A NON-TARGETED APPROACH TO THE DEVELOPMENT OF A FOOD ADDITIVE CCS SCREENING LIBRARY AND ITS APPLICATION

Michael McCullagh[1]; Mike Wilson[1]; Severine Goscinny[2]; Jeff Goshawk[1]; Kenneth Rosnack[3]
[1]Waters Corporation, Wilmslow, United Kingdom; [2]Sciensano, Brussels, Belgium; [3]Waters Corporation, Milford, MA.

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

OVERVIEW

- **A non-targeted UPLC-MS ion mobility strategy to generate HDMS^E precursor ion, product ions and collision cross section (CCS) values to create a food additives MS library has been performed.**
- **The food additives library generated incorporates, sweeteners, food colourings, antioxidants and preservatives.**
- **Extracts of food and drink products labelled as containing food additives have been screened to test the robustness of the CCS library generated.**
- **Compared to the food additive CCS library, $^{TW}CCS_{N2}$ delta values of < 2% have been obtained routinely using both positive and negative ion modes.**

INTRODUCTION

Although the use of food additives is strictly regulated under various European Union (EU) acts,(1) national authorities have the responsibility to assure effective controls and monitor the consumption of food additives (FAs) within their respective populations.(2) To fulfil these two requirements, analytical methods are compulsory and have to be able to quantify these substances in various types of foodstuffs for a large number of items available in the marketplace. Many analytical applications are already successfully implemented, but generally cover very few additives and/or few food matrices. As a result, it is very challenging and expensive to control the levels in foods considering the large availability of products on the market. An approach to make the analytical process more efficient can be achieved through the development of a more versatile, high-throughput multi-method, which in turn can flexibly cover in one analysis the largest number of FAs. Such methods will promote better coverage of foods that are required to be controlled, and additionally can also be used for exposure assessment to multiple FAs providing a unique analysis per sample. Hence, the development of a multi-method is two-fold.

According to the EU legislation, FAs are "*any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value*". These substances are authorized by the European Commission after being subjected to a safety assessment by the European Food Safety Authority (EFSA) demonstrating no health hazard outcomes and if their use comply with the EU legislation conditions (e.g. technological need, the advantages and benefits for the consumers). Enforcement of the legislation through implementation of national food control systems, should ideally cover all food marketed within the country. Like-wise for risk assessment, the analysis of large numbers of products are necessary, to accurately estimate the daily intake of FAs. To deal with the increasing number of sample matrices and the large number of FAs (authorized/un-authorised), it is essential to develop effective and reliable analytical methods.

We have investigated the utility of mass spectrometry libraries incorporating a CCS metric. UPLC-IM comprises ion mobility (gas phase separation prior to MS analysis) coupled with UPLC (neutral species separation). The timescale UPLC (seconds), IMS (milliseconds) and time-of-flight MS (microseconds) are compatible with the requirement of high throughput analysis of complex samples. Ion mobility separation of compounds result from gas phase ions being separated within a gas filled travelling wave ion mobility (TWIM) RF ion guide of the mass spectrometer, prior to the mass analyser. Mobility separation is obtained by driving packets of ions through an inert buffer gas (typically nitrogen) using a relatively weak electric field. The resultant separation depends on factors such as the mass of the ion, charge and shape. It provides an added dimension of separation to that of LC (hydrophobicity) and MS (*m/z*), in addition to CCS (collision cross section), a complimentary identification metric.

Illustration of strategies to incorporate ion mobility CCS as an additional cumulative metric for pesticide screening assays have previously been illustrated.[3-5] The routine use of CCS for small molecule analysis has increased across multiple areas of research including pharma (metabolism, metabolomics, lipids), forensic toxicology, food safety (veterinary drugs, mycotoxins, steroids, steviol glycosides, natural product screening, natural toxins).[6-9] CCS searchable libraries have been generated, where use of a CCS metric can be used to increase cumulative specificity of identification as well decrease false detections.

Application of a non-targeted CCS library building strategy,[10] addition of a CCS metric to enhance specificity in combination with a multi-method for the analysis of FAs has been explored.

