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Dr. Hendrik Schulte, Shimadzu Europa GmbH
Image Credit: daco/stock.adobe.com
The occurrence of the banned insecticide, ethylene oxide (EtO), and its transformation product, 2-chloroethanol (2-CE), has recently been reported in various food commodities. In this study, two alternative approaches based on gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) were developed to control maximum residue limit (defined as the sum of ethylene oxide and 2-chloroethanol expressed as ethylene oxide). The first approach offered a rapid screening of 2-CE (the contamination marker) in an aqueous acetonitrile extract purified by dispersive solid-phase extraction (dSPE). The total EtO was determined by the second approach, which involved conversion of EtO to 2-CE by acid hydrolysis in the presence of chloride; the ethyl acetate extract was purified prior to instrumental analysis by dSPE. The achieved limit of quantification for EtO (the sum of EtO and 2-CE expressed as EtO) was low enough to ensure compliance with regulatory requirements.

The accuracy of the results was successfully verified by analysis of the EUPT test material (EUPT-SRM16 - 2021).

Ethylene oxide (EtO) is a gaseous disinfectant that was, due to its toxicity, banned in the EU as a pesticide in 1991 and as an output product in food and feed in 2011. It is classified as a category 1 carcinogen by the International Agency Research of Cancer (IARC). In September 2020, Belgium reported via the Rapid Alert System for Food and Feed (RASFF) an occurrence of EtO in sesame seeds. The concentration of this active ingredient of insecticidal fumigants exceeded the maximum residue level (MRL) of 0.05 mg/kg set for this commodity in the Regulation (EC) NO 396/2005 (1) by six hundred times (the residue definition involves not only EtO but also its transformation product, 2-chloroethanol [2-CE], expressed as EtO). Up to September 2021, 629 notifications in RASFF related to the occurrence of EtO, mostly in seeds (particularly sesame seeds) and spices, have been registered. Contamination of various food additives, (for example, E410 and E416) and various food items, such as ice creams and desserts, milk products, pastries, breads, and a number of other products containing ingredients illegally processed with EtO, was also reported.

Due to a high reactivity, EtO used to be applied in the sterilization of health care material surfaces and for the treatment of spices and seeds in some countries (2–4). If the aeration step needed for spices and seeds is not properly performed, residues of EtO may spontaneously react with chloride ions commonly present in a particular matrix. In this way, 2-CE originates during storage, and thus becomes a suitable marker of the use of EtO for fumigation.

Until now, a laboratory-performed EtO control in foodstuffs has not been routinely performed, and only a few methods describing an analytical procedure have been published. Unfortunately, most methods are based on time-consuming sample preparation steps, including conversion of EtO to 2-CE using sodium iodide prior to analysis (5,6).

This study presents two simple validated gas chromatography tandem mass spectrometry (GC–MS/MS) methods, the first being dedicated to a rapid screening of 2-CE (the marker of EtO-based fumigation) in sesame seeds and spices,
the second one to enable determination of total EtO concentration, in line with the regulatory requirements (MRL control).

**Materials and Methods**

**Chemicals:** Standards of EtO and 2-CE were purchased from Sigma Aldrich. 2-chloro-1,1,2,2-tetradeuterioethanol (D$_2$-2-CE), an internal standard, was purchased from HPC Standards GmbH. Analytical-grade ethyl acetate and acetonitrile were obtained from Sigma Aldrich. Analytical-grade ethyl acetate and sulphuric acid (96%) were purchased from HPC Standards GmbH. Analytical-grade ethyl acetate from Sigma Aldrich. C18 and PSA sorbents were supplied by Agilent Technologies. Sodium chloride and sulphuric acid (96%) were purchased from Lach-ner and Penta, respectively.

Two sets of calibration solutions with 2-CE concentration 2, 12, 20, 50, 100, 200, 500, 1000, and 2400 ng/mL were prepared in ethyl acetate and acetonitrile. Each calibration level contained 100 ng/mL of D$_2$-2-CE.

To test EtO conversion to 2-CE, an EtO standard solution of 5000 ng/mL methanol was prepared.

**Samples:** Spice samples (ground pepper and paprika) and hulled bio sesame seeds obtained at a retail market were used for procedure optimization and validation experiments. For the quality control (QC) test material, the proficiency testing sample (sesame seeds) obtained from EUPT-SRM16 - 2021 was used (7).

**Sample Preparation:**

**Solid-Phase Microextraction:** A 1 g measure of the sesame seeds sample was mixed with 2 mL of 0.2 M H$_2$SO$_4$ and 0.5 mL of a saturated solution of NaCl. The samples were spiked by 2-CE at different concentration levels from 0.006 to 0.6 mg/kg and analyzed by solid-phase microextraction and gas chromatography coupled to high resolution mass spectrometry (SPME–GC–HRMS).

**Strategy No. 1: Extraction/Purification:** A 2 g amount of the representative sample (D$_2$-2-CE used as a surrogate was added at concentration 0.5 mg/kg) was mixed with 10 mL (v/v) 95:5 acetonitrile–water in a polypropylene centrifuge tube and shaken for 30 min. The tube was then centrifuged (5 min, 10,000 rpm, Rotina 35R, Hettich Zentrifugen). A 3 mL measure of supernatant were transferred into the upper organic layer and 0.075 g of C$_{18}$ and 0.2 g of MgSO$_4$ were added. The mixture was shaken for 2 min and centrifuged (3 min, 10,000 rpm). Finally, the extract was transferred into vials for GC–MS/MS analysis.

To test the yield of EtO conversion to 2-CE during acid hydrolysis, a sample of sesame seeds was spiked at a concentration of 0.05 mg/kg and analyzed using the above mentioned procedure.

During the sample preparation process, blanks were also prepared consisting of all the steps except the addition of the sample.

**Analyte Separation and Detection:**

**SPME–GC–HRMS:** For a headspace solid-phase microextraction (HS–SPME), a 75 µm Carboxen/polydimethylsiloxane (CAR/PDMS) fibre obtained by Supelco was used. The optimized HS-SPME extraction conditions were: incubation time, 120 min; incubation temperature, 50 °C; agitator speed, 500 rpm; extraction time, 20 min; desorption temperature, 250 °C; desorption time, 2 min; and the analytes were extracted in headspace. EtO analysis by SPME was performed using a GC–MS system consisting of a 7890A gas chromatograph (Agilent Technologies) equipped with a multifunctional autosampler MPS 2 (Gerstel) and coupled to Pegasus HRT ultrahigh-resolution time-of-flight mass spectrometer (Leco). For the separation, a 50 m × 0.20 mm, 0.20-µm HP-INNOWax capillary column (column no. 1) (Agilent...
Technologies) was used. A sample was injected in split mode (1:5). The oven temperature programme was as follows: 35 °C (8 min), 50 °C/min to 220 °C (hold 10 min). The temperatures of the transfer line and ion source were 220 and 230 °C, respectively. The mass spectrometric detector was operated in the electron ionization mode. The mass range was 35–550 m/z and the resolution of the mass analyzer was set > 25,000 (FWHM).

Strategy No. 1 and 2: GC–MS/MS:
A GC–MS/MS system consisting of an Agilent 7890A gas chromatograph equipped with multimode inlet and triple quadrupole mass spectrometer Agilent 7000b MS operated in EI mode was employed for the analysis of 2-CE.

The separation of EtO and 2-CE was performed on a 30 m × 0.2 mm, 1.12-µm HP-VOC capillary column (column no. 2) (Agilent Technologies) using the oven temperature program 45 °C (2 min), 20 °C/min to 240 °C (5 min). A 2 µL sample was injected in splitless and split mode (1:4) at 250 °C.

For the analysis of 2-CE after both types of extraction techniques, a 30 m × 0.25 mm, 0.25-µm HP-INNOWax capillary column (column no. 3)
TABLE 1: Performance characteristics of the analytical method (recoveries [rec], repeatability expressed as RSD and LOQs) for the analysis of EtO and 2-CE in sesame seeds, ground pepper, and paprika

<table>
<thead>
<tr>
<th>Matrix</th>
<th>EtO (0.5 mg/kg)</th>
<th>2-CE (0.012 mg/kg)</th>
<th>2-CE (0.06 mg/kg)</th>
<th>LOQ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rec %</td>
<td>RSD (%)</td>
<td>Rec (%)</td>
<td>% RSD (%)</td>
</tr>
<tr>
<td>Sesame seeds</td>
<td>99</td>
<td>10</td>
<td>101</td>
<td>11</td>
</tr>
<tr>
<td>Ground pepper</td>
<td>92</td>
<td>9</td>
<td>116</td>
<td>14</td>
</tr>
<tr>
<td>Ground paprika</td>
<td>73</td>
<td>9</td>
<td>119</td>
<td>20</td>
</tr>
</tbody>
</table>

FIGURE 3: Chromatographic record (GC–MS/MS) of calibration solution in ethyl acetate containing 2-CE (c = 50 ng/mL) and D₄-2-CE (c = 100 ng/mL).

