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On the applicability of comprehensive two-dimensional gas chromatography combined with a fast-scanning quadrupole mass spectrometer for untargeted large-scale metabolomics☆

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ABSTRACT

Comprehensive two-dimensional gas chromatography mass spectrometry (GC × GC–MS) offers excellent chromatographic separation performance and superior sensitivity. As such, it is eminently suitable for the analysis of complex biological samples. The applicability of a GC × GC instrument equipped with a fast-scanning qMS detector for large-scale untargeted metabolome analyses was investigated. We optimized the dimensions of an apolar × medium-polar column combination in order to meet detector requirements and to compromise between separation performance and analysis time. The final method enabled a sufficient separation ($R \geq 1.2$ or higher) of approx. 90% of all analytes detected in urine within less than 1 h. Using the qMS at maximum scan speed (20,000 u/s) and choosing a scan range of *m/z* 60–550, a data acquisition frequency of 33 Hz and usually at least 10–13 data points per ^{2}D peak above the baseline were achieved. Peak area as well as peak height could thus be determined precisely (mean RSD 2.5%). Spectral skewing was limited regarding the data points covering the upper peak half. As a consequence, peak apex spectra could be used for the alignment of analytes in different samples. The linear dynamic range was 1–2.5 orders of magnitude, depending on the analyte. In addition, the slow transition into saturation beyond the linear dynamic range made it possible to exploit an extended "working range" for relative quantification. Long-term stability of the system was demonstrated by the analysis of more than 300 human urine study samples for which detailed repeatability and intermediate precision data are provided. In summary, the GC × GC–qMS system proved to be applicable for untargeted large scale metabolome analyses.

1. Introduction

Comprehensive two-dimensional gas chromatography (GC × GC) is an analytical technique that enables chromatographic separation of all analytes in a sample based on two different and ideally independent separation mechanisms. The principal items of a comprehensive GC × GC system are two serially connected GC columns with different stationary phases and the modulator, a transfer device located between the two columns [1]. Because of its high separation performance and inherently high sensitivity, GC × GC is nowadays widely used for the analysis of complex mixtures in different disciplines. As GC × GC peaks are usually very narrow (^{2}D peak width 100–500 ms), a fast detector is required to obtain a sufficient number of data points per peak and to capture the peak profile quantitatively. For this reason, GC × GC is most often combined with Time of Flight-MS (TOF-MS) detection which enables data acquisition rates of more than 100 Hz [2] which in turn facilitates spectral deconvolution. In contrast, quadrupole MS (qMS) instruments achieve lower data acquisition rates due to the operating principle of the quadrupole mass analyzer but are well-known to be robust and affordable. Therefore, in case of GC × GC–qMS, a reasonable compromise between scan speed and the number of data points per peak on the one hand and the width of the scan range on the other hand has to be found. Another problem with GC × GC–qMS is spectral quality. Due to analyte refocussing by the modulator and the very short second column, the analytes reach the qMS as narrow bands causing fast changes of analyte concentration in the ion source. This leads to spectral skewing, i.e. a dissimilarity of mass spectra across a peak, especially if the spectra of data points at both peak flanks are compared. Hence, there is a clear relationship between spectral skewing and detector speed. While previous generations of qMS instruments achieved

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scan speeds of up to 10,000–12,500 u/s, modern fast-scanning qMS devices can achieve 20,000 u/s, allowing for a wider scan range or a higher data acquisition rate. The applicability of GC × GC systems equipped with a fast scanning qMS for targeted analyses has been investigated and confirmed several times [3–6].

One of the most challenging applications of GC × GC–MS is metabolomics, an approach which aims to detect and quantify as many metabolites in a biological sample as possible. Metabolomics or metabolome analyses differ from conventional targeted analyses in several aspects: Firstly, the majority of the detected analytes are a priori unknown and mostly relative differences between sample groups (e.g. treated vs. untreated, healthy vs. diseased) are of interest. Thus, a relative quantification is mainly used which can be associated with a higher analytical variation. Secondly, in order to ensure comprehensiveness of analysis, sample preparation methods are unselective resulting in a high matrix load which causes instrument contamination. Finally, if the differences between groups are expected to be small, large sample numbers have to be analyzed to obtain the desired statistical power. This requires a good long-term precision of the analysis. While GC–qMS, GC-TOF-MS and GC × GC-TOF-MS have been widely used for metabolome analyses [7–19], the applicability of a fast-scanning GC × GC–qMS for untargeted large-scale metabolomics has, to our knowledge, not been evaluated. Only Bressanello et al. [20] used a qMS instrument with moderate scanning speed but in a combined GC × 2GC–MS/FID setup.

Due to its robustness, GC × GC–qMS appears to be a promising platform for untargeted large-scale metabolomics. Therefore, we assessed the applicability of a GC × GC system equipped with a fast scanning qMS instrument for such studies. The first aim was to find a column combination which is compatible with detector requirements and enables a sufficient separation of the analytes in an acceptable time. Next, we evaluated qMS performance with respect to the number of data points per ^{2}D peak, precision of peak area and peak height determination as well as spectral quality and linear dynamic range. Finally, we used the system for the analysis of urine samples obtained in a large human metabolomics study and assessed repeatability and intermediate precision.