METHODS

UPLC-MS:

MS System: Waters Synapt G2-*Si*

Electrospray positive (ESI+) and ESI negative (ESI-). Desolvation Temperature:550°C. Acquisition Modes: HDMS^E. Mass Range: 50-1200 Da. Acquisition rate: 10 spectra/second. Capillary Voltage: 3 kV (+ve) and 2.2kV (-ve). Cone Voltage: 30V. Drift Gas: N_2. Collision Energy Ramp: 10-45 eV.

Default IMS screening parameters were utilised: IM resolution was ≈ 40 Ω/ΔΩ. IMS Wave Velocity Range: 650 m/s. IMS Wave Height: 40 V. IMS Gas Flow: 90 mL/min. Lockmass: leucine enkephalin ($C_{28}H_{37}N_5O_7$ (*m/z* 556.2766 for ESI+ and *m/z* 554.2620 for ESI-). CCS calibrant for $^{TW}CCSN_2$ calculations was performed using an IMS/ToF Calibration Kit (Waters Corp. UK).

FOOD COMMODITY ANALYSIS UPLC METHOD

LC System: Waters ACQUITY UPLC I-Class

For the analysis of substances relevant to food additives analysis, UPLC chromatographic separation was achieved using Waters ACQUITY UPLC HSS C18 column (150 mm × 2.1 mm, 1.8 µm).

Mobile phase (A): water with 10 mM ammonium acetate (0.1% formic Acid)
Mobile phase (B): methanol/acetonitrile (1:1) with 10 mM ammonium acetate (0.1% formic Acid).

UPLC gradient: Reverse phase separations (0.4 mL/min) at 45°C were performed using the gradient: 0–0.5 min isocratic at (95:5(A:B)); 6.0 min (0:100); 9.0 min (0:100) 9.5 min (95:5) 11.0 min (95:5). Injection volume:10 µL.

LIBRARY GENERATION UPLC METHOD

LC System: Waters ACQUITY UPLC I-Class
Column: Waters ACQUITY UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm)
Column temperature: 45°C Flow: 0.4 mL/min
Mobile phase: (A) water (0.1% formic Acid) (B) acetonitrile (0.1% formic Acid).
UPLC gradient: Reverse phase separations (0.45 mL/min) at 45°C were performed using the gradient: 0–0.14 min isocratic at (99:1 (A:B));0.42 min (85:15); 0.83 min (50:50) 1.25 min (95:5) 1.26 min (99:1) 2.5 min (99:1). Injection volume:10 µL.

Extraction and Sample Prepraration:

Food commodities screened for food additives: red fruits yoghurt (YB); strawberry yoghurt (YS); energy drink (D1); "zero" lemon drink (D2); "zero" strawberry and kiwi drink (D3); colourless tonic drink (D4); sparkling lemonade drink (D5). (zero=no added sugars).
Sample preparation.
Soft drinks: Dilution 10:1 and 100:1 (using H_2O).

Yogurt Extraction method: Yogurt samples (15 g) were weighed into 50-mL screw-cap centrifuge tubes (Waters, Milford, USA). A 10-mL volume of acetonitrile in 1% acetic acid was added as an extraction solvent and the tube then mixed vigorously for 1 min using a vortex mixer. Anhydrous $MgSO_4$(6 g) and sodium acetate (1.52 g) were added to the tube, to induce phase separation. Samples were immediately shaken for 1 min, and then centrifuged for 5 min at 1500 rcf at 4°C. Dispersive-SPE (dSPE) of the samples was carried out by pouring the supernatant (8 mL) into a centrifuge tube (50 mL) containing $MgSO_4$(1.2 g), PSA (410 mg) and C18 (404 mg). The sample was vortexed for 1 min and centrifuged for 5 min at 1500 rcf at 4°C.

DATA PROCESSING

All data processing was performed using UNIFI 1.9.2.

RESULTS AND DISCUSSION

A collision cross-section library for LC-MS amenable food additives has been developed using the non-targeted library generation protocol. The strategy applied, determines the most applicable ionisation mode, precursor ion/product ions and collision cross section values. The library generated contains a CCS metric for food additive classes colourings, preservatives, antioxidants and sweeteners.

