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Two-dimensional comprehensive gas chromatography coupled to time of flight mass spectrometry in doping control: Evaluation of the chromatographic plane organization

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Abstract

The GC×GC-TOFMS has already being used in different analytical fields with good results, but in doping control the approach was not extensively investigated yet. The goal here was to evaluate the level of chromatographic structuration using an orthogonal column system. The endogenous steroids usually employed in the profile were used as a model. Two different derivatization approaches were adopted (OTMS and MOTMS derivatives). The GC×GC-TOFMS system was equipped with a secondary oven and a non-moving quad-jet dual-stage modulator. The first column was 100%-polymethylsiloxane (16.3 m x 0.2 mm x 0.11 µm) and the second was a poly-14%-cyanopropylphenyl-86%-dimethylsiloxane (1m x 0.11 mm x 0.1 µm). The results indicate a high structuration in the chromatogram with great precision in the retention time measurements. In the second dimension, the elution order of the analytes is highly dependent to the polarity. An organization similar to the “roof-tile” effect was observed. The chromatographic plane was highly sensitive to structure nuances, including number of polar groups and double bonds. MOTMS derivatives allow the differentiation between keto and hydroxyl analytes. The knowledge relative to the chromatographic plane could be a relevant analytical information. However, better software tools and chemometric approaches are still needed to support an efficient non-target analysis. The features concerning the GC×GC coupling with the TOFMS analyzer suggests its used in non-target analysis. However the massive amount of information obtained is a challenge when the aim is to recognize unknown substances in the chromatographic plane.

Introduction

The two-dimensional comprehensive gas chromatography coupled to time of fight mass spectrometry (GC×GC-TOFMS) approach has been successfully used in different fields as petrochemical, phytochemical and metabolomics. Relative to doping control application a limited number of publications is available [1-4]. The principle of the technique is described elsewhere [5]. The power of the technique relies on the ultimate chromatographic separation, fast data acquisition (until 500 Hz) and software deconvolution tools. As a result of the synergism of these characteristics, GC×GC-TOFMS arises as a tool with high selectivity and specificity [4], able to provide high peak capacity and full spectra acquisition. Since the GC×GC could be coupled with a full spectral acquisition device (TOFMS analyser), it has potential to be used for non-targets analyses. Nevertheless, the high amount of information available from the high peak capacity and the full spectra acquisition is a challenge itself if the goal is to find out something new (prohibited) in the chromatographic plane. To reach out this goal, the development of better software tools and the application of chemometric approaches become necessary. Meanwhile, all relevant information available should be utilized. A GC×GC feature not yet exploited in doping control is the so-called “structured chromatogram”. This phenomenon arises from the orthogonality between the two columns features used in the procedure. Our goal was to evaluate the level of structuration obtained in the chromatographic plane for urine samples, using the endogenous analytes typically monitored in the steroid profile as a model.

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Experimental

Sample preparation
A mix of standards containing the endogenous steroids typically evaluated of profile were derivatized following two (classic) derivatization procedures: (i) For silylated derivatives (O-TMS): Addition of 100 µL MSTFA–NH_4I–2–mercaptoethanol (1000:2:6, v:w:v), incubation at 60ºC / 20 min. (ii) For metoxime-silylated derivatives (MO-TMS): 50 µL O-methylhydroxylamine hydrochloride/pyridine (8 %) at 60ºC / 30 min. Pyridine removed under N_2 flow. Addition of 100 µL TMS-imidazole/MSTFA (2 %) at 60ºC / 20 min.