2. Materials and methods

2.1. Chemicals

Methanol and ethanol (both GC grade) were purchased from Merck (Darmstadt, Germany). Heptane (>99.0%) and pyridine (>99.5%) were from Carl Roth (Karlsruhe, Germany). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was supplied by Macherey-Nagel (Düren, Germany). *O*-Methoxylamine hydrochloride was purchased from Chemos (Regenstauf, Germany). Internal standards as well as standards used for the preparation of the in-house mass spectral library were from different companies, mostly Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), ABCR (Karlsruhe, Germany), Merck (Darmstadt, Germany), TCI (Zwijndrecht, Belgium), Alfa-Aesar (Ward Hill, USA), and ChromaDex (Irvine, USA). All standards had a purity of at least 95%.

2.2. Urine samples

In order to prepare a representative, pooled urine sample for method development and performance evaluation of the GC × GC–qMS system, spot urine samples were donated by 30 male volunteers aged 15–65 after fasting overnight. Equal volumes of these samples were combined to create the pooled sample. The creatinine content was determined photometrically using the Urinary Creatinine Detection Kit (Arbor Assays, Ann Arbor, USA). The sample was aliquoted and stored at −24 °C.

The 24 h urine samples obtained in the Karlsruhe Metabolomics and Nutrition (KarMeN) study, a large human metabolomics study comprising 312 volunteers, were analyzed by GC × GC–qMS in order to evaluate the long-term stability and performance of the system. Samples were preprocessed as follows. After determination of urine volume, 14 ml of urine were transferred to a 50 ml falcon tube and centrifuged for 10 min at 4 °C and 1850 × *g* in order to remove insoluble matter. The supernatant was then aliquoted and stored until analysis at −190 °C in a specialized cryo storage system (a large dewar vessel partly filled with liquid nitrogen in which the samples are stored in the gas phase). A pooled quality control (QC) sample was prepared by combining a representative number of study samples. Creatinine content was determined as mentioned above. The entire study protocol was approved by the ethics committee of the State Chamber of Physicians of Baden-Württemberg (F-2011-051).

2.3. GC × GC–qMS system and analysis

The GC × GC–qMS system consisted of a gas chromatograph combined with a fast-scanning quadrupole mass spectrometer (Shimadzu GCMS QP2010 Ultra) and an AOC-5000 autosampler. Cold split injection (split 1:10) was done using an OPTIC4 system (GL Sciences, Eindhoven, The Netherlands). The injector temperature was ramped immediately after injection from 90 °C to 280 °C (60 °C/s) where it was held until the end of the run. A deactivated split liner with a glass frit and deactivated quartz wool was used. Helium was used as carrier gas at a column head pressure of 75 kPa. A non-polar Rxi-5SilMS (^1d_c = 0.25 mm, ^1d_f = 0.25 μm; Restek, Bellefont, USA) was chosen for ^{1}D. The ^{2}D column was a medium-polar BPX50 (^2d_c = 0.15 mm, ^2d_f = 0.15 μm; SGE, Milton Keynes, UK). Both columns were connected with a SilTite MiniUnion (SGE). The column lengths were optimized in order to find a compromise between separation performance and analysis time. Finally, we chose a comparatively short first column (^1L = 15 m plus 5 m of an integrated pre-column) and a second column of moderate length (^2L = 2.6 m), including 1.5 m for the modulator loop ($^2L_{loop}$) and 1.1 m for the "separation segment" ($^2L_{sep}$). The following GC temperature ramp was used: 90 °C → 2 °C/min → 100 °C → 3.5 °C/min → 200 °C → 4 °C/min → 268 °C → 42 °C/min → 320 °C (2.43 min) which resulted in a total run time of 54 min. Both columns were housed in the same oven. A cryogenic two-stage loop-type modulator based on pressurized air was used (ZX2, ZOEX Corp, Houston, USA). The modulation period was 6 s and the hot jet duration was 375 ms. The temperature of the modulator's hot jet was programmed stepwise and kept always at least 50 °C above oven temperature. The transfer line was held at 320 °C. The qMS was operated at maximum scan speed (20,000 u/s) covering a scan range of *m/z* 60–550 which corresponds to a data acquisition rate of 33.3 s^{-1} (Event Time: 0.03 s). An ion source temperature of 200 °C was chosen and 70 eV EI spectra were recorded.

2.4. Evaluation of qMS performance

The performance of the qMS instrument was examined in several ways. First of all, the number of data points per peak was determined for 20 selected analytes in a representative urine chromatogram, considering all modulated peaks. To ensure comparability, we counted (i) all data points clearly lying above the baseline, omitting those at the beginning and the end of the peak, (ii) all data points lying above 10% of peak height, and (iii) all data points covering the upper peak half, i.e. above 50% of peak height or at full width at half maximum (FWHM).