as huilmo and fava bean plants, sprouted seeds of broccoli, alfalfa, pea and daikon, not shown for brevity. Bacteria samples, as well as samples of urine, are currently under investigation using this GC × GC/TOFMS technique and will be the topics of future publications.

To emphasize the dynamic range of the samples studied, the region of Sample 1 around peak (a) is shown in Fig. 7C as a TIC surface plot. Peaks initially not easy to see in Fig. 6A are now readily apparent in Fig. 7C. In an effort to better visualize the lower intensity peaks some of the high-intensity peaks are off scale in Fig. 7C. Indeed, the GC × GC/TOFMS instrumentation is better suited to capture the concentration dynamic range and response of the constituents in the extract samples, often better than the available software.

4. Conclusions

Analysis of metabolites by GC × GC/TOFMS was demonstrated herein using standard mixtures of amino and organic acid derivatives. Initial studies of plant metabolites were undertaken using extracts of perennial rye grass samples in evaluating pre and post harvest physiology. The importance of using a GC × GC separation approach for the analysis of complex biological samples, such as metabolite extracts, has been demonstrated. It has further been demonstrated that this type of analytical approach is capable of detecting and describing the dynamic nature of the system under study. Currently, work is being undertaken to quantitatively study metabolite samples using this technique. This novel application of 2D GC combined with time-of-flight mass spectrometry promises to be a powerful technique for rapid and comprehensive metabolite analysis both in terms of target analysis, looking for specific analytes, as well as for pattern recognition and fingerprinting studies, looking for cause and effect relationships. GC × GC/TOFMS is at an early stage in its use for metabolic research, but it appears to have much to offer in the expanding field of metabolomics.

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