Apparatus
The GC×GC-TOFMS system used was a Pegasus 4D (Leco, St. Joseph, MI, USA) composed of an Agilent 6890 GC (Palo Alto, CA, USA) equipped with a secondary oven and a non-moving quad-jet dual-stage modulator and a time-of-flight mass spectrometer Pegasus III (Leco, St. Joseph, MI, USA). ChromaTOF® software version2.32 (LECO Corp., St. Josephs, MI) was used for data acquisition and processing. The first column (^{1}D) was an ultra-1 (Agilent Technologies, Inc., St. Clara, CA, USA), 100%-polymethylsiloxane, 16.3 m, 0.2 mm I.D., film thickness 0.11 µm and the second column (^{2}D) was an OV-1701 (MEGA, Milan, Italy), poly-14%-cyanopropylphenyl-86%-dimethylsiloxane, 1 m, 0.11 mm I.D., film thickness 0.1 µm. The second column was linked to TOFMS by an empty deactivated capillary, 0.50 m, 0.25 mm I.D. Split injection of 3 µL (1:10) at 280°C. Gas flow rate 1.2 mL/min using helium as carrier gas. Primary oven temperature program was 140°C for 1 min, ramped at 40°C/min to 180°C, then at 3°C/min to 280°C, then ramped at 40°C/min to 330°C. Secondary oven at 20°C higher than the first oven, but they stopped at the same end temperature. The modulation period was 6 s with 0.8 s hot pulse duration and a 45°C modulator temperature offset versus the primary oven temperature. The MS transfer line was held at 280°C. The TOFMS was operated in the electron ionization mode with collected mass range of *m/z* 50-750. Ion source temperature 230°C, detector at 1600 - 2000 V, applied electron energy 70 eV and acquisition rate 100 spectra/s.

Results and Discussion

The usefulness of any analytical data relies in its consistency, which in qualitative applications could be translated as precision. The information about the structured chromatogram could be expressed using the retention times (RT_1 and RT_2). Table 1 presents the repeatability obtained for the O-TMS steroids.

Endogenous steroids (O-TMS derivatives)	Relative standard deviation (%)			
	RT1	RT2	Area	Ratio analyte/ISTD*
5α-androstanediol bis OTMS	0.00	0.64	13.5	4.0
Androsteronebis OTMS	0.00	0.00	3.6	12.7
5β-androstanediol bis OTMS	0.00	0.84	14.9	4.9
DHEA bis OTMS	0.00	0.58	13.9	4.5
Testosterone bis OTMS	0.29	0.00	12.7	4.9
Epitestosterone bis OTMS	0.00	0.57	9.0	6.8
Etiocholanolone bis OTMS	0.00	0.00	6.7	4.3
11β-hidroxyandrosteronetris OTMS	0.00	0.00	7.7	3.3

*Internal Standard, methyltestosterone.

Table 1: Precision data considering retention times and area measurements.

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Its content represents the instrumental precision (repeatability), since the same sample was injected consecutively (n=6). The RT in both dimensions show very consistent results, as usually observed in GC/MS system. 5β-Androstanediol bis-OTMS presented the higher variation in RT_2 (0.84%). The RT_2 precision is critical for the structuration principle, since the chromatographic runs last only 6 seconds (modulation period). The good precision with the full spectra allows the evaluation of the chromatographic organization in a proper and reliable way, opening the possibility of the use of the approach in qualitative applications. Nevertheless, the precision relative to the area measurement presents values above 5% for the majority of the steroids evaluated. About 50% present imprecisions above 10%. The use of the ratio analyte/ISTD brings down the values to around 5%. These values are still systematically higher than those observed in the GC-MS system and hinders the steroid profile interpretation, considering all variation are related to the equipment (instrumental precision). The optimization of the acquisition ratio could improve the precision, but the global sensitivity should be carefully evaluated; especially for the analysis of other analytes (exogenous) in low concentration levels.
A very typical chromatographic structuration in GC × GC is known as “roof-tile” effect. In it, a side-by-side (or diagonal) band distribution appears throughout the chromatogram, usually corresponding to groups of isomers. It reveals a common behavior for similar compounds in the chromatographic system, especially in the second dimension. Since the number of isomers in urine is limited (compared to, *i.e.*, geochemical samples), it will be described here as “roof-tile”-like effect. Figure 1 presents the results for the isomers androsterone/etiocholanolone bis-OTMS (Figure 1A) and DHEA/epitestosterone/testosterone bis-OTMS (Figure 1B).

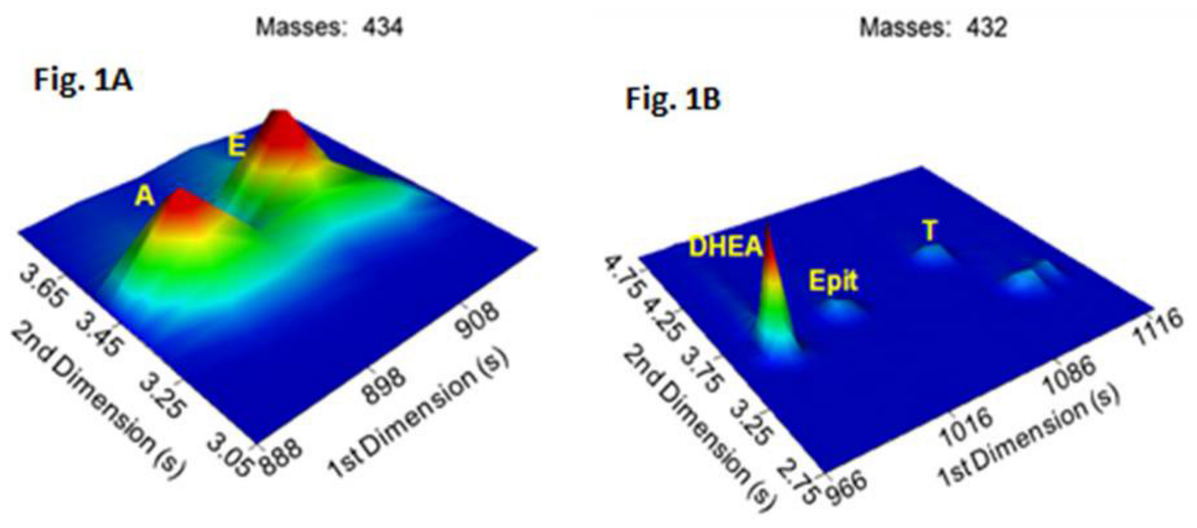

Figure 1: Extracted ion chromatograms from fragments m/z 434 and m/z 432. Observation of the so called "roof-tile" - like effect. Androsterone/etiocholanolone bis OTMS (Figure 1A). DHEA/epitestosterone/testosterone bis OTMS (Figure 1B).

As expected, the resolution among the analytes in the first dimension was observed, a in the GC-MS. The key point is the similarity among the RT_2, consequence of the similarity of physic-chemical properties among the compounds. Observing the experimental conditions, in particular the orthogonal features resulting from the differences in the columns polarity, the main contribution for the structuration is the analyte polarity. Table 2 presents the elution patterns of the OTMS analytes investigated. The more polar is the analyte (*i.e.*, 11β-hydroxy-androsterone), the less polar is the OTMS derivative, resulting in early elution in the ^{2}D. On the other hand, testosterone bis-OTMS presents the latest elution. The organization of the chromatographic plane obtained seems to be sensitive even to the number of double bonds. The RT_2 of the testosterone bis-OTMS (3.85 s / 2 double bonds) elutes later than the androsterone bis–OTMS (3.45 s / 1 double bond).

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This small difference has potential for diagnostic purposes since the precision of these RT_2 is remarkable, as aforesaid. OTMS derivatives show structured motifs, but small differences in the locations at the chromatographic plane, justified by the similar polarity among the derivatives (DRT_2 = 0.55 s, Table 2).

O-TMS Derivates	Structures	RT_1(s)	RT_2(s)
11β-OHA tris O-TMS		1092	3.30
5β-diol bis O-TMS		930	3.35
A bis O-TMS		894	3.45
Etio bis O-TMS		906	3.50
DHEA bis O-TMS		984	3.65
Epit bis O-TMS		1014	3.75
T bis O-TMS		1074	3.85

Table 2: Retention times in the first and second dimensions for the O-TMS derivatives of typical endogenous steroids. The second dimension is presented in increasing order.

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Optimizations could be foreseen aiming to improve the better use of the chromatographic plane. However, it should be done carefully, trying to avoid the wrap around profile. The relationship between the polarity and the chromatographic structuration was validated using a second derivatization strategy. Table 3 presents the elution patterns of the MOTMS analytes. Again, the elution order was directly linked to the polarity of the derivatized steroids. Indeed, the formation of metoxime group (from oxo steroids) creates interesting possibilities to differentiate steroids (DRT_2 = 1.68 s, Table 3), especially between keto and hydroxy analytes.

MO-TMS Derivates	Structures	RT_1(s)	RT_2(s)
5α-diol Bis O-TMS		924	3.60
Ptriol Tris O-TMS		1254	3.64
Pdiol Bis O-TMS		1224	3.98
11β OHA MO-bis OTMS		1134	4.06
A MO-TMS		900	4.18
DHEA MO-TMS		978	4.50
T MO-TMS		1074	4.78
11-Keto MO-TMS		1032	5.28

Table 3: Retention times in the first and second dimensions for the MO-TMS derivatives of typical endogenous steroids. The second dimension is presented in increasing order.

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The 11-ketoetiocholanolone elutes later (RT_2 = 5.28 s) due to the metoxime group than 11βOHA (RT_2 = 4.06 s) who presents a hydroxy group in position 11. Figure 2 presents the ^{2}D top view obtained for the MOTMS derivatives.

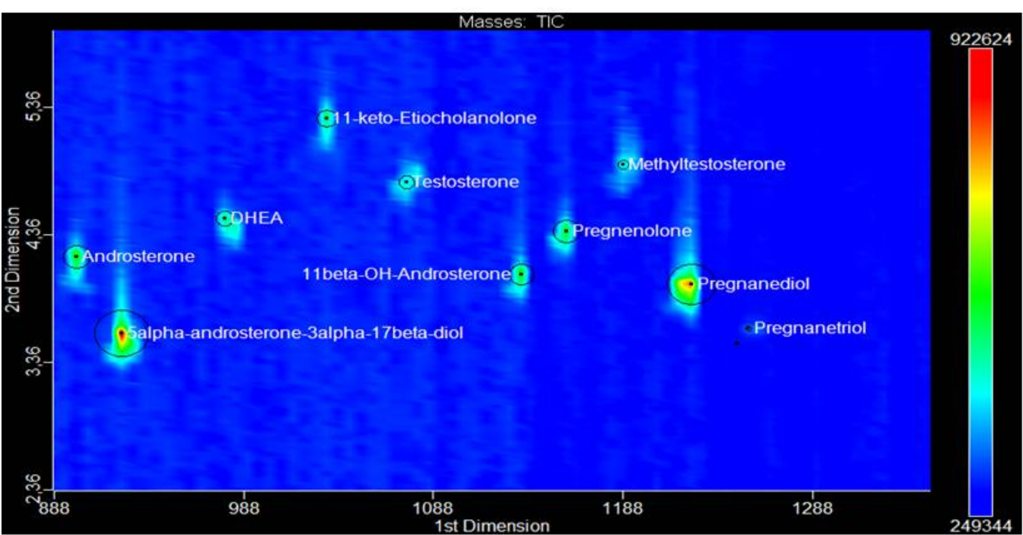

Figure 2: Top view obtained for the MOTMS derivatives of endogenous steroids.

Possible drawbacks that could increase the number of information (peaks) observed in the chromatogram are: i) formation of syn/anti isomers for the MOTMS derivatives and ii) incomplete derivatization reactions, especially for OTMS derivatives. Regarding the last one, hypothetically, the presence of non-derivatized groups should result in a ^{2}D retention time far away from the expected for the per-derivatized analytes. However, this hypothesis needs further validation. The structured chromatogram allows, as demonstrated, a direct relationship of the analyte feature (here, polarity) and the analyte chemical structure (*i.e.*, functional group or number of double bonds in the steroid hydrocarbon skeleton). This relationship could be helpful to do structural inference of unknown analytes. Further investigation is necessary to propose a mathematic model able to do such correlation in a robust way. Full characterization will be necessary to confirm the structure of any non-cataloged substance.

Conclusions

The level of structuration at the chromatographic plane obtained by the GC×GC-TOFMS was investigated. Steroids typically used to monitor the endogenous profile were used as model. The experiment was very sensitive regarding nuances in the steroid structure in regards to functional groups present or number of double bonds. The availability of the full spectra, high acquisition rate, high chromatographic resolution and the structuration of the chromatographic plane creates good

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perspectives for the GC×GC-TOFMS system in non-target analysis. Due to the huge amount of information obtained in each run, as a result of the improvement of peak capacity and full spectra acquisition, powerful software tools and chemometric approaches are necessary to develop the whole potential of the technique in this application.

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