Seven food samples labelled as containing a variety of food additives including sweeteners, preservatives and food colourings, were purchased from Belgian supermarkets. The sample analysis was used to test the robustness of the CCS library generated. UPLC HDMS^E data was acquired in positive and negative ion modes, enabling comparison of the precursor ion, ion mobility product ions and CCS of the food additives library.

For food commodity D2 (lemon soft drink), three sweeteners (neohesperidin, acesulfame and sucralose) and a food preservative (citric acid) were correctly detected and identified. The corresponding negative ion HDMS^E precursor ion/mobility product ion spectra and CCS values are presented in Figure 1. No food colourings were detected.

A food preservative and sweeteners were (citric acid, aspartame, acesulfame K, and sodium cyclamate) detected in food commodity D3 (strawberry and kiwi drink). The detection results are presented in Figure 2, with the corresponding negative ion HDMS^E ion mobility precursor/product ion spectra for aspartame. No false detections were obtained for the food commodities screened using the food commodity CCS MS library generated. To further illustrate the robustness of the library food commodities analysed were also spiked with a series of food additive colourings and sweeteners.

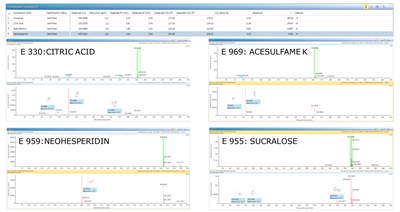

Figure 1. Negative ion HDMS^E precursor/product ion spectra for sweetener and preservative FAs detected in a lemon soft drink (D2).

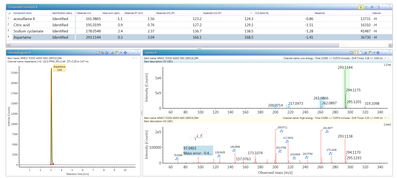

Figure 2. Negative ion HDMS^E precursor/product ion spectra for aspartame detected in a strawberry and kiwi soft drink (D 3). Colouring and sweeteners identified.

Food commodity D4 is a colourless tonic drink for which it would be expected that that no food colouring additives would be detected, as was the case as can be seen from Figure 3 where the negative ion HDMS^E precursor/product ion spectra for acesulfame K (E969) is shown and as well as detection of E 330 and E 955. The soft drink (D4) was spiked with a series of additional sweeteners and colourings for which the component summary detection results are shown in Figure 4, where the illegal sweeteners, alitame and glycyrrhizin were correctly detected.

Figure 3. Negative ion HDMS^E precursor/product ion spectra for acesulfame K detected a colourless tonic drink (D 4). Additives E 330, E 955 and E969 identified.

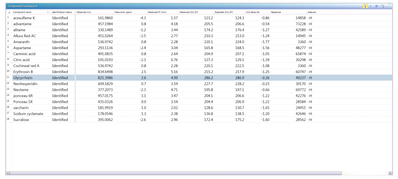

Figure 4. Negative ion HDMS^E detections for additional legal/illegal food additives spiked into a colourless tonic drink (D4), illustrating retention time, accurate mass measurement and expected/observed $^{TW}CCSN_2$.

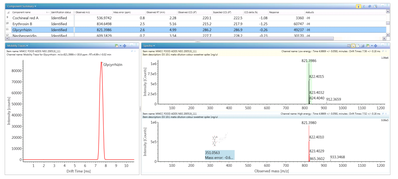

Figure 5. Negative ion HDMS^E precursor and ion mobility product ion spectra for illegal glycyrrhizin food additive spiked into a colourless tonic drink (D 4). Observed $^{TW}CCSN_2$=286.2Å^2 (CCS delta= -0.26%).

It s possible for some analytes more than one CCS value may be observed as reported for the discovery of pesticide and fluoroquinolone protomer's (charged isomers, where more than one site of ionisation is observed).(4,7) Two ion mobility separated species have been observed for the same isomeric mass measurement for tartrazine (209.2 Å^2 and 217.2 Å^2) and neohesperidin (238 Å^2 and 228.2 Å^2). The pairs of ion mobility separated species are presented in Figures 6 and 7. Each respective pair of ion mobility separated species produced matching product ion spectra, indicating that conformers may have been observed (further investigations to be performed). It is the case that whether protomer's, conformers or epimers result in two observed ion mobility species, two CCS values can be added to library database to further increase cumulative specificity for the analytes concerned.

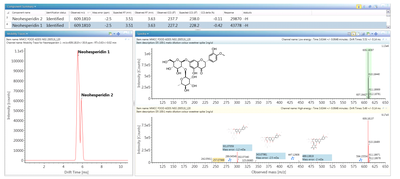

Figure 6. Negative ion HDMS^E precursor/product ion spectra for neohesperidin food additive, where two mobility separated species have been observed ($^{TW}CCS_{N2}$ = 228.2 and 238 Å^2).

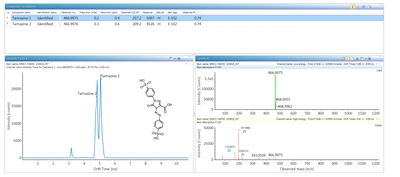

Figure 7. Negative ion HDMS^E precursor and ion mobility product ion spectra for food additive library constituent tartrazine, where two mobility separated species have been observed ($^{TW}CCS_{N2}$ = 209.2 and 217.2 Å^2).

CONCLUSION

- **A non-targeted ion mobility mass spectrometry library building strategy has been used to generate a food additives library comprised of sweeteners, preservatives, antioxidants and food colourings.**
- **The CCS library building strategy has been shown to be robust and has been applied to the screening of food commodities containing food additives; no false detections were observed.**
- **Stress testing of the food additives library generated, has resulted in the correct detection of legal/illegal food additives, within a 2% CCS tolerance.**
- **The use of a CCS metric offers the potential to reduce the initial specificity of applied screening parameters and reduce false detections in complex matrices, where generic extraction methods have been utilised.**
- **CCS of the food additives can be used as a confirmatory metric to increase confidence in identification.**
- **Food additive charged isomer/conformer species have been identified for the first time, where two CCS values can be used to increase cumulative specificity in analyte identification.**

References

1. Regulation (EC) No 882/2004.

2. Directives 94 / 35 / CE (Article 8), 94 / 36 / CE (Article 6) and 95 / 2 / CE (Article 7).

3. A novel approach to the reduction of false positive and negative identifications in screening of pesticide residues in food analysis. S. Goscinny and M. McCullagh. Proceedings of the 61st ASMS Conference on Mass Spectrometry and Allied Topics, Minneapolis, Minnesota. June, 2013.

4. Discovery of pesticide protomers using routine ion mobility screening. M. McCullagh, and S. Goscinny. *Waters Appl. Note* 2014, *720005028E*, 1–7.

5. Towards the use of ion mobility mass spectrometry derived collision cross section as a screening approach for unambiguous identification of targeted pesticides in food. S. Goscinny, M. McCullagh, J. Far, E. De Pauw and G. Eppe. *Rapid Commun. Mass Spectrom*. 2019;1–15.

6. Investigations into cross-platform and long-term robustness of a ccs metric. M. McCullagh, M. Wood, N. Mistry, S. Goscinny, D. Douce and P. Dalsgaard. 67th ASMS Conference on Mass Spectrometry and Allied Topics, Atlanta, GA, June 2-6, 2019.

7. Investigations into the performance of travelling wave enabled conventional and cyclic ion mobility systems to characterise protomers of fluoroquinolone antibiotic residues M. McCullagh, K. Giles, K. Richardson, S. Stead and M. Palmer. *Rapid Commun Mass Spectrom*. 2019;1–11.

8. Use of ion mobility mass spectrometry to enhance cumulative analytical specificity and separation to profile 6-*C*/8-*C*glycosylflavone critical isomer pairs and known–unknowns in medicinal plants. M McCullagh, C. A. M. Pereira and J. H. Yariwake. *Phytochemical Analysis*. 2019;1–13.

9. Exploring the complexity of steviol glycosides analysis using ion mobility mass spectrometry. M. McCullagh, D. Douce, E. Van Hoeck and S. Goscinny. *Anal. Chem.* 2018; 90,4585−4595.

10. The Development Of A Natural Products Library Using Ion-Mobility Enabled Mass Spectrometry. J. Goshawk, Gitte Barknowitz and M. McCullagh. 67th ASMS

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS