Targeted Analysis and Non-Targeted Discovery of Steroids in Urine Using the ecTOF

Introduction

The identification of disease biomarkers is one of the most important goals of bioanalytical chemistry. As a non-invasive and a cost-effective tool for screening, diagnosis and monitoring of diseases, urine analysis is the second most frequently performed medical analysis used for diagnostic purposes after the analysis of blood [1,2]. Urinary metabolites such as steroids give indications on diseases and tissue injuries, which can be used to determine for example indicate the type of pathology, environmental influences, toxins, nutritional problems, cancer, or diabetes [3].

Mass spectrometry (MS) in combination with chromatographic separation is one of the major techniques for the analysis of biomolecules in complex biological matrices with gas chromatography mass spectrometry (GC-MS) as one of the most important techniques [4]. This research study will highlight the potential of the ecTOF coupled with a gas chromatograph (GC-ecTOF) for steroid profiling. The ecTOFs unique feature of acquiring both chemical ionization (CI) and 70 eV electron ionization (EI) mass spectra in parallel in one chromatographic experiment provides additional information needed for the comprehensive analysis of complex samples [5]. We performed both targeted analysis for quantitation of 40 target analytes and non-targeted analysis for global profiling of additional biomolecules [6,7].

Keywords:
Steroid profiling, urine analysis, ecTOF, non-targeted, compound identification, EI&CI, GC-HRMS, dual ionization

Marleen Vetter[1], Steffen Bräkling[1], Joël Fritsch[1] and Sonja Klee[1], Arnd Ingendoh[2]
1: TOFWERK, Thun, Switzerland
2: Bruker Applied Mass Spectrometry, Bremen, Germany

Methods

A standard of 40 endogenous steroids was purchased from Steraloids, Newport RI, USA. An anonymized urine sample was also provided by the Inselspital (Insel Gruppe AG, Bern, CH). Sample preparation was performed in the Department of Nephrology and Hypertension of the Bern University Hospital (Inselspital) [8].

The instrumental conditions for the GC, as routinely employed at Inselspital, as well as the ecTOF conditions can be found in Table 1. Medical-ethical guidelines from the Swiss Academy of Medical Sciences as implemented at the Inselspital were followed during sample collection and analysis.

Table 1. GC-ecTOF method parameters.

Analyte	MRM Transition (*m/z*)
Injection	2 µL (direct splitless)
Inlet Temperature	260°C
Carrier Gas Flow	1.2 mL/min He
Purge Flow	10.0 mL/min
Column	HP5-1MS (15 m, 0.25 mm, 25 µm; Agilent Technologies)
Septum Purge	3.0 mL/min
Temperature Program	50 °C for 2 mins, 30 °C/min to 210 °C, 2 °C/min to 265 °C
Flow Split	1:1 CI:EI
Heated Transfer Line Temperature	280°C
Source Temperature EI	280°C
Source Temperature CI	300°C
Ionization Sources	StarBeam 70 eV EI source, HRP CI source reagent: N_2H^+ (N_2) [9]
Mass Range	1-700 *m/z*

Results: Employing the GC-ecTOF for known and unknown identification

Figure 1. shows the EI and CI traces of both the reference standard (a) and the urine sample (b). For ease of visualisation, the EI extracted ion chromatograms of *m/z* 73.0472 are shown in Figure 1 (c,d). The fragment ion arises from the derivatization reagent (N-trimethylsilylimidazole (TMSI)). Compounds containing at least one trimethylsilyl (TMS) group show a prominent *m/z* 73.0472 ($Si(CH_3)_3^+$). The specific formation mechanism of the fragments is described in the literature [10,11]. The 40 target steroids are highlighted as green. As can be seen in Figure 1, the ecTOF was able to identify and detect all 40 steroids, demonstrating that the ecTOF can be used for routine targeted analysis (Table 2, Figure 1).

The same chromatographic run of the ecTOF can also be used to provide information on additional steroids and other compounds present in the urine samples. Conventionally, unknowns are identified using comparison to standards, EI information and database matches. However, when no standard is available and/or the library match is insufficient, the unique feature of the ecTOF to also provide CI information is invaluable. This is exemplified using two example compounds from the urine sample. Potential steroids were initially identified using exclusion factors (signal higher than *m/z* 400 in the CI data, presence of the *m/z* 73.0474 ($Si(CH_3)_3$) fragment as well as a neutral loss of *m/z* 90.0500 (SiC_3H_9OH) in the EI as an indication of derivatisation). In Figure 1b, these potential steroids are highlighted in orange, two of which are discussed in more detail (highlighted in red as "1" and "2").

Table 2. Targeted steroids

Nr.	Trivial Name	Abbreviation
1	Androsterone	ANDRO
2	Etiocholanolone	ETIO
3	Dihydroandrosterone, Androstanediol	DH-ANDRO
4	Dehydroepiandrosterone	DHEA
5	Androstenediol	ANDRO-DIOL
6	5α-Dihydrotestosterone	5a-DHTST
7	11-oxo-Etiocholanolone	11-OXO-ETIO
8	17β-Estradiol	17b-ESTRADIOL
9	Testosterone	TESTOSTERONE
10	11β-Hydroxyandrosterone	11b-OH-ANDRO
11	11β-Hydroxyetiocholanolone	11b-OH-ETIO
12	17-Hydroxypregnanolone	17-HP
13	16α-Hydroxy-DHEA	16a-OH-DHA
14	Pregnanediol	PD
15	Pregnanetriol	PT
16	Androstenetriol	5-AT
17	Tetrahydro-11-deoxycortisol	THS
18	Tetrahydrodeoxycorticosterone	THDOC
19	Estriol	ESTRIOL
20	Pregnanetriolone	PTONE
21	Pregnenetriol	5-PT
22	Tetrahydrocortisone	THE
23	Tetrahydro-11-dehydrocorticosterone	THA
24	Tetrahydrocorticosterone	THB
25	5α-Tetrahydrocorticosterone	5a-THB
26	Tetrahydrocortisol	THF
27	5α-Tetrahydrocortisol	5a-THF
28	α-Cortolone	a-CORTOLONE
29	Tetrahydroaldosterone	THALDO
30	β-Cortol	b-CORTOL
31	β-Cortolone	b-CORTOLONE

Nr.	Trivial Name	Abbreviation
1	α-Cortol	a-CORTOL
2	Cortisone	CORTISONE
3	Cortisol	CORTISOL
4	20β-Dihydrocortisone	20b-DHE
5	20α-Dihydrocortisone	20a-DHE
6	20β-Dihydrocortisol	20b-DHF
7	6β-Hydroxycortisol	6b-OH-F
8	18-Hydroxycortisol	18-OH-F
9	20α-Dihydrocortisol	20a-DHF