(Agilent Technologies) was used. A 2 µL volume was injected in splitless mode at 250 °C. The oven temperature programme was as follows: 57 °C (1 min), 20 °C/min to 130 °C, 5 °C/min to 140 °C (post run at 230 °C for 25 min).

Triple quadrupole was operated in multiple reaction monitoring (MRM) mode detecting transitions for EtO (44→29, 44→28, 44→19), 2-CE (80→31, 82→31, 80→43), and D₄-2-CE (84→34, 86→47, 86→34, 84→47) at collision energy 5 eV.

To verify a yield of EtO to 2-CE conversion, the sample of sesame seeds was spiked with EtO at concentration level 0.05 mg/kg, respectively. The method, including acid hydrolysis followed by ethyl acetate extraction for the analysis of EtO (conversion to 2-CE), was validated at the concentration level 0.05 mg/kg.

To comply with the EtO MRL definition (the Regulation [EC] NO 396/2005) involves free EtO and EtO converted into 2-CE. While EtO is a reactive compound with a low boiling point (10.4 °C), the value for 2-CE is more than ten times higher (129 °C). To the best of our knowledge, both EtO forms may occur in tested samples; nevertheless, 2-CE dominates. It is worth noting that simultaneous determination of these two analytes, EtO and 2-CE, by GC is a difficult task due to fairly differing physicochemical and thus chromatographic properties.

In our preliminary experiments, SPME was used for analyte headspace sampling followed by GC separation and MS detection (a high-resolution TOF mass analyzer was used for screening purposes). In the case of EtO, critical matrix interference occurred under testing conditions; the target analyte coeluted with acetaldehyde, a compound with the same elemental composition (C₂H₄O), similar Kovats retention index (RI) (EtO: 680, acetaldehyde: 690, both related to polyethylene glycol stationary phase), and a very similar mass spectrum. Moreover, in spite of the advanced instrumental technique used, the limit of quantification (LOQ, detectability of confirmation ion 80.0023 required) of 2-CE (0.05 mg/kg) was not low enough due to relatively high “chemical noise” (see Figure 1).

To overcome the issue of EtO and acetaldehyde coelution and to achieve a lower LOQ, column no. 2, with a thick film of stationary phase suited for the analysis of compounds with low boiling points, was used and an acetonitrile–water extract.
was injected into the GC–MS/MS system in split injection mode. Acetaldehyde and EtO were successfully separated; however, the lowest LOQs for EtO and 2-CE (see Figure 2) were only 0.5 and 0.2 mg/kg, respectively, and so did not enable MRL control (sesame seeds, MRL = 0.05 mg/kg for EtO – sum of EtO and 2-CE expressed as EtO; spices, MRL = 0.1 mg/kg for EtO – sum of EtO and 2-CE expressed as EtO). For lower LOQs, the splitless injection mode was also tested, but this injection technique negatively affected a peak shape of EtO and its detectability.

As the simultaneous GC–MS analysis of EtO and 2-CE was found to not be easily feasible, in the next phase, we focused on (i) screening of 2-CE in suspect samples and (ii) accurate quantification of total EtO (targeted as 2-CE) in seeds and spices. Column no. 3 was employed for GC separation. Sample preparation strategy no. 1 used aqueous acetonitrile for 2-CE extraction and dSPE-based cleanup with Z-Sep + MgSO₄. In this way, fast screening of low levels of 2-CE in contaminated samples was enabled. Although free EtO was not determined by this strategy, the available results confirmed that “positive” samples always contain detectable 2-CE, in quantities fairly high compared to free EtO; in other words, this “bound” EtO was, practically without exception, the dominating component.
of residue. To determine EtO residue in line with the MRL definition, conversion of EtO to 2-CE was performed by acid hydrolysis in the presence of chloride ions. In this case, ethyl acetate was used for target analyte extraction from an aqueous acidic solution. To remove co-extracted, less polar compounds, including various plant pigments, a mixture containing PSA/C18/MgSO4 was employed.

For the maximum correction of matrix effects, D4,2-CE was used as an internal standard (see Figure 3).

Table 1 summarizes the outcome of the validation of the latter strategy used for analysis of sesame seeds, ground pepper, and paprika spiked at levels 0.05 mg/kg for EtO and 0.012 mg/kg and 0.06 mg/kg for 2-CE. The recoveries ranged in tested matrices from 73 to 99%, from 101 to 119%, and from 86 to 109%, respectively. The repeatability (n = 6) expressed as relative standard deviation was in the range of 2 to 20%. This key performance characteristic met the criteria applied in generated data by this new GC–MS/MS method, EU proficiency test material, EUPT-SRM16 - 2021 test material (sesame seeds), all the performance characteristics were in line with guidance document DG SANTE/12682/2019 recommendations.

To calculate recovery of the EtO conversion to 2-CE, six spiked (0.05 mg/kg) sesame seed samples were analyzed by acid hydrolysis followed by ethyl acetate extraction. Recovery of the EtO to 2-CE conversion was 50%, with repeatability at 11%.

To demonstrate the accuracy of the generated data by this new GC–MS/MS method, EU proficiency test material, EUPT-SRM16 - 2021 (sesame seeds), was analyzed. Using classification based on the z-score for EtO (sum of EtO and 2-CE), a satisfactory result was obtained (z-score = 0.35). Figure 4 presents the chromatographic record of 2-CE in EUPT-SRM16 - 2021 at concentration level 5.07 mg/kg and 2-CE in ground pepper at concentration level 0.02 mg/kg.

Conclusion
This article has presented new implemented and validated analytical approaches based on GC–MS/MS for the determination of EtO and 2-CE in sesame seeds and spices. For rapid contamination control based on targeting 2-CE as a marker, the analysis of aqueous acetonitrile extract purified by dSPE can be used. To determine total EtO content, that is, both free and transformed EtO (in compliance with MRL definition, Regulation [EC] No 396/2005), acid hydrolysis in the presence of chloride ions followed by ethyl acetate extraction and dSPE purification was the best choice. The accuracy of generated data by the latter method was proven through analysis of EUPT-SRM16 - 2021 test material (sesame seeds). All the performance characteristics were in line with guidance document DG SANTE/12682/2019 recommendations.

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References

Michal Stupák is an assistant professor at the Department of Food Analysis and Nutrition, UCT Prague. His research is focused on the use of GC–MS in food quality, safety, and authenticity, as well as in natural products.

Maria Filatova is a PhD student at the Department of Food Analysis and Nutrition, UCT Prague. Her main focus is on GC–MS analysis of volatile compounds in food and natural products.

Vladimir Kocourek is a full professor at the University of Chemistry and Technology Prague (UCT Prague) and a quality manager of the accredited Metrological and Testing Laboratory, Department of Food Analysis And Nutrition.

Jana Hajšlová is a professor at UCT Prague. She is the head of ISO 17025/2018 accredited laboratory and also heads a research group concerned with separation science in the field of food/environmental analysis. She is the chair of a series of prestigious international symposia—Recent Advances in Food Analysis (RAFA, www.rafa2021.eu).
Cannabis Metabolomic Data Processing: Challenges to be Addressed

Kamila Bechynska, Vit Kosek, Marie Zlechovcova, Petra Peukertova, and Jana Hajšlová, University of Chemistry and Technology, Department of Food Analysis and Nutrition, Prague, Czech Republic

The interest in cannabis products is growing exponentially, because they are expected to provide a number of beneficial and therapeutic effects. Cannabis plants contain a number of unique biologically active secondary metabolites, with their pattern largely dependent not only on a respective variety or chemotype but also on several other factors. This article presents the challenges that arise during cannabis metabolomics analysis using ultrahigh-performance reversed-phase liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC–reversed-phase–HRMS/MS). For this purpose, ethanolic extracts of two cannabis varieties were measured twice within one month and the obtained datasets were chemometrically analyzed with respect to the analysis date. Rather surprisingly, the effect of time variability was observed at the level of expression of relative abundance of features as well as in multivariate models, where not only variety clustering but also time-dependent clustering occurred. The data handling workflow, within time variability can be revealed and removed by advanced normalization method, is described.

Cannabis sativa L. (Cannabaceae) is an annual plant that has been used for its therapeutic and other effects for a thousand years. Up to now, more than 750 secondary metabolites have been identified in cannabis plants, with over 120 of them representing unique C21 terpenophenolic constituents named phytocannabinoids (1–3). Tetrahydrocannabinivar, cannabionil, cannabinil (CBD), cannabidivar, cannabinol (CBG), delta-9-tertrahydrocannabinol (Δ9-THC), and cannabinemone with its acidic forms (the respective carboxylic acids) are currently of major concern when assessing cannabis quality. The other bioactive metabolites that occur in a complex mixture belong to flavonoids, alkaloids, stilbenoids, and phenols (4). It should be noted that the cannabis metabolome is highly variable, depending not only on the respective variety or chemotype (5) but also on growing conditions (6). Moreover, cannabis post-harvest processing, including drying, extraction, and other practices may have a significant impact on product composition and thus overall biological activity. Although such variability offers diversity in their uses, unexpected treatment effects should be taken into consideration as well (7). In any case, effective tools for fit-to-purpose laboratory control of cannabis-based products should be available.

Currently, gas chromatography (GC) is the method of choice for the characterization of volatile terpenes in cannabis and products thereof (8). For a long time, it also used to be the primary analytical method for major phytocannabinoids quantification (9,10). However, due to the thermolability of acidic forms (their decarboxylation to neutral forms takes place at elevated temperatures), time-consuming derivatization should be used for accurate analysis. As these limitations are not encountered in liquid chromatography (LC), this technique has become the gold standard in this field (11). Recently developed methods using ultrahigh-performance liquid chromatography (UHPLC) coupled to diode-array detection (DAD) or mass spectrometry (MS) make reliable quantification of up to 18 major phytocannabinoids (using analytical standards available for the compounds mentioned above) in various matrices possible (12–14). However, considering the complexity of the cannabis metabolome and the limited stability of some components, more comprehensive information is needed to characterize cannabis samples. Target analysis of major phytocannabinoids was shown...
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The advantage of target analyses is the curve of the analytical standards (including components quantification based on the calibration developing cannabis analyses, one discovery of cannabis chemovars and so untargeted metabolomics was investigated by several research teams (15). “Phytocannabinomics” appears to be a promising approach, enabling the discovery of cannabis chemovars and varieties with unique phytocannabinoid profiles (16). Although UHPLC coupled to high-resolution mass spectrometry (HRMS) or tandem mass spectrometry (MS/MS) accounts for a significant proportion of applications (17,18,19), nuclear magnetic resonance (NMR) (20,21) or GC coupled to a flame ionization detector (FID) (22) can be easily compared. The opposite situation occurs for screening and metabolomics-type analyses, where the unavailability of analytical standards makes the transfer of peak intensities to any reference value impossible, and so these results are usually expressed as relative abundances. This relativization is called total ion current (TIC) normalization and is the most common normalization technique. The process of converting the absolute intensities to relative ones by forcing all the sample to have equal total intensity aims to eliminate sources of systematic variation between sample profiles. TIC normalization works well, until the ion intensities exceed the linear range of detector and inconsistent nonlinear changes are observed across peaks (25).

However, during LC–MS measurement, specific deviations may occur, which differ between features. Thus, an algorithm called Quality Control-Robust LOESS Signal Correction (QC-RLSC), developed by Dunn et al., was introduced. During QC-RLSC, a correction curve is interpolated for each feature through the whole analytical run to which the feature is normalized (26). Quality control (QC) samples are usually created by pooling aliquots of all samples in sequence and injecting them during the whole analytical run at the same intervals (every 10 injections) (27). As LC–MS measurement is batch-wise, such prepared QC can be used as a reference level between batches using the same normalization correction procedure, QC-RLSC (28).

Metabolomics analysis generates big data and their processing should be carefully performed to obtain unbiased interpretation.

This study presents a solution to a problem encountered when handling the data obtained by UHPLC–HRMS/MS analysis of cannabis extracts of two varieties in different time slots. The importance of choosing the relevant chemometric tools for the correct results interpretation is emphasized.

Materials and Methods

Chemicals: All of the LC–MS-grade chemicals (ethanol [EtOH], acetonitrile, 2-propanol [iPrOH]) and mobile phase modifiers (ammonium acetate, acetic acid) were purchased from Merck. Deionized water (H₂O) was obtained from a Milli-Q Integral system (Millipore).

Sample Preparation: For the experiment, 30 samples of dried and homogenized cannabis plants (15 samples of Santhica variety and 15 samples of Bialobrzeskie variety, harvested during July–October 2016 in Olomouc and Sumperk locality) were used. A representative sample (0.5 g) was weighed into a centrifuge tube (50 mL), mixed with 20 mL of EtOH, and shaken for 30 min at 240 rpm. The extracts were centrifugated (5 min, 10,000 rpm) and filtered into a 50 mL flask. The solid residue was extracted again using the same extraction procedure and the final pooled extract was made up to 50 mL. Aliquots were transferred into vials for UHPLC–HRMS/MS analysis. All samples were measured in duplicates (in randomized order). QC samples were prepared by pooling 100 µL of each sample and were

FIGURE 1: PCA score plot and loadings plot of cannabis metabolomics data with visible time variability.

![PCA score plot and loadings plot of cannabis metabolomics data with visible time variability.](image-url)
Instrumental Analysis: For the metabolomics analysis, an UHPLC system (Infinity 1290, Agilent) coupled with a high-resolution tandem mass spectrometer (6560 Ion Mobility QTOF LC–MS, Agilent) was employed. Chromatographic separation was performed using a 2.1 × 150 mm, 1.7-μm Acquity BEH C18 column (Waters). The mobile phase consisted of (A) 5 mM ammonium acetate in a mixture of 95.5% O–acetonitrile (v/v) with 0.1% acetic acid and (B) 5 mM ammonium acetate in a mixture of 75:20:5 iPrOH–acetic acid and (35% A, 0.3 mL/min), 4.0 min (35% A, 0.3 mL/min), 8.0 min (22.5% A, 0.2 mL/min), 13.0 min (0% A, 0.2 mL/min), 13.0 min (0% A, 0.3 mL/min), 18.0 min (0% A, 0.35 mL/min), 18.0 min (100% A, 0.3 mL/min), 20.0 min (100% A, 0.3 mL/min). The column temperature was maintained at 60 °C, the injection volume was set to 1 µL. The autosampler was kept at 10°C. The mass spectrometer was operated in the positive ionization mode (ESI+) and negative ionization mode (ESI−) using auto MS/MS acquisition mode in the mass range 100–1000 mass-to-charge ratio (m/z) under the following conditions: gas temperature, 280/300 °C; drying gas, 12 L/min; nebulizer pressure, 40/25 psig; sheath gas temperature, 360/370 °C; sheath gas flow, 12 L/min; nozzle voltage, 400 V; fragmentor voltage, 380 V; octopole radiofrequency voltage, 750 V; capillary voltage, 3500 V.

Results and Discussion

As mentioned earlier, metabolomic fingerprinting represents a challenging tool for a comprehensive characterization of cannabis samples and their authentication. The working plan was to analyze samples (ethanolic extracts obtained from two sets of cannabis varieties, Santhica and Bialobrzeskie) two times to control the variability of measurements. The data obtained by the first and second measurement, which was realized three weeks later, were processed together, and after alignment the final data matrix contained 834 features: 290 and 544 features were detected in ESI+ and ESI−, respectively.

Time Variability in Multivariate Analysis:
A PCA score plot was used to overview the multivariate data. A standard preprocessing procedure, including TIC normalization, logarithmic transformation, and Pareto scaling, was used (29). Surprisingly, instead of two expected clusters defined by cannabis varieties, four clusters were found (Figure 1). This is very confusing in terms of authentication as it seemed that two other cannabis chemotypes were analyzed. PCA revealed that in addition to the biological variability between Santhica and Bialobrzeskie varieties, which is described by the first principal component and covers 62.1%
of the total variance, the instrumental/time variability represents the second largest part of variance (6.7%) in the data. The complementary PCA loadings plot shows the distribution of features. While the points on the first principal component (left and right side) correspond to *Bialobrzeskie* and *Santhica* markers, the direction of the second principal component (upward and downward) indicates the increasing effect of time difference on them. In order to calculate the amount of time-related features whose intensity has changed between batches, a paired $t$-test was performed. There were 486 features significantly differing (FDR, [see Materials and Methods]) ($p$-value < 0.01) between QC samples from the first and second batch.

**Different Relative Abundance of Features in Time:** When investigating the reasons for time-dependent separation, the problem of different relative peak intensities between batches was observed. As the linear range of the time-of-flight mass analyzer used in this study is relatively narrow, the most intensive ions outside of the linearity in the first measured batch could easily get into the linear range in the later date second measurement because of the decreased total ion current. Different peak profiles (caused by peak oversaturation) lead to different peak parameters (area/height), thus the relative abundance of features changes. Figure 2 illustrates the most intensive peak oversaturation in batch 1 and its subsequent fit into linear range in batch 2. As the peak description was inaccurate in batch 1 (the area of the highest peak was significantly decreased by 11%), less abundant peaks were therefore enhanced by 2% and 6%.

Time dependence could also be observed on the total ion current, which differed not only between cannabis varieties but also with regard to date of analysis. Figure 3 presents the decrease of total ion current in the second batch of the dataset. Based on the paired $t$-test, the decrease of intensity was significant for both varieties ($p$-value < 0.01).

**Quality Control-Robust LOESS Signal Correction (QC-RLSC) Method:** As the connection between the data processing and the following statistical evaluation is very close, the time variability present in the data should be removed. That's why more advanced normalization techniques were applied. Our developed normalization procedure is based on the QC-RLSC method introduced by Dunn et al. (26). This strategy is based on QC samples that are injected at the same interval throughout the whole sequence. In the case of a larger sample set that cannot be measured in a single run, the same QC sample must be used in all batches. The assumption is still the same sample composition of QC sample through the batch(es) and all changes between individual injections are therefore caused by instrumental variability. In other words, the injection profile serves to monitor these intensity changes. The most pronounced advantage of QC-RLSC is the correction of nonlinear changes, where each variable profile is considered individually. In the first step, only QC sample features intensities are used for locally weighted scatter plot smoothing (LOESS). The fitted curve estimates the values of correction factors for feature intensity in QCs, which are then used for the calculation of correction factors for the rest of the samples using the quadratic spline function. Unlike the QC samples, these analytical samples differ due to the biological variability, and the instrument variability is, depending on the current situation, less or more visible. Their correction factors are therefore estimated based on the QC injection profile. Except for the first and last section of the sequence, the two foregoing and two oncoming QC samples correction factors are always used for polynomial function calculation. The first and last section is resolved with the first and last free QC samples, respectively. Finally, the correction factors for all the samples and features are obtained, and the measured intensities can be divided by them.

**Application of QC-RLSC on Cannabis Fingerprints:** After application of QC-RLSC normalization on the data, the effect of time variability was removed. On the PCA score plot (Figure 4[a]), there is no sign of time variability described by the first two principal components. The total ion current between batches is straightened (Figure 4[b]) and there is also no significant difference between batch 1 and 2 according to the paired $t$-test, where for *Santhica* and *Bialobrzeskie* the $p$-value was equal to 0.238 and 0.503, respectively. Even when a paired $t$-test was again used to reveal time-related markers, none of the features
Conclusion

Analytical methods based on UHPLC–
HRMS/MS cannot be easily compared, because the metabolomic fingerprint does not have any reference value to which the measured ion intensities could be linked and thus make them time-dependent. Although the measurement settings were still the same, the condition of the instrument (the status of the ion source and chromatographic system) differ in time and cause the time variability. In initial phase, time dependency was visible after application of the common normalization technique, as exceeding the linear range of the mass analyzer detector occurred. This risk must always be considered. Nevertheless, the correction can be achieved by employing QC-RLSC, a method enabling the removal of undesired time variability by taking each feature intensity profile into account and smoothing it.

Acknowledgement

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Benefits of Integrating Travelling Wave Ion Mobility Spectrometry into Liquid Chromatography and Mass Spectrometry Workflows for Steroid Analysis

Maykel Hernández-Mesa1,2, Gaud Dervilly1, and Bruno Le Bizec1, 1Oniris, INRAE, LABERCA, Nantes, France, 2Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Granada, Spain

The advantages provided by the implementation of ion mobility spectrometry (IMS), and in particular travelling wave ion mobility spectrometry (TWIMS), in traditional liquid chromatography–mass spectrometry (LC–MS) systems are discussed. TWIMS provides new analytical information on the structure of ions, the so-called collision cross section (CCS), which in combination with retention indices and mass spectra, increases confidence in compound identification. The use of this parameter for identification purposes is quite recent and the development of CCS databases that can be subsequently applied in both targeted and non-targeted analysis is required. In the case of steroids, a TWCCS database for 300 compounds, including endogenous and exogenous steroids, has recently been reported. From the point of view of compound separation, the integration of TWIMS as a third separation dimension in LC–MS workflows increases the resolving power of the method, allowing the separation of isobaric and isomeric steroids. Furthermore, it also allows the separation of the steroids from the chemical background related to the urine sample, improving the limits of detection (LODs) between 2 and 7 times.

Steroid analysis is of special interest in multiple fields, including medicine, food safety, and doping control applications. These biochemical molecules play an important role in human metabolism and are indicators of health and disease (1). Furthermore, the monitoring of steroid levels in professional athletes or animals intended for human consumption is of special relevance to identify their fraudulent use to increase sport performance or as growth promoters in animals, respectively. Traditionally, the analysis of steroids has been approached by gas chromatography–mass spectrometry (GC–MS), although currently the use of liquid chromatography (LC)–MS for this purpose has become widespread (2). Despite the multiple options available to carry out the determination of steroids, it still represents an analytical challenge because of the large number of molecules that make up this chemical family of compounds, their low concentration levels in biological matrices, and the presence of isomers that hamper molecular identification.

The recent commercialization of hyphenated ion mobility-mass spectrometry (IM-MS) instruments offers new possibilities to enhance traditional LC–MS workflows by overcoming the aforementioned challenges. Ion mobility spectrometry (IMS) is a post-ionization separation technique in which ions are separated according to their size, shape, and charge in a gas phase because of the action of an electric field (3). Integration of IMS into LC–MS workflows is possible as separation in the three dimensions occurs on different timescales, namely minutes in LC, milliseconds in IMS, and microseconds in MS. In this sense, a high acquisition rate is required in the MS dimension, so IM-MS instruments are usually based on time-of-flight (TOF)-MS technology. Regarding IMS, there are
several available technologies, each of them presenting advantages and disadvantages with respect to the others (4,5). For non-targeted analysis, which is increasingly applied in holistic studies of the steroidome, three different IMS technologies have been successfully implemented, namely drift tube ion mobility spectrometry (DTIMS), travelling wave ion mobility spectrometry (TWIMS), and trapped ion mobility spectrometry (TIMS).

DTIMS, TWIMS, and TIMS provide information on the collision cross section (CCS) of ions, which represents additional information on the retention indices and mass spectra of the molecules, and which can be used to obtain molecular identification with a higher level of confidence. In IMS, the CCS represents a momentum transfer between the ions and the buffer gas particles in the ion mobility cell, and is derived quantitatively from the mobility of ions (K) via mathematical models such as the Mason-Schamp equation (6). However, this mathematical approach, which is widely used, is only applicable when using DTIMS in a specific operating mode. Consequently, CCS values are generally obtained from calibration curves generated with compounds of known CCS values.

The correlation between CCS and mass-to-charge ratio (m/z) parameters is obvious and undeniable (7). In this framework, there is an open discussion about the advantages provided by the implementation of this molecular characteristic for identification purposes (8). Nevertheless, certain ions have been shown to exhibit unexpected CCS values compared to their chemical and structural analogues (9). It is in these cases where the CCS values are complementary data to m/z, being of special interest for the identification of isobars and isomers that present different CCS under the same experimental conditions. In general, CCS values can be experimentally obtained, computationally generated, or predicted by the use of machine learning approaches (10). Regarding experimentally derived CCS values, an increasing number of CCS databases have been reported in recent years, including a CCS compendium (11,12). In general, early inter-laboratory studies indicate that CCS databases can be applied in laboratories equipped with the same IMS technology as that used for database generation (13,14). However, more research is still needed to evaluate whether extrapolation of CCS values between IM-MS platforms based on different IMS technologies is possible (15).

The use of CCS as an identification parameter in LC–IM-MS-based methods has been shown to reduce the number of false positives/negatives observed in LC–MS analyses (16), but it is not the only advantage provided by IMS. As the movement of ions through the ion mobility cell depends on their conformation in the gas phase, isobars and isomers can be separated depending on the resolving power (R N) of IMS technology (3). Therefore, this results in a significant improvement in the selectivity of the analytical method. In general, the integration of IMS into LC–MS workflows increases the peak capacity, allowing the resolution of a greater number of ions (5). Moreover, IMS also contributes to the reduction of chemical background noise, improving concentration sensitivity. As a consequence, lower LODs can be reached and compounds can be detected at lower concentration levels in complex matrices (17).

This article covers and groups our most relevant findings related to the analysis of steroids by IMS using TWIMS technology (9,14,17), as well as related computational studies (18). It shows how TWIMS can improve the performance characteristics of LC–MS methods traditionally used for the analysis of steroids, particularly in urine samples. In this sense, it is demonstrated that the integration of TWIMS into LC–MS workflows improves method selectivity and sensitivity, allowing the differentiation of isomeric and isobaric steroids and the detection of steroids at lower concentration levels. Furthermore, the development of a CCS database for steroids, and its subsequent cross-validation by three external laboratories, provides a new tool for steroid identification, especially in non-targeted analysis. In addition, computational studies have been conducted and the application of machine learning tools accessible online has been investigated to provide more confidence in the reported CCS values. Therefore, our work covers the three strategies currently used to generate CCS values. Finally, this article reports the first results obtained for the separation of steroids by cyclic-TWIMS. They demonstrate how this new commercial technology provides the opportunity to achieve separation of pairs of steroids with close CCS values that could not have been separated by TWIMS before.

**Experimental**

**Chemicals:** Water (HiperSolv Chromanorm for HPLC) was supplied by WWR International. Methanol, propan-2-ol, and acetonitrile (LC–MS Chromasolv-grade) were provided by Sigma-Aldrich. Ethanol (Promochem, HPLC-grade) was acquired from LGC Standards GmbH. Formic acid (eluent additive for LC–MS) was purchased from LGC Standards GmbH. Mass calibration was carried out using a solution of sodium formate (0.5 mM in 90:10 (% v/v) propan-2–ol–water). MS calibration solution was prepared from sodium hydroxide (1 M, Fisher Scientific) and formic acid (Promochem) supplied by Fisher Scientific and LGC Standards, respectively. The Major Mix IMS/TOF Calibration Kit from Waters was used for CCS calibration. Leucine-enkephalin (2 μg/mL) in 50:50 (% v/v) water–acetonitrile solution containing 0.2% (v/v) of formic acid was used as internal standard.
acid was used as a lock mass standard. Leucine-enkephalin standard was acquired from Waters. Steroid standards were obtained from Steraloids, Sigma-Aldrich, and the National Measurement Institute. In general, steroid stock solutions were prepared in ethanol at 100 μg/mL or 1 mg/mL. Working standard solutions (10 μg/mL) were prepared by diluting stock standard solutions with methanol and kept at −20 °C in amber glass vials.

Sample Preparation: Urine samples from bovines (adult animals) and calves were selected from the LABERCA biobank. Urine samples (n = 9) were thawed at room temperature and subsequently prepared following a "dilute-and-shoot" protocol. Briefly, urine samples were submitted to centrifugal filtration for 10 min at 7800 g and 15 °C using centrifugal filters (polyethersulfone membrane, molecular weight cut-off of 10 kDa; VWR International). After filtration, the samples were spiked with working standard solutions consisting of selected mixtures of steroids to achieve a steroid concentration of 2 μg/mL. The spiked samples were subsequently diluted 10-fold with 0.1% (v/v) aqueous formic acid, and submitted to analysis.

Ultrahigh-Performance Liquid Chromatography (UHPLC) Separation: UHPLC separation was performed on an Acquity UPLC system (Waters) equipped with a 2.1 × 100 mm, 1.7-μm Acquity UPLC BEH C18 column (Waters). Mobile phase consisted of water (A) and acetonitrile (B), and both solvents contained 0.1% (v/v) formic acid. The gradient flow rate was 600 µL/min. The following concentration gradient program was selected for the analysis of steroid standard solutions (except for steroid esters and progestagens) and urine samples: 95/5 (A/B, v/v) between 0 and 0.3 min, 57/43 at 9.6 min, 0/100 from 13.5 to 15.5 min, and 95/5 from 16 to 19.5 min. In the case of steroid ester and progestagen standards, the concentration

![FIGURE 1](image1.png) Correlation between TWCCSN and m/z of all monomers and singly charged ions resulting from the ionization of androgens (n = 127), estrogens (n = 80), and progestagens and corticosteroids (n = 89) under ESI(+) conditions.

![FIGURE 2](image2.png) (a) Molecular structure of androstanedione isomers, and the information related to the FIA analysis of standards of both of them by IMS. (b) Mobility spectra obtained from the analysis of androstanedione isomers by traditional TWIMS (max. CCS/ΔCCS = 40). (c) Mobility spectra from the analysis of androstanedione isomers by cyclic-TWIMS (1 pass).

![FIGURE 3](image3.png) Analysis of a mixture of 14α-hydroxytestosterone (a) and 16α-hydroxytestosterone (b) by cyclic-TWIMS-TOF-MS selecting (a) a single pass, (b) 5 passes, and (c) 10 passes through the mobility cell.
gradient program consisted of 50/50 (A/B, v/v) between 0 and 2 min, 90/10 at 9.6 min, 0/100 from 13.5 to 15.5 min, and 50/50 from 16 to 19.5 min. The column temperature was kept at 50 °C and 5 µL of samples was injected onto the column.

**IM-MS Conditions:** IMS analyses were performed on a hybrid quadrupole (Q)-TWIMS-TOF-MS instrument (Synapt G2-S HDMS, Waters) equipped with an electrospray ionization (ESI) interface. Analyses were performed in ESI+ and ESI- modes, acquiring continuous data in the range 150–1200 m/z at 2.5 Hz. The TOF analyzer was operated in high resolution mode. Source and desolvation temperatures were set at 150 and 350 °C, respectively. Nitrogen was used as cone and desolvation gas and was supplied at 50 and 1000 L/h, respectively. Nebulizer pressure was set at 6.0 bar. Cone voltage and source offset were set at 31 and 40 V, respectively. Capillary voltage was set at 3.0 and 2.5 kV for ESI+ and ESI- modes, respectively. A maximum tolerance of 10 ppm was established for identification of ions based on mass accuracy.

Regarding IMS conditions, nitrogen was used as trap and IMS buffer gas and was supplied at 50 and 1000 L/h, respectively. Nebulizer pressure was set at 6.0 bar. Cone voltage and source offset were set at 31 and 40 V, respectively. Capillary voltage was set at 3.0 and 2.5 kV for ESI+ and ESI- modes, respectively. A flow rate of 20 µL/min was established for the lock mass standard solution, and MS data were acquired every 15 s at 5 Hz (3 scans to average). LockSpray capillary voltage was set at 3 kV and 4.0 V, respectively. IMS DC bias and trap cell, wave velocity and height were set at 311 m/s and 4.0 V, respectively. In the transfer cell, wave velocity and height were set at 219 m/s and 4.0 V, respectively. IMS DC bias and trap DC bias were set at 3.0 and 47.0 V, respectively. For analyses in ESI+ mode, IMS wave velocity and height were set at 1000 m/s and 40.0 V, respectively. For analyses in ESI- mode, IMS wave velocity and height were set at 550 m/s and 40.0 V.

**TmCCS_N Database:** For CCS characterization of steroids, steroid standard solutions (10 µg/mL) were injected into the IMS instrument applying a flow injection analysis (FIA) method. The Acquity UPLC System was used for this purpose, although a stainless steel flexible capillary for flow restriction (2 m × 0.12 mm, with 1/16 in female connector on both ends; Agilent Technologies) was selected instead of an LC column. CCS characterization of steroids was performed under IMS conditions similar to those mentioned above. Specific details, including FIA conditions, can be found in the related published paper (9).

**Cross-Validation of CCS Values:** Cross-validation of CCS values in the reported TmCCS_N database (9) was carried out in three external laboratories located at the University of Geneva (Geneva, Switzerland), Waters Corporation (Wilmslow, UK), and the INRAE-BIA platform BIBS (Nantes, France). These three laboratories are equipped with IMS instrumentation based on TWIMS technology. The INRAE-BIA platform BIBS was equipped with a Synapt G2-Si instrument (Waters), while Vion instruments (Waters) were available at both the University of
**TABLE 1:** $^{14}$CCS$_{N_2}$ values of specific steroid ions reported in the $^{14}$CCS$_{N_2}$ database, and their comparison with the average $^{14}$CCS$_{N_2}$ values obtained from measurements performed in the context of the cross-validation study. CCS comparison with computationally derived CCS values and CCS values predicted by machine learning approaches.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Androstanedione</th>
<th>Testosterone</th>
<th>Fluoxymesterone</th>
<th>Methylchlorotestosterone</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion</td>
<td>[M+H]$^+$</td>
<td>[M+H]$^+$</td>
<td>[M+H]$^+$</td>
<td>[M+H]$^+$</td>
<td>[M+H]$^+$</td>
</tr>
<tr>
<td>$^{14}$CCS$_{N_2}$ (Å$^2$)$^{a}$</td>
<td>176.9</td>
<td>173.0</td>
<td>178.3</td>
<td>180.8</td>
<td>191.6</td>
</tr>
<tr>
<td>$^{14}$CCS$_{N_2}$ (Å$^2$)$^{b}$</td>
<td>176.0</td>
<td>171.7</td>
<td>177.5</td>
<td>179.5</td>
<td>189.9</td>
</tr>
<tr>
<td>$\Delta$CCS/CCS</td>
<td>-0.5%</td>
<td>-0.9%</td>
<td>-0.5%</td>
<td>-0.7%</td>
<td>-0.9%</td>
</tr>
<tr>
<td>MN15$^{c}$</td>
<td>CCS$_{tho}$ (Å$^2$)</td>
<td>175.2</td>
<td>170.0</td>
<td>175.7</td>
<td>179.6</td>
</tr>
<tr>
<td></td>
<td>$\Delta$CCS/CCS</td>
<td>-1.0%</td>
<td>-1.7%</td>
<td>-1.5</td>
<td>-0.7%</td>
</tr>
<tr>
<td>wB97X-D$^{c}$</td>
<td>CCS$_{tho}$ (Å$^2$)</td>
<td>175.8</td>
<td>170.4</td>
<td>176.1</td>
<td>180.2</td>
</tr>
<tr>
<td></td>
<td>$\Delta$CCS/CCS</td>
<td>-0.6%</td>
<td>-1.5%</td>
<td>-1.2%</td>
<td>-0.4%</td>
</tr>
<tr>
<td>MetCCS$^{d}$</td>
<td>CCS$_{pred}$ (Å$^2$)</td>
<td>167.4</td>
<td>166.0</td>
<td>170.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\Delta$CCS/CCS</td>
<td>-5.4%</td>
<td>-4.1%</td>
<td>-4.2%</td>
<td>-</td>
</tr>
<tr>
<td>AllCCS$^{d}$</td>
<td>CCS$_{pred}$ (Å$^2$)</td>
<td>174.0</td>
<td>174.7</td>
<td>182.9</td>
<td>182.1</td>
</tr>
<tr>
<td></td>
<td>$\Delta$CCS/CCS</td>
<td>-1.6%</td>
<td>1.0%</td>
<td>2.6%</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

Geneva and Waters Corporation. The experimental conditions selected for the cross-validation of the $^{14}$CCS$_{N_2}$ database were similar to those LC–IM-MS conditions described above. Details and modifications of these conditions can be found in the related published paper (14).

**Cyclic-TWIMS Experiments:** Pairs of isomeric steroids were prepared at 62 ng/mL in 1:1 water–acetonitrile containing 0.1% (v/v) formic acid. These stock solutions were infused directly into a Waters cyclic ion-mobility enabled quadrupole time-of-flight (Q-cIM-oaToF) mass spectrometer fitted with an ESI source. The quadrupole was used to select the ions of interest, which were then passed into the cIM region. Scalable IM separation was employed in a stepwise fashion to observe the arrival time distribution (ATD) profile for zero passes around the cIM device (no IMS separation), followed by one, two, three, and subsequent multiple passes, up to a total of 10 passes. Selection of a narrow portion of ions, followed by fragmentation of those ions, was also carried out.

**Computational Studies:** The MobCal software was used to calculate the CCS values of the selected steroids whose structures were optimized by density-functional theory (DFT) calculations, using the Gaussian 16 program (19). The wB97X-D exchange-correlation functional and the Minnesota MN15 functional were used for conformational analysis. The geometry of the various potential ionized structures was fully optimized in vacuo to identify preferred ionization sites. CCS estimations for the various relevant conformers were carried out with the modified version of MobCal incorporating a N$_2$-based trajectory method (TM) algorithm. Finally, CCS values were weighted according to the relevance of the conformers obtained for each steroid.

**Results and Discussion**

**Increase of Confidence in the Identification:** CCS parameter has recently emerged as a novel molecular characteristic to support compound identification in combination with retention indices and mass spectra (16). Consequently, a large number of CCS databases are currently being reported in order to overcome the lack of information on this parameter for a wide range of molecular ions (12). In this context, CCS databases should cover the most common and abundant ions found in real sample analysis ([M+H]$^+$, [M+Na]$^+$, [M-H]$^-$, among other relevant ions). However, there are many works in the literature that have addressed the separation of molecular dimers due to their significant differences observed during CCS characterization. From a practical point of view, the analysis of molecular dimers is of little interest since these ions are not always formed during the analysis of complex samples, such as biofluids. Furthermore, if they are formed, detected, and identified on the mass spectrum, they are usually present in much lower abundance than other monomer ions observed for the same molecule. Therefore, to extend the use of IMS beyond the field of academic research, CCS characterization, as well as ion separation by IMS, must focus on those ions that are likely to be detected in the analysis of real samples.

**$^{14}$CCS$_{N_2}$ Database for Steroids:** The CCS database for steroids includes up to 300 compounds, such as protonated and deprotonated molecules and sodium adducts, among many others, and covers endogenous compounds, such as glucuronide and sulfate metabolites, and...
exogenous substances, such as steroid esters, as well as deuterated steroids (9). It is the first large CCS database reported for this family of compounds and CCS values have been obtained using nitrogen as buffer gas in a TWIMS-TOF-MS system. Therefore, following currently accepted nomenclature (6), this database is referred as T^α/β CCS-N database for steroids. It is important to indicate the type of IMS technology and buffer gas used for CCS characterization, since the CCS of compounds depends on experimental conditions, such as the type of gas in the IMS cell, temperature, and pressure. Furthermore, it is currently not demonstrated that CCS databases can be extrapolated and applied in instruments based on different IMS technologies.

The T^α CCS-N database for steroids covers a m/z range between 241.1956 and 543.2126, while T^α CCS-N ranges from 157.3 to 264.8 Å² if only monomers and singly charged ions are selected. Figure 1 shows the relationship between T^α CCS-N and m/z observed for positively charged steroid ions. Although both parameters cannot be considered orthogonal, the observed CCS differences for ions with identical or close accurate mass are sufficient to distinguish these ions. For example, the sodium adduct of 6β-hydroxyetiocholanolone (m/z 329.2087) exhibits a totally different T^α CCS-N (190.3 Å²) from the T^α CCS-N observed for the positional isomers 11β-hydroxyetiocholanolone and 16α-hydroxyetiocholanolone (m/z 329.2087; 199.3 Å² and 195.1 Å², respectively). The T^α CCS-N of sodium adducts also differs greatly in the case of other steroids, such as 18-hydroxycortisol (m/z 401.1935; 200.4 Å²) and 6β-hydroxycortisol (m/z 401.1935; 211.4 Å²). These examples show how IMS can be a powerful parameter to contribute in compound annotation in non-targeted analysis or to improve confidence in identifying compounds in targeted analysis, especially to distinguish between isomers.

Cross-Validation of CCS Values:
Cross-validation of the reported CCS databases seems necessary to extend their use beyond the laboratories where they have been generated. As mentioned earlier, CCS values can be obtained experimentally, computationally calculated, or predicted using machine learning approaches (10). Initially, the T^α CCS-N database for steroids was cross-validated experimentally by three external platforms using TWIMS technology, specifically in a Synapt G2-Si and two Vion instruments (14). For this purpose, 97 steroids included in the original T^α CCS-N database were selected. The selection encompassed compounds from the different classes of steroids, such as androgens, n = 42; estrogens, n = 28; progestogens, n = 8; and corticosteroids, n = 19, and involved a maximum of 167 ions under study. In general, the T^α CCS-N measurements performed on the Synapt G2-Si, Vion #1, and Vion #2 instruments were biased within the ±2.0% range of the T^α CCS-N values in the database for 98.8, 79.9, and 94.0% of the ions detected by each platform (a minimum of 142 ions), respectively. This bias is within the expected bias threshold, as a threshold of ±2.0% is widely accepted for T^α CCS-N measurements compared to T^α CCS-N values in databases. Therefore, the T^α CCS-N database for steroids has been successfully cross-validated.

Nevertheless, and in order to reduce the bias between T^α CCS-N values measured in other external laboratories and reference values, a T^α CCS-N database with average T^α CCS-N values from the four different laboratories (LABERCA, University of Geneva, Waters Corporation, and the INRAE-BIA) has been reported (14). In this database, the relative standard deviations (RSDs; n = 12) were below 1.5%. The bias between the average T^α CCS-N values (or reference values) and the T^α CCS-N measurements performed by each laboratory was within the range of ±1.5% for 96.8% of the total ions (n = 142).

Computational studies and the application of machine learning approaches were carried out for a smaller number of steroids to verify the potential of both methodologies to generate CCS values and, consequently, be used as tools for cross-validation of the reported CCS databases. First, computationally-derived values were obtained for the selected 20 steroids (involving 23 ions) applying two different approaches (18), as specified in the experimental section. In this sense, slightly better performance was observed for the MN15 functional in comparison to that of the wB97X-D functional. In general, high correlation was observed between experimental (9) and computationally-derived CCS values (R² = 0.9696 for MN15 functional). Furthermore, the CCS value for almost 80% of the studied compounds was properly calculated within a ±2% error range from the observed data. Therefore, although time-consuming, computational studies are suitable for cross-validation of CCS databases, as well as generating information on the CCS of compounds that are not available as chemical standards and cannot be characterized. Finally, two machine learning tools, MetCCS (20) and AllCCS (21), which are accessible online, were evaluated for the prediction of steroid CCS values. Table 1 shows the CCS values of a selection of five steroids obtained by the three different approaches currently available to generate CCS values, including by machine learning tools. Although machine learning approaches are useful tools for rapidly generating CCS values compared to time-consuming computational approaches, they cannot be as precise regarding experimentally measured CCS values. Obviously, as can be seen from Table 1, the precision of machine learning
approaches depends on the model itself, such as the parameters used to build the model, as well as the compound dataset used to generate the model. **Matrix Effect:** In order to implement the use of the CCS values as an identification parameter, it seems important to demonstrate that there is no influence of the sample matrix on the CCS measurement of the CCS of the analyte of interest. Such effect matrix was evaluated for phase II metabolites of androgens and estrogens (n = 25) in urine samples, studying both positive and negative ionization mode (17). All steroids were detected as the deprotonated ion under ESI(-) conditions, whereas only 21 steroids were detected either as the protonated molecule or as a sodium adduct under ESI(+). In total, 1086 TWCCS\(_N\) measurements were carried out over four months, and high accuracy was observed for TWCCS\(_N\) values of steroids in urine samples compared to the TWCCS\(_N\) values included in the database. More than 77% of TWCCS\(_N\) measurements for steroids in urine samples were within an error of ±0.5%, while this error was only greater than ±1.0% in 1.4% of the TWCCS\(_N\) measurements. Therefore, the urine matrix did not show any influence on the TWCCS\(_N\) measurement of steroids, since the TWCCS\(_N\) values obtained in the presence of matrix matched with the TWCCS\(_N\) values in the database within the extensively applied error threshold of ±2.0% for the comparison of measured TWCCS values and twCCS values in databases (22).

**Selectivity Improvement:** The low \( R_p \) of TWIMS technology available as part of IM-MS instruments has traditionally been a drawback to achieving the separation of isomers and isobars showing close CCS values. In some ways, this fact has probably been the main limitation for the development of applications using this analytical technique. For compounds with CCS values around 200 Å\(^2\), only those compounds that show a difference in terms of CCS of at least 2.5% can be separated in a TWIMS dimension with a \( R_p \) of 40 (23). However, the introduction of cyclic-TWIMS instrumentation has brought the possibility of separating with TWIMS technology those complex mixtures of isomeric and isobaric compounds that present smaller differences.

In the case of steroids in urine samples, we have observed that only those ions that differ in CCS values by at least 4%, such as the deprotonated molecules of eetocholanolone glucuronide (\(^{tW}CCS_N = 206.9\) Å\(^2\)) and epiandrosterone glucuronide (\(^{tW}CCS_N = 221.4\) Å\(^2\)) or 19-noretocholanolone glucuronide (\(^{tW}CCS_N = 204.2\) Å\(^2\)) and 19-norandrosterone glucuronide (\(^{tW}CCS_N = 213\) Å\(^2\)) can be separated by the Synapt G2-S system. Even in spite of that, partial or no separation was observed for other steroid isomers, even when they showed a CCS difference greater than 4%. For example, androstenedione isomers, which differ in the \( \alpha \) or \( \beta \) position of the proton at C5 and show a CCS difference of 5.8% for their protonated forms (Figure 2[a]), were only partially separated in the TWIMS dimension of the Synapt G2-S instrument (Figure 2[b]). However, these ions have been separated at the baseline by a cyclic-TWIMS system, even passing the ions only once through the ion mobility cell (Figure 2[c]). In this sense, the \( R_p \) of the cyclic-TWIMS selecting a single pass is already greater than that of the Synapt instruments (\( R_p \) of 65 vs. 40) (24).

Other isomeric pairs of steroids, such as 14\( \alpha \)-hydroxytestosterone and 16\( \alpha \)-hydroxytestosterone, did not separate on either the Synapt G2-S system or the cyclic-TWIMS instrument by selecting only a single pass. In fact, the protonated ions of both molecules (m/z 305.2111) show close \(^{tW}CCS_N\) values, 174.2 and 176.1 Å\(^2\), respectively, requiring a high resolving power of the TWIMS dimension to be separated. Since the resolving power of the cyclic-TWIMS system depends on the number of passes selected, up to 10 passes were evaluated to achieve baseline separation of these ions with a CCS difference of 1.1%.

Figure 3 shows how both ions practically separate at the baseline when they pass through the cyclic-TWIMS path five times. In this sense, it could be argued that the separation would improve if they were made to pass a greater number of times through the mobility cell. However, this is not without its drawbacks, as ions cannot be passed through the cyclic-TWIMS cell indefinitely. In addition to a significant loss of ions in the successive passes through the cell, the increasingly narrow selection of the range of mobility to separate in each pass leads to obtaining broader peaks and poorer quality analytical information. As can be seen in Figure 3(c), a significant depletion of signal intensity is observed when ions move along the cyclic-TWIMS path ten times instead of five times; broader peaks can also be observed. Therefore, the number of passes to be selected in cyclic-TWIMS implies a compromise between the resolving power to be achieved and signal intensity and/or peak shape.

**Sensitivity Enhancement:** One of the main advantages of integrating IMS into LC–MS workflows is that it generally improves method sensitivity. This third dimension brings the possibility of selecting drift time regions (or CCS values) of interest in the acquired data, which implies the subtraction of the analytical signal of the analytes from the chemical background noise. Consequently, lower LODs can be achieved. Figure 4 shows the improvement in terms of LODs observed for various phase II steroid metabolites when the same urine samples were analyzed by LC–TWIMS-MS and LC–MS (TWIMS cell was inactivated but allowed
ion transmission). In general, signal-to-noise ratio (S/N) was improved from two- to sevenfold by selecting the drift time region of the target analytes. As can be seen in Figure 4, this improvement in concentration sensitivity is crucial for the determination of compounds at concentration levels close to the LOD of the method (S/N = 3). They can be determined at concentration levels even higher than the limit of quantification (LOQ) (S/N = 10) when including the IMS as a third separation dimension, therefore, allowing their quantification.

Conclusions

TWIMS or other IMS technologies, such as DTIMS or TIMS, are called to revolutionize current LC–MS methods, or other methods that involve chromatographic separations and MS detection. As observed for this particular case of steroids, it introduces an extra separation dimension in LC–MS workflows that enhances concentration sensitivity and increases the resolving power of the method. Furthermore, the molecular ions of isomers, such as those of steroids, can be distinguished according to their CCS values. Isomer differentiation has been a traditional analytical challenge because they tend to show similar mass spectra and can coelute in the chromatographic dimension. As shown in this work, IMS is a powerful analytical technique to overcome that challenge.

Although low resolving power has traditionally been attributed to IMS-based systems, in recent years, technological improvements have been made regarding this drawback. Consequently, there are IM-MS instruments currently available that allow the separation of compounds showing similar ion mobility or close structural conformation in the gas phase. Therefore, ions with close CCS values are now susceptible to being separated by IMS. In this sense, in the coming years a greater implementation of the use of the CCS in both targeted and non-targeted approaches is expected with the aim of improving confidence in the identification of compounds. However, it is still necessary to characterize a wide range of molecules in terms of CCS to generate large CCS databases that can be applied routinely in compound determination. It is desirable that such CCS databases are based on measurements carried out in different laboratories, thus reducing any laboratory bias. Also, computational calculations or even proper machine learning tools are suitable approaches for generating CCS values and expanding the number of entries in CCS databases, especially for those compounds for which analytical standards are not available.

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References

19) M.J. Frisch, G.W. Trucks, H.B. Schlegel, et al., Gaussian 16, revision C01; Gaussian, Inc.: Wallingford, CT, (2016).
Q. Why is the analysis of residues of prohibited pharmaceutical active substances (PPAS) in milk important?
A: There is strict EU legislation regarding the use of pharmaceutical active substances in food production, and some substances are even marked as prohibited by Commission regulation (EU) No 37/2010.

Q. How do PPAS enter the food chain?
A: PPAS are rarely used and found in food. However, many substances that have a pharmaceutical effect can be used to treat (sick) animals. For example, nitroimidazoles are an important antiparasitic agent with a high biological activity and are therefore very effective. However, nitroimidazoles are prohibited because there are concerns about toxicity, mutagenicity, and genotoxicity.
In general, some substances are marked as prohibited by the EU due to potential public health risks as a result of exposure to toxic residues or related metabolites and the potential for development of antimicrobial resistance. Antimicrobial resistance could lead to ineffective antibiotics and reduce treatment options in human and animal health.

Q. You worked on a method to analyze residues from five different classes of these substances in a single method. What analytical techniques were used previously?
A: Before this new multi-class method was developed and implemented in routine analyses, five different single-class methods were used. This means a method, including a complete sample cleanup and liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis, (i) for the group of nitrofurans, (ii) for the group of nitroimidazoles, (iii) for dapsone, (iv) for chloramphenicol, and (v) for chlorpromazine. The sample preparation for 20–50 samples using five different methods takes an analyst five times one and a half workdays. This is followed by 5 times 8–20 min analysis time on a LC–MS/MS instrument for each sample. With this new method, the sample preparation time is reduced to only one and a half workdays to prepare 20–50 samples for all 16 PPAS and 14 min analysis time on the LC–MS/MS instrument.

Q. What were the main challenges you encountered to develop a single method to analyze this wide combination of analytes?
A: The wide combination of PPAS makes the sample preparation challenging, because the different physicochemical properties limits the possibilities for single method cleanup. On the one hand, sufficient recovery of all PPAS is required, and on the other hand you want to remove as much unwanted matrix compounds as possible. With a liquid–liquid extraction using ethyl acetate we were able to create sufficient recovery for all PPAS. However, the matrix compounds in the milk caused an unwanted gel formation during this extraction step. We found that this gel formation could be prevented by using the AOAC dispersive SPE kit while maintaining sufficient recovery.

Developing the LC–MS/MS analysis was also challenging because all PPAS are prohibited and therefore zero-tolerance for the presence of these substances is applicable. A zero-tolerance policy requires high sensitivity. For a sensitive detection, two of the 16 PPAS included have to be measured in the negative ionization mode of the mass spectrometer. While the highest sensitivity is achieved in the positive mode for two other compounds. Therefore, an instrument was needed that was fast enough to quickly switch between both ionization modes. Additionally, an optimized chromatographic method ensured separation of most PPAS. In combination with the scheduled multiple reaction monitoring (MRM) detection, this method resulted in a more sensitive detection.

Q. What is novel about your approach?
A: Within the field of food safety, a lot of research has been done. Many papers have been published on the analysis of a single class of residues of regulated and/or prohibited pharmaceutical active substances. Several methods are even published about the combination of PPAS in a single method. However, to our knowledge, no fully validated method in milk has previously been reported that includes 16 compounds belonging to five different classes. This new approach results in a far more cost-effective surveillance of the PPAS in milk, using one multi-class method instead of multiple single-class methods.
High Sensitivity Measurement of Off-Flavours in Drinking Water Samples by Solid-Phase Microextraction (SPME) Using GCMS-TQ8050 NX

Dr. Hendrik Schulte, Shimadzu Europa GmbH

Introduction
Geosmin, 2-Methylisoborneol (2-MIB), and 2,4,6-Trichloroanisole (2,4,6-TCA) are well known off-flavours in drinking water. While Geosmin and 2-MIB are naturally occurring, 2,4,6-TCA originates from 2,4,6-Trichlorophenol (2,4,6-TCP). To ensure high water quality these compounds are monitored routinely.

As the odour threshold value for these compounds is very low, a highly sensitive and robust method is needed to monitor these off-flavours. This application describes the highly sensitive measurement of 2,4,6-TCA, 2-MIB, Geosmin, and 2,4,6-TCP at trace levels down to 1.33 ng/L by means of solid-phase microextraction (SPME) immersion technology.

Sample Preparation
A stock solution containing 1 µg/L of Geosmin, 2-MIB, 2,4,6-TCA, and 2,4,6-TCP in demineralized water was prepared. Sequential dilution was performed to create seven calibration levels from 1.33 ng/L to 100 ng/L. The pH-value of each sample was adjusted to 4 to prevent dissociation of 2,4,6-TCP. In addition, 5% (v/v) of methanol (MeOH) was added to prevent adsorption of the phenolic compounds to the glass walls of the vials. Sample preparation was done in 10 mL magnetic screw cap vials with a final volume of 7.5 mL.

Sample Measurement
Sample measurement was performed using the Shimadzu GCMS-TQ8050 NX equipped with AOC-6000 for automatic sample introduction. The SPME fibre was introduced after immersion into a split/splitless injector for desorption. Measurement parameters are summarized in Table 1.

Sensitivity
Method sensitivity was evaluated by determination of signal-to-noise ratio (S/N) at the lowest calibration level using a root mean square approach (range = 0.1 min). Figure 1 shows peak profiles at 1.33 ng/L. Table 2 gives the S/N ratios.

Calibration Data
Instrument calibration was performed using an external standard approach. Seven levels between 1.33 ng/L and 100 ng/L were measured for calibration and resulted in an $R^2$ value above 0.9990 for all compounds. Figure 2 shows the acquired calibration curves and the $R^2$ values.

Reproducibility
Reproducibility of the method was evaluated by three consecutive injections of the lowest standard at 1.33 ng/L.

Table 1: Analytical conditions

<table>
<thead>
<tr>
<th>Sampler</th>
<th>AOC-6000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>SPME Immersion</td>
</tr>
<tr>
<td>Fibre type</td>
<td>SPME DVB/PDMS/Carbon WR (P/N: 227-35316-01)</td>
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<td>Incubation time</td>
<td>5 min</td>
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<tr>
<td>Extraction time</td>
<td>25 min</td>
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<td>Extraction temp.</td>
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<td>Stirring speed</td>
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<td>Desorption time</td>
<td>3 min</td>
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<tr>
<td>Desorption temp.</td>
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<tr>
<td>Fibre cleaning</td>
<td>10 min at 270 °C</td>
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<td>GC–MS System</td>
<td>GCMS-TQ8050 NX</td>
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<tr>
<td>Injector</td>
<td>SPL-2030</td>
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<tr>
<td>Injector temp</td>
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<tr>
<td>Liner</td>
<td>SPME liner (P/N: 227-35322-01)</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Splitless</td>
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<tr>
<td>Linear velocity</td>
<td>35 cm/s</td>
</tr>
<tr>
<td>Column</td>
<td>SH-Rxi-5MS, 30 m, 0.25 mm, 0.25 µm (P/N: 221-75940-30)</td>
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<tr>
<td>GC oven program</td>
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<tr>
<td>Transfer line temp.</td>
<td>280 °C</td>
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<tr>
<td>Ion source temp.</td>
<td>200 °C</td>
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<tr>
<td>Emission current</td>
<td>150 µA</td>
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<tr>
<td>Ionization mode</td>
<td>EI, 70 eV</td>
</tr>
<tr>
<td>Acquisition mode</td>
<td>MRM</td>
</tr>
</tbody>
</table>

2-MIB

| 107.0>91.0 (15 eV) | 95.0>55.0 (15 eV) |
| 2-MIB |

2,4,6-TCA

| 212.0>197.0 (15 eV) | 212.0>169.0 (25 eV) |
| 2,4,6-TCA |

Geosmin

| 112.0>83.0 (15 eV) | 112.0>97.0 (15 eV) |
| Geosmin |

2,4,6-TCP

| 160.0>97.0 (21 eV) | 196.0>132.0 (12 eV) |
| 2,4,6-TCP |
Table 2: S/N ratios (RMS) at 1.33 ng/L

<table>
<thead>
<tr>
<th>Compound</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-MIB</td>
<td>11</td>
</tr>
<tr>
<td>2,4,6-TCA</td>
<td>303</td>
</tr>
<tr>
<td>Geosmin</td>
<td>72</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>420</td>
</tr>
</tbody>
</table>

Table 3: % RSD at 1.33 ng/L (three injections)

<table>
<thead>
<tr>
<th>Compound</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-MIB</td>
<td>5.2</td>
</tr>
<tr>
<td>2,4,6-TCA</td>
<td>3.4</td>
</tr>
<tr>
<td>Geosmin</td>
<td>4.8</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Figure 1: Peak profiles at 1.33 ng/L.

Table 3 shows excellent % RSD for all compounds at this low sample concentration.

**Conclusion**

Excellent sensitivity combined with a high linear dynamic range was shown for the detection of typical off-flavours in water samples. SPME technology in combination with the Shimadzu GCMS-TQ8050 NX leads to a very good reproducibility for this quantitative approach.

Figure 2: Calibration curves from 1.33 ng/L to 100 ng/L.

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- **Pink** Original, Non-buffered 10x ECMSSC50CT - QuEChERS 4g MgSO4 / 1g NaCl, 50mL CT
- **Green** AOAC 10x ECMSSA50CT - QuEChERS 6g MgSO4 / 1.5g NaOAc, 50mL CT
- **Orange** European 10x ECQUEU750CT - QuEChERS 4g MgSO4 / 1g NaCl / 500mg Na2Cit / 1g Na3Cit, 50mL CT

dSPE (2mL Centrifuge Tubes)
- **Pink**: 20x CUMPS2CT - QuEChERS 150mg MgSO4 / 50mg PSA, 2mL CT
- **Green**: 20x CUMC182CT - QuEChERS 150mg MgSO4 / 50mg C18, 2mL CT
- **Blue**: 20x CUMPSC18CT - QuEChERS 150mg MgSO4 / 50mg PSA / 50mg C18, 2mL CT
- **Brown**: 20x CUMPSGGC182CT - QuEChERS 150mg MgSO4 / 50mg PSA / 50mgChloroFiltr / 50mg C18, 2mL CT
- **Yellow**: 20x CUMPSC1875CB2CT - QuEChERS 150mg MgSO4 / 50mg PSA / 50mg C18 / 7.5mg GCB, 2mL CT

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