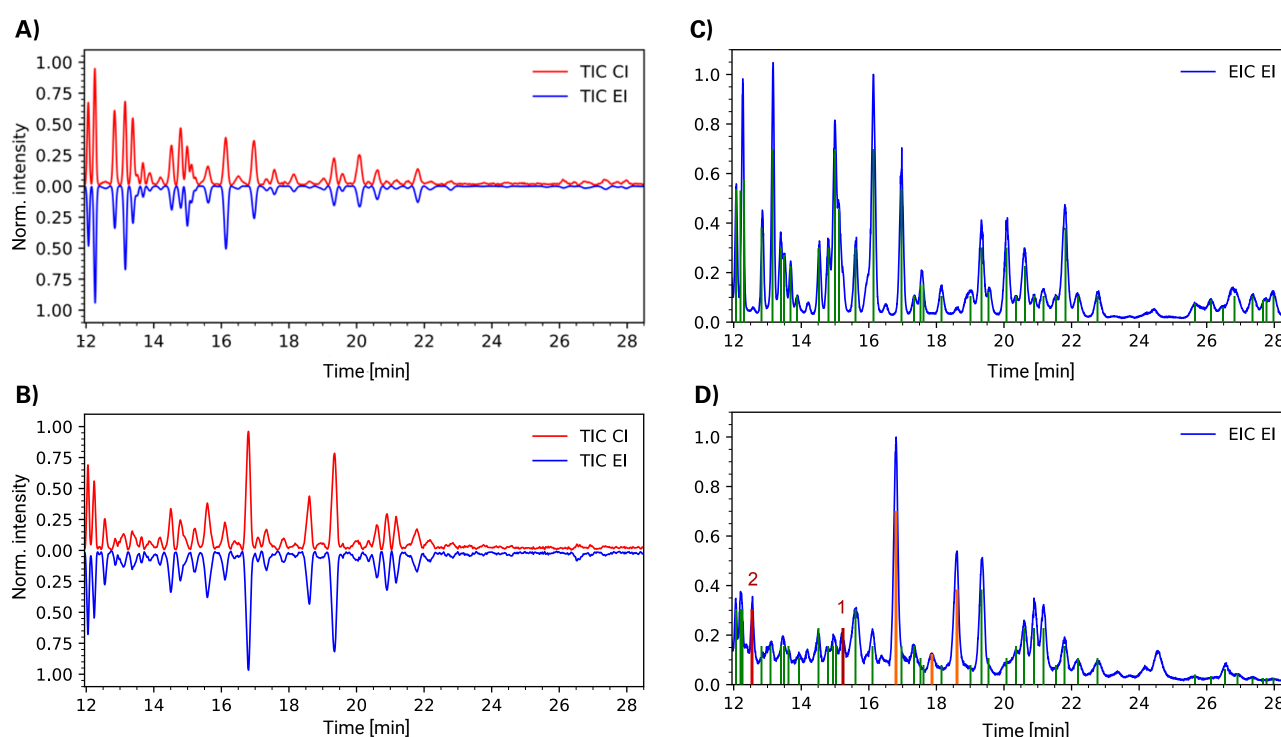

Figure 1. Head to tail total ion chromatograms of EI and CI mass spectra for a) the standard and b) the urine sample. Extracted ion chromatographic traces of mass *m/z* 73.0472 of c) the standard and d) the urine sample. The 40 steroids contained in the standard are highlighted in green, potential steroids in orange and the two steroids further discussed in detail in red.

The first peak (Peak "1" in Figure 1d) elutes at 15.2 minutes. Figure 2a shows the EI and corresponding CI mass spectra for this peak. The NIST hit provides a fair match (match factor 765, reverse match factor 788), and the probability of 95.9 % for enterodiol (2,3-bis(3-hydroxybenzyl) butane-1,4-diol tetratms) (Figure 2). Using the NIST hybrid similarity search without additional CI information, the derivatized enterodiol is still the best match, however match factors do not considerably improve (match 748, reverse match 762, hybrid search match factor (o. match) 688) with a delta mass of *m/z* - 90.0500. The main issue with the match results is the missing $M-CH_3$ peak at *m/z* 575.2821 in the EI mass spectrum, and the loss of molecular mass information using 70 eV ionization for derivatized enterodiol. Including the additional CI information in the NIST hybrid similarity search, the match factor for derivatized enterodiol can be improved to 830, reverse match factor to 847 and o. match to 768 and both the $[M-CH_3]^+$ as well as the $[M+H]^+$ can be identified. In conjunction with the accurate mass and isotopic ratios information (Figure 2c,d), this information can be used to support and improve identification confidence in the NIST library search results.

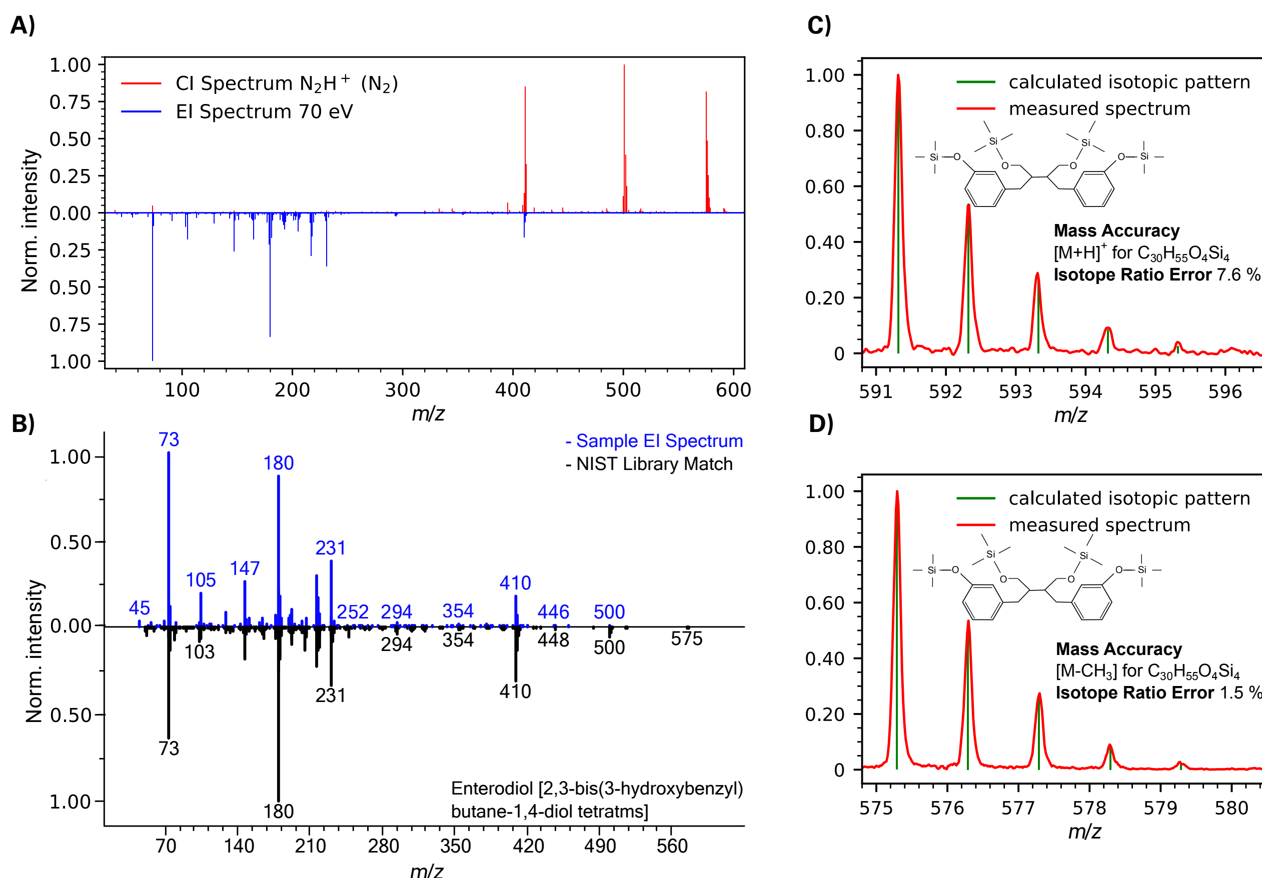

Figure 2. Using the ecTOF to increase identification confidence. a) CI and EI mass spectra of the peak "1" at 15.2 minutes. b) NIST comparison of the EI mass spectrum with best NIST library hit: derivatized enterodiol ($C_{30}H_{55}O_4Si_4$). c) isotopic ratio fit of the CI accurate masses found at *m/z* 591.3134 with $[M+H]^+$ for $C_30H_{55}O_4Si_4$ as well as d) its $[M-CH_3]^+$ mass match at *m/z* 575.2821.

Furthermore, the combination of EI and CI information can be used to provide sum formulas and structures for compounds not in the NIST library. As an example, we will discuss the peak at 12.5 minutes (Peak "2", Figure 1d, Retention Index of 2646). The EI and CI mass spectra for this compound are found in Figure 3a. The best EI NIST library hit is thioinosine, 3 TMS (Figure 3b). However, this identification is not supported by the CI information which indicates $[M+H]^+$ of *m/z* 484.2311 and $[M-CH_3]^+$ of *m/z* 468.2005 (a typical fragment in CI for TMS derivatized compounds). The EI does not yield any molecular ion. Since no EI library hit corresponds to the CI derived sum formula, the target compound is probably not listed in the library. Including the information of the molecular mass to the hybrid similarity search option of the NIST (Version 2.4) search software, thioinosine with 3 TMS groups (due to derivatization) still provides the best hit but does not explain the delta mass of -17 Da observed (Table 3). The results of the hybrid similarity search are given in Table 3. Appling the nitrogen rule the molecular ion suggests an uneven number of nitrogen atoms in the molecule, which is not the case for thioinosine, 3 TMS [12]. Using the accurate mass information of the CI spectrum, we can deduce that the SH group was replaced by a NH_2 group, thus explaining the mass difference of -17 Da to the library hit (Table 3). Since the purine derived group ($C_5H_3N_4S$ *m/z* 151.0078) does not greatly contribute to the fragmentation pattern of thioinosine, 3 TMS, these adenosine derivates show very similar EI mass spectra.

Table 3. Reverse NIST hits for the compound at 12.5 mins. The replacement of functional group highlighted in orange accounts for the detected delta mass in comparison the library hit.

Reverse NIST Hit	Name and Retention Index	Structure	Match Factor/ o. Match Factor	Delta mass
1	Thioinosine, 3TMS derivative (RI 2760)	HS, N, N, N, N, O, O, O, O, Si, Si, Si	918/808	-17 (-SH replaced with $-NH_2$)
2	1-Methyladenosine, tris(trimethylsilyl) ether (RI 2669)	HN, N, N, N, N, O, O, O, O, Si, Si, Si	887/808	-14 ($-CH_3$ replaced with -H)
12	5′-Methylthio-adenosine, 2 TMS derivative (Not available)	H_2N, N, N, N, N, O, O, O, S, Si, Si	829/564	42 ($-SCH_3$ replaced with $-OSi(CH_3)_3$)

The same procedure can be applied to other hits of the hybrid search result. 1-methyladenosine, tris(trimethylsilyl) ether, the second suggested NIST hit in the hybrid search show a discrepancy between the peak at *m*/*z* 178 ($C_7H_8N_5O$) of the 1-Methyladenosine, tris(trimethylsilyl) ether and the *m*/*z* 164 found in the compound spectra. Here, delta mass of -14 can be explained by the $-N-CH_3$ group being reduced to a -NH on the purine structure. This NIST hit also has a very similar retention time index to that obtained for the compound of interest (Table 2, Figure 3c). Furthermore, the base structure of adenine can be corroborated by match hit 12 5′-methylthioadenosine, 2 TMS derivative. Here, the delta mass of 42 can be explained by the $-SCH_3$ group being replaced with a $-OSi(CH_3)_3$ on the ribose part of the molecule (Table 2, Figure 3d). Using this approach, the mass differences in most initial NIST hits can be explained.

With this, a sum formula of $C_{19}H_{37}N_5O_4Si_3$ is highly likely. The accurate mass and isotope ratio (1.7 % error) of the $[M+H]^+$ ion further supports this (Figure 4). Combining all the information from above, a tentative identification for this peak is adenosine 3TMS. Only the 4TMS derivative of adenosine can be found within the NIST library. Indeed, adenosine 4TMS was also found in the urine sample data at 13.6 minutes, suggesting that the derivatization process for adenosine was incomplete (Figure 5).

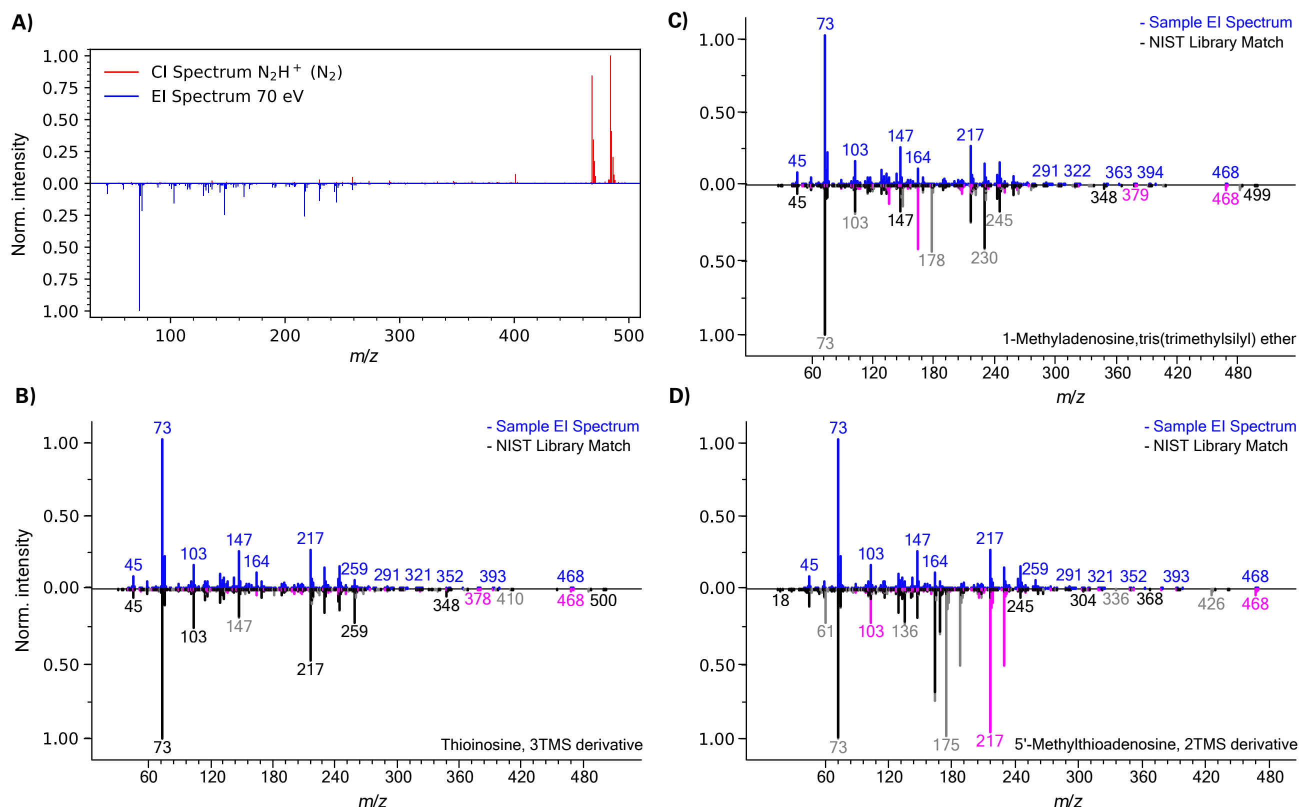

Figure 3. a) EI and CI mass spectra for the peak found at 12.5 minutes. Comparison of EI spectrum with NIST hybrid search results of b) thioinosine, 3TMS derivative, c) with 1-methyladenosine, tris(trimethylsilyl) ether and d) 5'-methylthioadenosine, 2TMS derivative. Masses with a grey line are those present in the NIST spectra but not in the sample and masses with a pink line those found in the compound spectra indicating the delta mass.

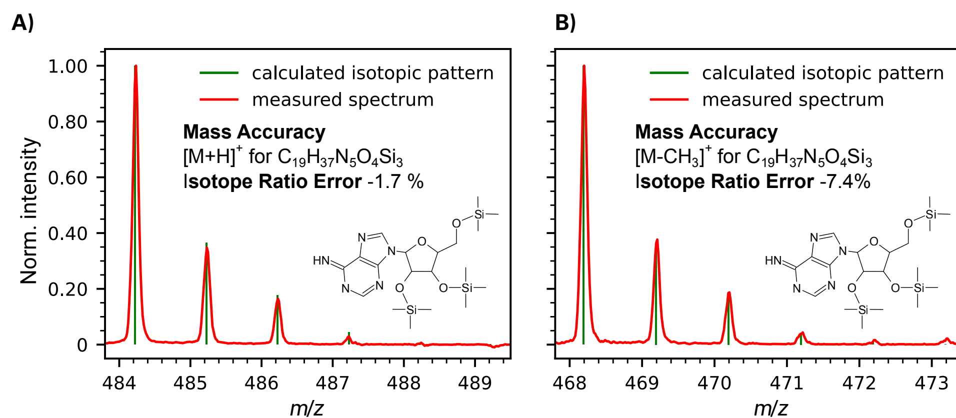

Figure 4. a) Isotopic ratio fit of the CI accurate masses found at *m/z* 484.2311 with $[M+H]^+$ for $[C_{19}H_{38}N_5O_4Si_3]^+$ as well as its $[M-CH_3]^+$ mass match at *m/z* 468.2005.

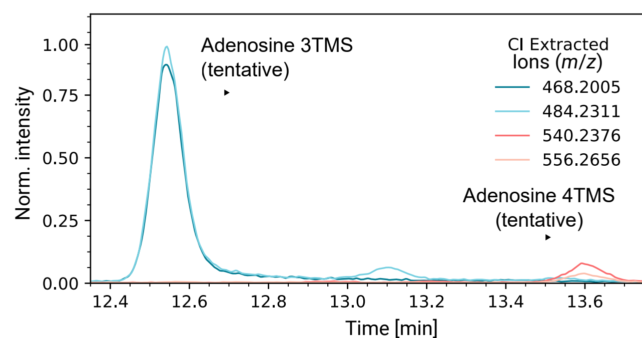

Figure 5. CI extracted ion chromatograms for *m/z* 468.2005 $[M-CH_3]^+$ and *m/z* 484.2311 $[M+H]^+$ (adenosine 3TMS) and *m/z* 540.2376 $[M-CH_3]^+$ and *m/z* 556.2656 $[M+H]^+$ (adenosine 4TMS).

Conclusions

These research examples demonstrate the potential of the GC-ecTOF to identify additional compounds of interest within a urine sample. The ecTOF increases identification confidence in potential NIST hits and enables tentative identification of compounds not within the library. This can be done in parallel to routine analysis, or this additional information can later be investigated. With this, biomedical research laboratories can obtain a comprehensive overview of the compounds present in a sample which in turn enables the search for novel disease biomarkers.

References

[1] K. K. Pasikanti et al., "Noninvasive Urinary Metabonomic Diagnosis of Human Bladder Cancer," Journal of Proteome Research, vol. 9, no. 6, pp. 2988–2995, Jun. 2010, doi: 10.1021/pr901173v.

[2] S. S. Dinges et al., "Cancer metabolomic markers in urine: evidence, techniques and recommendations," Nature Reviews Urology 2019 16:6, vol. 16, no. 6, pp. 339–362, May 2019, doi: 10.1038/s41585-019-0185-3.

[3] A. Zhang et al., "Urine metabolomics" Clinica Chimica Acta, vol. 414, pp. 65-69, Dec. 2012, doi: 10.1016/j.cca.2012.08.016.

[4] D. J. Beale et al., "Review of recent developments in GC-MS approaches to metabolomics based research," Metabolomics, vol. 14, no. 11, Nov. 2018, doi: 10.1007/S11306-018-1449-2.

[5] S. Bräkling et al., "Parallel Operation of Electron Ionization and Chemical Ionization for GC-MS Using a Single TOF Mass Analyzer," Anal Chem., vol. 94, no. 15, pp. 6057-6064, April 2022, doi: 10.1021/acs.analchem.2c00933

[6] O. Fiehn, "Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling," Curr Protoc Mol Biol, vol. 114, Apr. 2016, doi: 10.1002/0471142727.MB3004S114.

[7] M.M. Koek et al., "Semi-automated non-target processing in GC × GC–MS metabolomics analysis: applicability for biomedical studies" Metabolomics, vol. 7, pp. 1-14, Mar. 2011, doi: 10.1007/s11306-010-0219-6

[8] A. Bileck et al., "A comprehensive urinary steroid analysis strategy using two-dimensional gas chromatography-time of flight mass spectrometry," Analyst, vol. 143, no. 18, pp. 4484–4494, 2018, doi: 10.1039/c7an01990d.

[9] S. Bräkling et al., "Hydrogen Plasma-Based Medium Pressure Chemical Ionization Source for GC-TOFMS," J Am Soc Mass Spectrom, vol. 33, no. 3, pp. 499–509, Mar. 2022, doi: 10.1021/JASMS.1C00329.

[10] J. M. Halket and V. G. Zaikin, "Derivatization in Mass Spectrometry—1. Silylation," European Journal of Mass Spectrometry, vol. 9, no. 1, pp. 1–21, Feb. 2003, doi: 10.1255/ejms.527.

[11] A. G. Smith, S. J. Gaskell, and C. J. W. Brooks, "Trimethylsilyl group migration during electron impact and chemical ionization mass spectrometry of the trimethylsilyl ethers of 20- hydroxy-5α-pregnan-3-ones and 20-hydroxy-4-pregnen-3-ones," Biological Mass Spectrometry, vol. 3, no. 4, pp. 161–165, Aug. 1976, doi: 10.1002/bms.1200030404.

[12] T. Kind and O. Fiehn, "Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry," BMC Bioinformatics, vol. 8, no. 1, pp. 1–20, Mar. 2007, doi: 10.1186/1471-2105-8-105/TABLES/6.

Bruker Applied Mass Spectrometry is continually improving its products and reserves the right to change specifications without notice. © 2024, Bruker Applied Mass Spectrometry. AMS-015 1910127

For Research Use Only. Not for use in clinical diagnostic procedures.

Bruker Switzerland AG

Fällanden · Switzerland
Phone +41 44 825 91 11

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

marketing.bams.emea@bruker.com - www.bruker.com

Learn more about the product on our website: