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PEER REVIEW

GC×GC–TOF-MS and Comprehensive Fingerprinting of Volatiles in Food: Capturing the Signature of Quality

The strategic role and potential of comprehensive GC×GC combined with TOF-MS and pattern recognition using template matching for data processing to unravel the quality traits of high-quality food products is investigated.

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INTERVIEW

The Rising Role of Foodomics

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Read more: ***http://bit.ly/2msRG0s***

NEWS

Investigating the Effect of Kynurenic Acid in Alcoholic Beverages

Researchers from the Medical University of Lublin, in Poland, have investigated the presence of kynurenic acid (KYNA) in alcoholic beverages and the significance of its presence using HPLC.

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Improving Extraction Processes and Sample Preparation in Food Analysis

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Ion Mobility–Mass Spectrometry for Food Analysis: An Update

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In food analysis, many different biological matrices are investigated containing numerous compounds that can interfere with liquid chromatography–mass spectrometry (LC–MS) analysis. To overcome the challenges that arise with these highly complex matrices, the additional separation of analytes and matrix compounds complementing chromatographic separation is becoming more significant. In this article, the potential of ion mobility–mass spectrometry (IM-MS) to increase selectivity and for additional identity confirmation is investigated. An extensive evaluation of IM-MS instruments was performed on a broad test set of food safety contaminants. The tested IM-MS platforms were differential ion mobility spectrometry (DMS), travelling-wave ion mobility spectrometry (TWIMS), low field drift tube ion mobility spectrometry (DTIMS), and trapped ion mobility spectrometry (TIMS). Collision cross section (CCS) data were determined using the different instruments, and the ability to separate isomers and compounds of interest from sample matrix in the IM dimension was explored.

The field of food quality and safety analysis deals with many different biological samples, such as feed, urine, milk, and tissue. These complex matrices contain compounds that interfere with the analysis and cause challenges for identification and quantification of regulated compounds. The effect of interfering compounds can be minimized by application of highly selective separation techniques, such as gas chromatography (GC) and liquid chromatography (LC) (1,2). Nevertheless, the influence of the matrix may not be completely removed by the chromatographic separation when complex samples are considered.

A non-chromatographic technique offering possibilities for additional separation is ion mobility (IM). Ion mobility has also been coupled to, or fully integrated in, mass spectrometry (MS) instrumentation. The resulting ion mobility–mass spectrometry (IM-MS) instruments enable more in-depth investigation of ions using both ion mobility and mass spectrometric data. Ion mobility spectroscopy (IMS) has been used since the 1950s to study the mobility of ions in the gas phase and to investigate ion–molecule reactions of volatile compounds (3). A typical, drift tube-like, ion mobility system consists of a drift tube through which ions are transported using a weak electric field while a so-called *drift gas* is effectively static or flows in the opposite direction of ion transport. The mobility (K) of an ion can be determined by measuring the residence time of an ion in the drift tube (referred to as a *drift time*) and relating this to the applied field strength (V/m). The drift time is influenced by the shape, size, and charge of an ion, and also the interaction potential with the buffer gas. The more compact the shape and size of an ion is, the shorter the drift time (4). An experimentally obtained drift time of a compound can be used to calculate the rotationally averaged collision cross section (CCS, Ω), which is a two-dimensional (2D) representation of the ion's three-dimensional (3D) structure (5). While drift times are instrument- and condition-dependent, CCS values should be intrinsic properties of ions in a given buffer gas. Thus, when comparing the same ion species and confirmation, agreement across different IM techniques and conditions should be possible (6). The CCS of an ion of known mass-to-charge can be calculated using drift-tube ion mobility spectrometry (DTIMS) following the Mason-Schamp equation (7):

$$\Omega = \frac{3ze}{16N}\left(\frac{2\pi}{\mu k_B T}\right)^{1/2}\frac{1}{K_0} \qquad [1]$$

in which z is the charge state of the analyte ion, e is the charge of an electron (fundamental charge), N is the number density of the drift gas, μ is the reduced mass of the ion-neutral pair, k_B is the Boltzmann constant, T is the absolute gas temperature, and K_0 is the reduced mobility (the mobility

normalized to NIST standard temperature and pressure) (7,8). Structural isomers can have different CCS values and are therefore amenable for separation using IMS (9–12). Furthermore, confidence in analyte characterization can be increased by adding CCS as an additional identification point to support other identification points as retention time and MS parameters (13).

The IM principles can be subdivided into selective, separation-in-space, techniques; differential mobility spectrometry (DMS) and field-asymmetric ion mobility spectrometry (FAIMS), and dispersive, separation-in-time, techniques; DTIMS, travelling-wave ion mobility spectrometry (TWIMS), and trapped ion mobility spectrometry (TIMS) (8,12,14). While previous limitations have included the confinement of IM-MS instruments to research environments, recent developments of instruments has opened up possibilities to support applications involving analysis of small molecules in real samples. IM-MS can, for example, be used to separate analytes from isobaric interferences and thus decrease the limit of detection by improving the signal-to-noise (*S/N*) ratio (15–18,14).

Recently, a review article by Hernández-Mesa *et al.* (14) was published highlighting the most important developments, achievements, and limitations of IM-MS in food safety research. However, no current, experimental evaluation of the possibilities of IM-MS instruments has been published until now. This article strives to show both the potential of IM-MS in food safety and the limitations of IM-MS instruments in terms of analytical performance. Several mass spectrometers with ion mobility cells have therefore been evaluated for their ability to separate isomers and reduce matrix interferences.

FIGURE 1: DTIMS (left) and TWIMS (right) spectra of a mixture of cis- and trans-DES.

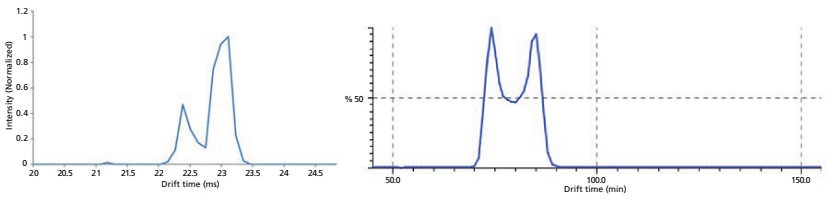
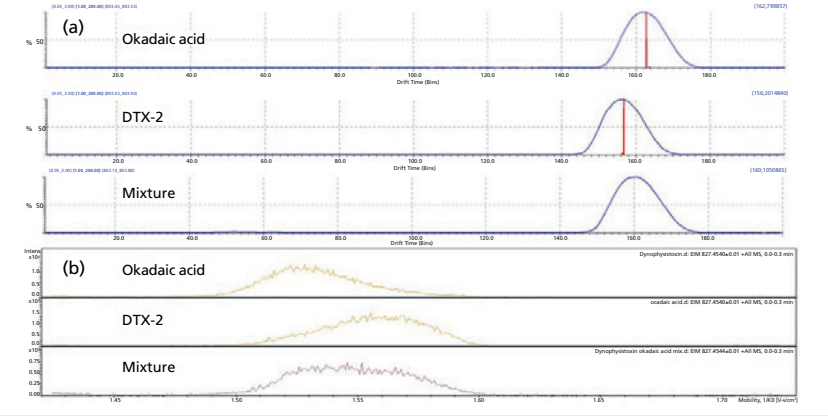


FIGURE 2: Mobility spectra of okadaic acid (top), DTX-2 (middle), and as mixture (bottom) on an (a) TWIMS and (b) TIMS instrument.



Experimental Conditions

DIMS: MS Settings: A QTRAP 6500 MS/MS system (Sciex) equipped with an electrospray ionization (ESI) source and a SelexION ion mobility cell was used in selected ion monitoring (SIM) mode at the specific mass-to-charge ratio (*m/z*) of each investigated compound. The heater gas temperature was 500 °C and the ion spray voltage was 5500 V. Regarding the mobility cell, the compensation voltage (COV) was scanned to obtain COV scans while different settings were varied to study the effect on the mobility separation: separation voltages (SV) ranged from 0 up to 4500; DIMS resolution enhancement (DR) was set to either off, low, medium, or high; and the use of 2-propanol as modifier with modifier composition (MDC) set to low or high.

DTIMS: MS Settings: Measurements were made using an Agilent 6560 IMS-QTOF mass spectrometer using ESI (Agilent G1607A dual Jetstream) and with electronic drift gas pressure control. Nitrogen was used as drying gas at a temperature of 360 °C, and a sheath gas flow rate of 13 L/min at a temperature of 225 °C. The MS capillary voltage was 3500 V, the nozzle voltage was 500 V with the nebulizer gas pressure set to 30 psi, and the fragmentor was set to 275 V. The scanned mass range was *m/z* 50 to 1700. The instrument was calibrated prior to measurements using the supplied tune mixture of the manufacturer and the mass spectrometer was tuned in the 2 GHz extended dynamic range mode. A trapping time of 10,000 µs was used for the IM separation with packages of ions released every 60 ms using a trap

FIGURE 3: (a) DTIMS spectrum of $[M+H]^+$ ions of levalbuterol and isoetharine; (b) DTIMS spectrum of sodium adducts of levalbuterol and isoetharine; (c) TWIMS spectrum of $[M+H]^+$ ions of GTX-2 and GTX-3; (d) TWIMS spectrum of sodium adducts of GTX-2 and GTX-3.

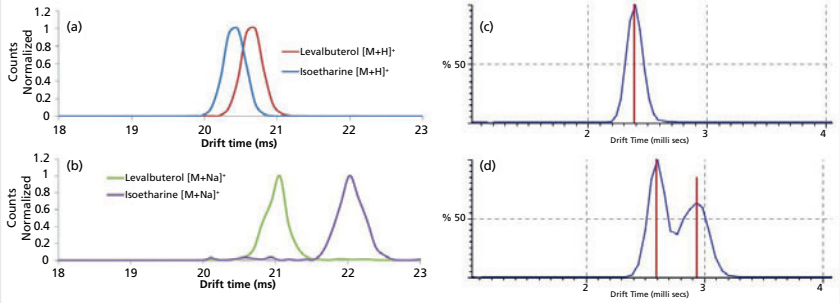


FIGURE 4: Mobility spectra of para-CAP and meta-CAP on an (a) DIMS ($[M-H]^-$), (b) TIMS ($[M+Na]^+$), (c) DTIMS ($[M-H]^-$), and (d) TWIMS ($[M+Li]^+$) instrument.

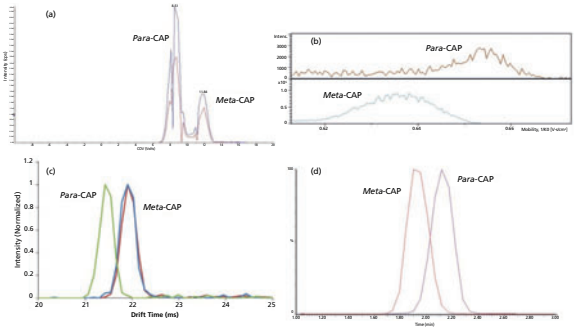
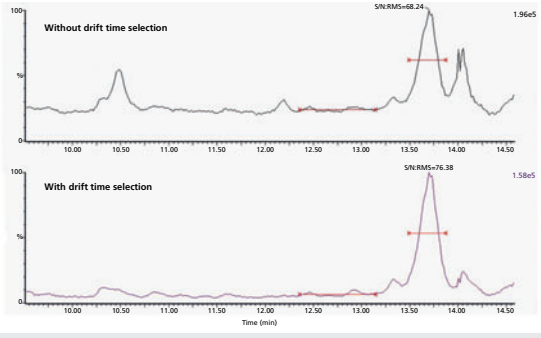


FIGURE 5: Extracted ion chronograms (EICs) of *m/z* 416.1 (+/- 0.5 Da) without (top) and with (bottom) drift time selection of 3.45 ms (+/- 0.2 ms).



release time of 150 µs. The drift tube was filled with nitrogen and operated with an absolute entrance voltage of 1574 V and an exit voltage of 224 V with a drift tube pressure of 3.95 Torr and a temperature of 25 °C. CCS calibration was performed in the single field mode using established conditional reference values from a recent interlaboratory study (19).

TIMS: MS Settings: A TIMS-TOF (Bruker Daltonics) equipped with ESI was used to obtain full scan measurements in positive and negative mode with a scan range of *m/z* 100–1000. The capillary voltage was 3200 V, the end plate offset was -500 V, the dry gas flow was 3.0 L/min, the dry heater was set to 200 °C, the nebulizer was at 0.3 bar, and the collision energy was 5.0 eV. The TIMS operation mode was set initially to survey mode where after TIMS settings (TIMS voltage range and accumulation time) were optimized for each ion to obtain optimal resolution in a narrow $1/K_0$ range. CCS calibration and calculation was performed using Compass data analysis with Agilent tuning mix as calibrant (*m/z* 322.0481 reference mobility 0.737; *m/z* 622.0290 reference mobility 0.994; *m/z* 922.0098 reference mobility 1.212).

TWIMS: MS Settings: A Synapt G2-S MS system (Waters) equipped with ESI was used to obtain full scan measurements in positive and negative mode with a scan range of *m/z* 200–900 in resolution mode. The source temperature was 150 °C, the capillary voltage was 3.0 kV, and the cone voltage was 20 V. The TWIMS cell was operated at a nitrogen gas flow of 90 mL/min, a wave velocity of 650 m/s, and a wave height of 40 V. CCS calibration and calculation was performed using DriftScope v2.7 (Waters) with polyalanine as calibrant.

LC–MS Method: For the implementation of IM into an LC–MS method, an Acquity ultra-performance liquid chromatography (UPLC) system (Waters) was coupled to a Synapt G2-S MS (Waters). A 4.6 mm × 150 mm, 5-µm Zorbax Eclipse XBD-C8 column (Agilent Technologies) was used for separation. Mobile phase A consisted of 0.2% formic acid in purified water, and mobile phase B of 0.2% formic acid in methanol. The column temperature was 50 °C, the flow rate 0.4 mL/min and an injection volume of 10 µL was used. Gradient elution was as follows: 0.5 min of isocratic elution at 100% A, followed by a gradient to 80% B in 1 min, to 90% B in the next 8.5 min, and finally to 100% B in 3 min. After 5 min at this state, the system

was re-equilibrated for 5 min at 100% A. Full scan IM-MS measurements with a scan range of *m/z* 200–900 in resolution mode were performed using a source temperature of 120 °C, capillary voltage 3.5 kV, and cone setting 30.

Results and Discussion

Test Set: To be able to assess the performance of IM-MS instruments for food safety applications, a test set of over 20 compounds was prepared. The test set of compounds represent a wide range of contaminants, toxins, and veterinary drugs that can be present in food or be used in the food industry, with low molecular weights (<1000 Da). Depending on their characteristics and availability of isomers, not all compounds in the test set were used for every aspect of this research: some compounds were mainly used for assessment of CCS determination under repeatability conditions of measurement, while others were used to determine isomer separation efficiency.

CCS Determination: Experimental CCS values of individual compounds can be determined using different strategies, such as using a primary method of measurement (stepped-field DTIM-MS), or using a secondary method following external calibration of the IM-MS instrument using a calibration mixture with best estimates of CCS values. In this research, only secondary approaches were used; CCS values were determined using a single-field DTIMS instrument and using a TWIMS instrument. Primary measurement of CCS values is only possible on a static drift tube instrument when all experimental parameters are well characterized, allowing the propagation of arrival times at different field strengths to be converted to CCS values (20). Previous work has shown excellent agreement (average bias <1%) between a primary stepped-field and a secondary single-field DTIM-MS method based on a derivation of the Mason Schamp equation (19). Indirect measurement is also performed using TWIMS or TIMS instruments using CCS values from DTIM-MS instruments as calibrants. However, unlike DTIMS, drift times obtained from TWIMS instruments cannot be directly converted into CCS values since the electric field in the TWIMS cell is constantly changing and thus no direct relationship between the obtained drift time and CCS can be made (21). The TWIMS drift times are therefore very much instrument- and parameter-dependent, making calibration of TWIMS instruments using a mixture containing calibrants with consensus CCS values crucial to obtain reliable CCS values (6).

It should be noted that no CCS calculation was performed using the TIMS and DIMS instruments. At the time when the TIMS instrument was used, CCS values could not yet be routinely obtained. This feature was only added in later software versions (22), and so could not be tested in this research. The correlation between CCS and K_0, and thus the possibility to calculate CCS from drift times, is only valid when the ratio between electric field strength and buffer gas density is small. In DIMS and FAIMS instruments, however, the electric field strength is larger, making it not possible to calculate CCS from experimental drift times. For this reason, the ion mobility cell in DIMS and FAIMS instruments is mainly used as a filter to separate analytes or remove undesirable interferences (8).

TABLE 1: Experimental, both DTIMS and TWIMS, CCS values, and deviation of tested compounds

Compound	Detected Ion	$^{DT}CCS_{N2}$ (Å²)	$^{TW}CCS_{N2}$ (Å²)	Δ_{CCS} (Å²)	% Difference
Intermedine	$[M+H]^+$	170.0	169.9	0.1	0.1%
	$[M+Na]^+$	171.8	174.6	2.8	1.6%
Okadaic acid	$[M+Na]^+$	285.0	291.6	6.6	2.3%
DTX-2	$[M+Na]^+$	281.9	289.9	8.0	2.8%
GTX-2 / GTX-3	$[M-H]^-$	179.6	177.8	1.8	1.0%
Clenpenterol	$[M+H]^+$	167.1	168.4	1.3	0.7%
	$[M+Na]^+$	180.6	181.9	1.3	0.7%
Procaterol	$[M+H]^+$	170.7	170.7	0.0	0.0%
Clenbuterol	$[M+Na]^+$	177.7	179.3	1.6	0.9%
	$[M+H]^+$	164.2	162.8	1.4	0.9%
α-zearalenol	$[M-H]^-$	180.0	175.9	4.1	2.3%
	$[M+Na]^+$	179.5	181.9	2.4	1.3%
β-zearalenol	$[M-H]^-$	179.0	175.3	3.8	2.1%
	$[M+Na]^+$	181.0	184.9	3.9	2.1%
RR-*p*-CAP	$[M-H]^-$	166.4	162.2	4.2	2.6%
	$[M+Na]^+$	169.3	175.8	6.5	3.8%
SS-*p*-CAP	$[M-H]^-$	166.5	163.4	3.1	1.9%
	$[M+Na]^+$	169.8	175.1	5.3	3.1%
Erythro-*m*-CAP	$[M-H]^-$	163.1	161.4	1.7	1.0%
	$[M+Na]^+$	171.8	168.7	3.1	1.8%
Clenhexerol	$[M+H]^+$	173.7	184.1	10.4	5.8%
	$[M+Na]^+$	187.7	184.7	3.0	1.6%
Clen-iso-hexerol	$[M+H]^+$	170.3	174.6	4.3	2.5%
	$[M+Na]^+$	185.2	186.9	1.7	0.9%
Isoetharine	$[M+H]^+$	156.8	157.0	0.2	0.1%
	$[M+Na]^+$	168.2	168.1	0.1	0.0%
Levalbuterol	$[M+H]^+$	158.5	157.6	0.9	0.5%
	$[M+Na]^+$	160.4	159.9	0.5	0.3%
Dobutamine	$[M+H]^+$	169.0	166.4	2.6	1.6%
	$[M+Na]^+$	167.4	163.6	3.8	2.3%
Erythro-isoxsuprine	$[M+H]^+$	172.3	179.1	6.8	3.9%
	$[M+Na]^+$	182.6	185.9	3.3	1.8%
Formoterol	$[M+H]^+$	179.4	187.5	8.1	4.4%
Phenylethanolamine A	$[M+H]^+$	175.1	194.9	19.8	10.7%
	$[M+Na]^+$	185.0	192.1	7.1	3.7%
Ractopamine	$[M+H]^+$	172.2	173.1	0.9	0.5%
	$[M+Na]^+$	178.2	180.0	1.8	1.0%
Isoxsuprine	$[M+H]^+$	172.5	171.0	1.5	0.9%
	$[M+Na]^+$	182.6	186.3	3.7	2.0%

Accuracy of CCS Determination:

CCS values for 22 compounds were obtained using both DTIMS and TWIMS instruments. The experimental CCS values are listed in Table 1. Compounds were measured in both positive and negative mode, and some compounds were additionally detected as sodium adduct.

As can be noted from Table 1, the difference in experimental CCS values obtained using instruments from different IM principles is generally lower than 3% for the large majority (82%) of the detected ions. The obtained CCS values differed by more than 10% for only one compound (phenylethanolamine A). The correlation found between DTIMS and TWIMS experimental CCS values is also in good agreement with literature reports. In several publications (11,23,24,21), deviations between DTIMS and TWIMS CCS values in the order of a few percent have been reported. In individual cases, however, larger differences could occur, as observed both in previous literature (23,21) and in this study. Possible explanations for this phenomenon is the occurrence of different ion conformations or even ion species in DTIMS and TWIMS experiments caused by protonation site isomers ("protomers") (25), or the mismatching of calibrants with the investigated structures in the case of TWIMS. The latter issue is described by Hines *et al.*, who demonstrated that selecting a suitable calibrant for the compounds of interest can be significant because the use of mismatched calibrants can result in larger CCS errors (26). The observation that not all compounds yield similar CCS values in DTIMS and TWIMS instruments shows that care has to be taken when identity confirmation of a compound relies on CCS data obtained using a different type of IM-MS instrument than the reference value.

Precision of CCS Determination Under Intermediate Precision Conditions of Measurement Using TWIMS: The precision of CCS determination under intermediate precision conditions of measurement was also explored. A smaller test set comprised of eight compounds with *m/z* ranging from 220 up to 803 was measured on seven different days using a TWIMS instrument. The instrument was CCS calibrated using polyalanine at the start of each day, polyalanine was also used as reference calibrant for CCS calculation using reference nitrogen CCS values. The resulting CCS values and relative standard deviations (RSDs) can be found in Table 2.

TABLE 2: Experimental CCS values obtained on seven different days using a TWIMS instrument and their RSDs

Compound	*m/z*	Average Experimental CCS (Å², n = 7)	RSD
Cimaterol	220.1450 ([M+H]⁺)	154.7	0.4%
Clenbuterol	277.0874 ([M+H]⁺)	162.8	0.6%
Isoxsuprine	302.1756 ([M+H]⁺)	174.6	0.6%
THC	315.2324 ([M+H]⁺)	187.7	0.4%
RR-*p*-CAP	321.0045 ([M-H]⁻)	160.7	1.1%
THC-COOH	343.1909 ([M-H]⁻)	195.4	0.6%
Salmeterol	416.2801 ([M+H]⁺)	210.0	0.3%
Okadaic acid	803.4582 ([M-H]⁻)	310.8	0.9%

All CCS values could be determined with a repeatability precision below 1%, with the exception of RR-*p*-CAP (1.1%). The found repeatability was in good consensus with the literature with several studies reporting CCS repeatability precision below 2% (27–29,6,30). While not investigated in this research, several other studies have reported CCS data on different instrument classes to be highly reproducible on instruments located in different laboratories, with interlaboratory relative standard deviations (RSDs) below 3% (31,19). The excellent repeatability of the CCS values obtained using TWIMS in this study support the notion that CCS can be used as a reliable identification point for the investigated compounds.

Isomer Separation: To evaluate the separation power of the different IM-MS concepts, several compounds were selected from the test set based on the availability of isomers and structural differences between those isomers. Even though most of these isomers can be separated using chromatography, the rise of ambient and direct ionization techniques, capable of high-throughput screening without chromatography, has evoked an interest in non-chromatographic separation of isomers. The isomers were first analyzed by IM-MS separately to determine the drift times and separation potential. When a notable difference was observed between the drift times of the isomers, the isomers were measured as mixtures to investigate the separation.

The separation of cis- and trans-diethylstilbestrol (DES) was investigated. Benigni *et al.* reported a CCS difference of 3 to 5 Å², and separation of the two isomers on a TIMS instrument with an ion mobility resolution of 70–120 (32). DES isomers were analyzed on the DTIMS, TWIMS, and DIMS instruments, where the latter did not result in any separation. DES isomers are preferably analyzed in negative mode and were thus not analyzed on the TIMS instrument since it could only be used in positive mode at that time. Using DTIMS and TWIMS, the DES isomers were clearly separated (Figure 1). The observed peak-to-peak resolution was below 1 for both DTIMS and TWIMS measurements, which is in good correspondence with the reports of Causon *et al.* stating that for a peak-to-peak resolution of 0.6, at which two components can be reliably determined, a CCS difference of 1.5–1.8% is needed. To obtain baseline separation with a peak-to-peak resolution of 1.5, a CCS difference of at least 3.7–4.4% is required (33), as previously described by Dodds *et al.* (34).

The necessity of a CCS difference of more than just a few percent to separate isomers on current IM-MS instruments was confirmed when analyzing compounds with limited structural differences, such as pyrrolizidine alkaloids and the marine toxins okadaic acid and DTX-2. No significant CCS difference was observed between the investigated pyrrolizidine alkaloids, while okadaic acid and DTX-2 yielded a minor difference of around 2.5% (310.8 and 303.2, respectively). Figure 2 shows okadaic acid and DTX-2 analyzed separately and as a mixture. While some difference in drift times could be noticed between the individual compounds, the mixture yielded only one coalesced peak in the ion mobility dimension.

While limited fine-tuning of the IM parameters was possible in the DTIMS, TWIMS, and TIMS instruments (at the time of this study), fine-tuning was possible with the DIMS instrument. While the other investigated techniques are incorporated into the MS instrument ion pathway, the DIMS mobility cell is placed in the ion source before the MS inlet and works as a filter based on mobility. The amplitude of

the separation voltage waveform, the residence time of the ions in the mobility cell, and the use of a modifier were most effective to achieve better separation. Higher separation voltages resulted in the somewhat increased separation of a mixture of four β-agonists with *m/z* varying from 220 to 416, mainly between the lowest mass compound, cimaterol, and the other three compounds (clenbuterol, isoxsuprine, and salmeterol). Longer residence time of the ions in the mobility cell resulted in narrower peaks, and thus an increase in IM resolution. The isobaric β-agonists clenpenterol and procaterol were used to test the separation power of the DIMS mobility cell. Although these isobars can be separated on a high-resolution MS instrument, mass separation is not possible on lower resolution MS instruments since their *m/z* difference is only 0.067 Da. While no separation was achieved using standard IM settings, the combination of a high separation voltage, 4000 V, and use of 2-propanol as modifier resulted in an almost baseline separation. Since clenpenterol contains two chlorine atoms, it has a $^{35}Cl^{37}Cl$-peak (*m/z* 293) at 65% of the $^{35}Cl_2$-peak (*m/z* 291). This phenomenon is clearly visible in the resulting scan: *m/z* 291 shows two peaks (both clenpenterol and procaterol), while *m/z* 293 shows only one major peak (clenpenterol). While these results look very promising, it should be noted that a more than 100-fold reduction of signal intensity was observed when applying these radical settings, thus hampering practical use of DIMS when high sensitivity is required.

Even though mobility spectra of structurally very similar isomers can show a detectable difference when analyzed separately, a mixture will often yield a single peak only as a result of the insufficient separation power of the IM-MS instrument. At this time, state-of-the-art chromatography is still a better option in most cases for separation of compounds with a small CCS difference. The investigated pyrrolizidine alkaloids, for example, could all be resolved using a 2D-LC method (35). Nevertheless, recent publications about novel high-resolution IM-MS designs showed very impressive preliminary results regarding mobility separations by use of structures for lossless ion manipulations (SLIM) (36) or a multi-pass cyclic ion mobility separator instrument (37). Here, very long ion pathways are created, raising the ion mobility resolution to such a degree that could be the key to lifting IM-MS to even higher levels, opening up the ability to separate isomers with very small CCS differences.

Another possibility to increase IM separation is the promotion of adduct formation. From the literature it is known that cationic adduct formation has the potential to improve the separation of isomers that are otherwise not separated (38–42). In this study, the effect of sodium cationization on IM separation was further investigated with the same isobaric β-agonists as described above: clenpenterol and procaterol. While protonated ions did not show any separation in the ion mobility dimension, sodium adducts showed a clear difference in drift time and were almost baseline separated. The effect of increased separation using sodium cationization was further observed for several other isomer pairs. The β-agonist isomers, levalbuterol and isoetharine, showed minor separation in their protonated form, but baseline separation as sodium cations (Figure 3[a] and 3[b]). Attempts to separate stereo-isomers gonyautoxin-2 (GTX-2) and gonyautoxin-3 (GTX-3), two marine neurotoxins, were previously described by Poyer *et al.* (5). While non-sulfated saxitoxin analogues could be separated on a TWIMS instrument, no separation was observed for GTX-2 and GTX-3. This finding was confirmed in this study, where no separation of the marine toxins was detected when analyzed as protonated molecules (Figure 3[c]). Promotion of sodium adduct formation by the addition of sodium acetate to the sample, however, resulted in separation of GTX-2 and GTX-3 as $[M+Na]^+$ ions (Figure 3[d]). Since these isomers were only available as mixture, it was not possible to assign the two peaks. Although no baseline separation was achieved, observed peak-to-peak resolution was approximately 0.8, with the obtained separation a huge improvement compared to the unseparated $[M+H]^+$ ions. Both from the literature (41) and from the data presented here, no general trend or rule for whether or not adduct formation will result in additional IM separation could be derived. The investigation of individual isomer pairs of interest is therefore inevitable but can result in the extra separation needed.

An interesting test case for IM-MS from food safety practice was the separation of different CAP isomers. Eight isomers of CAP exist, two positional isomers (*para*- and *meta*-CAP) with four stereoisomers each (RR-, SR-, RS-, and SS-CAP), but only one of those isomers has antimicrobial activity (RR-*p*-CAP) (43,44). In line with the findings presented above, no separation was observed between the stereoisomers (see Table 1) because their structural differences are very minor. *Para*- and *meta*-CAP, however, could be separated to a certain extent on each IM-MS instrument tested (Figure 4). Where the DTIMS instrument was able to separate the structural isomers ($[M-H]^-$, Figure 4[c]), a high separation voltage was needed on the DIMS instrument (Figure 4[a]). The TIMS

instrument was, as mentioned before, only used in positive mode and thus the separation of sodium adducts is shown in Figure 4(b). TIMS works with $1/K_0$ spectra, meaning that the spectra are reversed compared to direct drift time spectra, and sodium cationized *para*-CAP therefore had a shorter drift time than sodium cationized *meta*-CAP. On the TWIMS instrument, only separation for lithium adducts was observed (Figure 4[d]). Lithium cationized *meta*-CAP exhibited a shorter drift time compared to *para*-CAP, which is most likely caused by lithium forming a more compact adduct with *meta*-CAP.

Value of Additional IM Separation of Signal from Matrix: LC–MS: The additional value of IM to existing methods was investigated by adding IM to an LC–MS method. Extracted animal feed samples were analyzed on the TWIMS-MS instrument in full scan mode. The obtained data was afterwards evaluated with only *m/z* selection, and after additional drift time selection. The *m/z* window was set at 1 Da to mimic precursor ion selection in a triple quadrupole MS instrument, since these MS instruments are most commonly used for quantitative LC–MS analysis. As shown in Figure 5, selection of the drift time of an analyte can reduce the noise and filter interfering peaks from isobaric matrix compounds. The signal intensity of the peak of interest at 13.7 min decreased somewhat when drift time selection was applied: from 1.96×10^5 to 1.58×10^5 (19%). The *S/N* ratio, however, increased by 12% (from 68 to 76) yielding a higher sensitivity when drift time selection was used. A similar decrease of matrix interference by drift time selection was noted during a previous study (16) and the increased *S/N* ratio was also described in the literature (17,18,45,36). Next to the increased *S/N*, the other peaks at this specific *m/z*, most likely interfering compounds from the matrix, decrease to a much larger extent than the peak of interest and therefore the interference of these peaks during quantification of the targeted peak was reduced.

Conclusion

CCS values could be obtained from DTIMS and TWIMS instruments with good agreement and repeatability, even over extended periods of time and between the IM-MS instruments utilizing different IM principles. However, not all compounds yielded the same CCS values when analyzed on the different instruments, implying that caution has to be taken when experimental data are compared without analysis of reference compounds.

The IM-MS instruments offer somewhat limited possibilities for isomer separation when the CCS difference between the isomers is small. IM separation of stereoisomers was therefore found to be challenging, while positional isomers are more amendable for IM separation. Cationic adduct formation may offer a solution for non-separated isomers, since it was found that some isomers showed larger CCS differences when analyzed as sodium cations. Furthermore, it was shown that implementation of IM into existing LC–MS analysis can be a solution to reduce interferences from isobaric matrix components. By using drift time selection, additional selectivity can be obtained without large sensitivity losses, or even sensitivity gains due to increased *S/N*. This approach may therefore be promising for data-independent screening approaches for food analysis.

In summary, IM can be a valuable addition to current food safety analysis in terms of the separation of isomeric and isobaric compounds. However, to truly resolve closely related compounds, such as diastereomers, further advances in instrumentation with higher mobility resolution are required. This might be already on its way with the development of techniques such as SLIM and multi-pass cyclic IM (37,36).

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References

1) A. Masia, M.M. Suarez-Varela, A. Llopis-Gonzalez, and Y. Pico, *Anal. Chim. Acta* **936**, 40–61 (2016).
2) L. Mainero Rocca, A. Gentili, V. Perez-Fernandez, and P. Tomai, *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **34**, 766–784 (2017).
3) E.W. McDaniel, D.W. Martin, and W.S. Barnes, *Review of Scientific Instruments* **33**, 2–7 (1962).
4) C. Lapthorn, F. Pullen, and B.Z. Chowdhry, *Mass Spectrom. Rev.* **32**, 43–71 (2013).
5) S. Poyer, C. Loutelier-Bourhis, G. Coadou, F. Mondeguer, J. Enche, A. Bossee, P. Hess, and C. Afonso, *J. Mass Spectrom.* **50**, 175–181 (2015).
6) J. Regueiro, N. Negreira, and M.H. Berntssen, *Anal. Chem.* **88**, 11169–11177 (2016).
7) E.A. Mason and H.W. Schamp Jr, *Annals of Physics* **4**, 233–270 (1958).
8) F. Lanucara, S.W. Holman, C.J. Gray, and C.E. Eyers, *Nat. Chem.* **6**, 281–294 (2014).
9) B.M. Kolakowski and Z. Mester, *Analyst* **132**, 842–864 (2007).
10) P. Martinez-Lozano and J. Rus, *J. Am. Soc. Mass Spectrom.* **21**, 1129–1132 (2010).
11) J.P. Williams, M. Grabenauer, R.J. Holland, C.J. Carpenter, M.R. Wormald, K. Giles, D.J. Harvey, R.H. Bateman, J.H. Scrivens, and M.T. Bowers, *International Journal of Mass Spectrometry* **298**, 119–127 (2010).
12) R. Cumeras, E. Figueras, C.E. Davis, J.I. Baumbach, and I. Gracia, *Analyst* **140**, 1376–1390 (2015).
13) M. Hernandez-Mesa, B. Le Bizec, F. Monteau, A.M. Garcia-Campana, and G. Dervilly-Pinel, *Anal. Chem.* **90**, 4616–4625 (2018).
14) M. Hernández-Mesa, A. Escourrou, F. Monteau, B. Le Bizec, and G. Dervilly-Pinel, *TrAC Trends in Analytical Chemistry* **94**, 39–53 (2017).
15) H.S. Jonasdottir, C. Papan, S. Fabritz, L. Balas, T. Durand, I. Hardardottir, J. Freysdottir, and M. Giera, *Anal. Chem.* **87**, 5036–5040 (2015).
16) W.F. Duvivier, T.A. van Beek, and M.W. Nielen, *Rapid Commun. Mass Spectrom.* **30**, 2331–2340 (2016).

17) K.L. Arthur, M.A. Turner, A.D. Brailsford, A.T. Kicman, D.A. Cowan, J.C. Reynolds, and C.S. Creaser, *Anal. Chem.* **89**, 7431–7437 (2017).
18) K.L. Arthur, M.A. Turner, J.C. Reynolds, and C.S. Creaser, *Anal. Chem.* **89**, 3452–3459 (2017).
19) S.M. Stow, T.J. Causon, X. Zheng, R.T. Kurulugama, T. Mairinger, J.C. May, E.E. Rennie, E.S. Baker, R.D. Smith, J.A. McLean, S. Hann, and J.C. Fjeldsted, *Anal. Chem.* **89**, 9048–9055 (2017).
20) D.N. Mortensen, A.C. Susa, and E.R. Williams, *J. Am. Soc. Mass Spectrom.* **28**, 1282–1292 (2017).
21) L. Fiebig and R. Laux, *International Journal for Ion Mobility Spectrometry* **19**, 131–137 (2016).
22) M. Chai, M.N. Young, F.C. Liu, and C. Bleiholder, *Anal. Chem.* **90**, 9040–9047 (2018).
23) M.F. Bush, I.D. Campuzano and C.V. Robinson, *Anal. Chem.* **84**, 7124–7130 (2012).
24) J.G. Forsythe, S.M. Stow, H. Nefzger, N.W. Kwiecien, J.C. May, J.A. McLean, and D.M. Hercules, *Anal. Chem.* **86**, 436–4370 (2014).
25) J. Boschmans, S. Jacobs, J.P. Williams, M. Palmer, K. Richardson, K. Giles, C. Lapthorn, W.A. Herrebout, F. Lemiere, and F. Sobott, *Analyst* **141**, 4044–4054 (2016).
26) K.M. Hines, J.C. May, J.A. McLean, and L. Xu, *Anal. Chem.* **88**, 7329–7336 (2016).
27) J.C. May, C.R. Goodwin, N.M. Lareau, K.L. Leaptrot, C.B. Morris, R.T. Kurulugama, A. Mordehai, C. Klein, W. Barry, E. Darland, G. Overney, K. Imatani, G.C. Stafford, J.C. Fjeldsted, and J.A. McLean, *Anal. Chem.* **86**, 2107–2116 (2014).
28) G. Paglia, J.P. Williams, L. Menikarachchi, J.W. Thompson, R. Tyldesley-Worster, S. Halldorsson, O. Rolfsson, A. Moseley, D. Grant, J. Langridge, B.O. Palsson, and G. Astarita, *Anal. Chem.* **86**, 3985–3993 (2014).
29) L. Beucher, G. Dervilly-Pinel, S. Prevost, F. Monteau, and B. Le Bizec, *Anal. Chem.* **87**, 9234–9242 (2015).
30) J. Hofmann, A. Stuckmann, M. Crispin, D. J. Harvey, K. Pagel, and W.B. Struwe, *Anal. Chem.* **89**, 2318–2325 (2017).
31) G. Paglia, P. Angel, J.P. Williams, K. Richardson, H.J. Olivos, J.W. Thompson, L. Menikarachchi, S. Lai, C. Walsh, A. Moseley, R.S. Plumb, D.F. Grant, B.O. Palsson, J. Langridge, S. Geromanos, and G. Astarita, *Anal. Chem.* **87**, 1137–1144 (2015).
32) P. Benigni, C.J. Thompson, M.E. Ridgeway, M.A. Park, and F. Fernandez-Lima, *Anal. Chem.* **87**, 4321–4325 (2015).
33) T.J. Causon and S. Hann, *J. Chromatogr. A* **1416**, 47–56 (2015).
34) J.N. Dodds, J.C. May, and J.A. McLean, *Anal. Chem.* **89**, 952–959 (2017).
35) M.G.M. van de Schans, M.H. Blokland, P.W. Zoontjes, P.P.J. Mulder, and M.W.F. Nielen, *J. Chromatogr. A* **1503**, 38–48 (2017).
36) Y.M. Ibrahim, A.M. Hamid, L. Deng, S.V. Garimella, I.K. Webb, E.S. Baker, and R.D. Smith, *Analyst* **142**, 1010–1021 (2017).
37) K. Giles, J.L. Wildgoose, S. Pringle, D. Langridge, P. Nixon, J. Garside, and P. Carney, *Proceedings of the American Society for Mass Spectrometry and Allied Topics* (St. Louis, Missouri, USA, 2015).
38) Y. Huang and E.D. Dodds, *Anal Chem* **85**, 9728–9735 (2013).
39) T.G. Flick, I.D. Campuzano, and M.D. Bartberger, *Anal. Chem.* **87**, 3300–3307 (2015).
40) A. Troc, M. Zimnicka, M. Kolinski and W. Danikiewicz, *J. Mass Spectrom.* **51**, 282–290 (2016).
41) K.A. Morrison, B.K. Bendiak, and B.H. Clowers, *J. Am. Soc. Mass Spectrom.* **28**, 664–677 (2017).
42) X. Zheng, X. Zhang, N.S. Schocker, R.S. Renslow, D.J. Orton, J. Khamsi, R.A. Ashmus, I.C. Almeida, K. Tang, C.E. Costello, R.D. Smith, K. Michael, and E.S. Baker, *Anal. Bioanal. Chem.* **409**, 467–476 (2017).
43) L.M. Shabad, T.A. Bogush, I.A. Konopleva, and G.A. Belitsky, *Neoplasma.* **24**, 147–150 (1977).
44) B.J. Berendsen, M.L. Essers, L.A. Stolker, and M.W. Nielen, *J. Chromatogr. A* **1218**, 7331–7340 (2011).
45) A. Cohen, N.W. Ross, P.M. Smith, and J.P. Fawcett, *Rapid Commun. Mass Spectrom.* **31**, 842–850 (2017).

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Automated Gas and Liquid Chromatography Retention Time Modelling and Prediction Using Open-Access Molecular Database Structures and Quantitative Structure-Chromatography Retention Relationships

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Retention time modelling and prediction software was developed for gas chromatography (GC) and liquid chromatography (LC applications). This open access software incorporates balloon (3D modelling of compounds) and PaDEL (molecular descriptor calculation) as well as support vector machine regression (modelling). To generate models a list of retention times of known compounds is needed together with their molecular structures in simplified molecular-input line-entry system (SMILES) notation. For validation of the models, independent lists of other compounds are used to compare experimental and predicted retention times. The performance of the developed software was successfully demonstrated on three different data sets, including one independent external data set consisting of 507, 291, and 528 compounds, respectively. This software would be useful for the identification of unknown food contaminants and designer drugs in sports doping, and other applications where accurate mass and tandem mass spectrometry (MS/MS) data alone are inconclusive for the assignment of postulated molecular structures.

Retention time (RT) is one of the criteria presented in guidelines for the confirmation of the identity of compounds detected by liquid chromatography (LC) or gas chromatography (GC) systems coupled to mass spectrometry (MS) in fields such as food safety and sports doping (1,2). According to these guidelines, confirmatory methods should be based on target compounds for which standards are available for comparative analysis of RT and fragmentation in (tandem) MS.

However, in exploratory screenings of samples with separation methods coupled to full-scan accurate mass MS techniques, many signals will relate to compounds for which no standard is available. In practice, without a reference RT and fragmentation information, each accurate mass and elemental composition(s) thereof may be explained by multiple compounds. In these cases RT prediction may help to reduce the number of identification solutions. Aalizedeh *et al.* (3) modelled and predicted RTs for LC and subsequently used the predicted RTs to help exclude false-positive candidate compounds. The principle of this is represented by the infographics in Figure 1.

In many papers retention modelling and prediction has been performed based on quantitative structure-chromatographic retention relationships (QSRR) (4–6) for a set

FIGURE 1: Infographic representation of how RT modelling and prediction can help to eliminate false positive compound identifications.

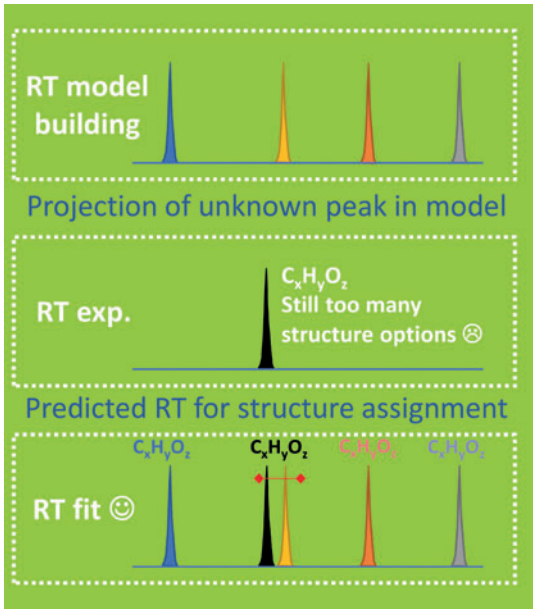
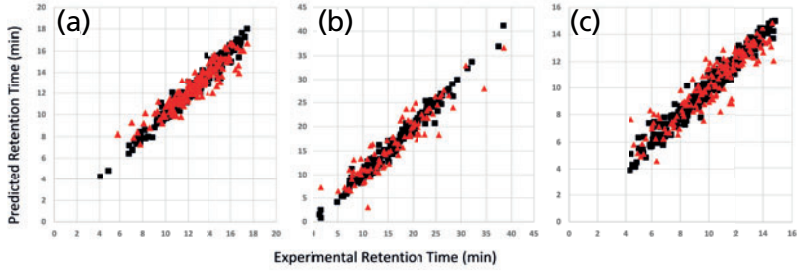


FIGURE 2: Examples of predicted retention time (average output from 240 generated models) vs. the experimental retention time for three different chromatographic separations using CalcRetModel software. (a) in-house GC data. (b) in-house LC data. (c) external LC data (3). Squares (black) represent data used for modelling (dependent data), triangles (red) represent data exclusively used for validation (independent data).



of compounds measured with LC–MS or GC–MS (3,7–21). It is beyond the scope of this article to review in-depth all the different software tools and complex chemometric methods described and applied in QSRR modelling and prediction. Basically, for each compound a structure notation (for example, simplified molecular-input line-entry system [SMILES] format) and retention time are needed. The SMILES is used to calculate molecular descriptors. A selection of molecular descriptors is made based on RT prediction performance. Chemometrics tools are then tested to obtain the best models for prediction by combining descriptors. Finally, in all cases, the validation of a model must be performed on an independent validation set with known RTs, not used for model building (3,7–21).

A major difficulty in using RT prediction in practice is that is that the cited studies (3,7–21), excluding reference 3, have either complex chromatography, a small number of compounds, or not enough information on SMILES and experimental RTs (Supplementary Material S1: Numbers.xlsx). As a result, benchmarking of new developments against previous work is difficult or almost impossible.

In order to make RT modelling and prediction more accessible, new open access software that can model and predict RTs for a wide range of chemically different compounds in GC and LC was developed. Large data sets and simple linear temperature or solvent gradients were used. As an independent validation one-third of all compounds were kept out of modelling and used to serve as substitutes for potential unknown compounds matching the experimental data. Furthermore, an entirely independent external data set from reference 3 was used to benchmark this newly developed software.

Experimental

Obtaining RTs for Compounds for In-House GC, In-House LC, and External LC:

In-House GC:

The retentions of 507 pesticides and contaminants were obtained using GC–MS (Pegasus-4D system, Leco) as described in detail in Lommen *et al.* (22). Only the first dimension GC retention data (10 m × 0.25 mm, 0.25-µm RTX-CL pesticides column [Restek]) were used. The GC oven temperature was linearly programmed from 60 °C to 320 °C at 15 °C /min. RTs of GC-stable

TABLE 1: Results of the modelling and prediction. 2D/3D: descriptors used, Mod: descriptor modification applied, R2_model: R^2 for modelling set, R2_val: R^2 for validation set, RMSE_model: root mean square or error for modelling set in minutes, RMSEP_val: root mean square or error of prediction for validation set in minutes. Rel_RMSE_model: relative RMSE_model: RMSE_model divided by separation time, Rel_RMSEP_val: RMSEP_val divided by effective separation time

(a) In-house GC data (effective 12 min separation): average of 240 models							
2D/3D	Mod.	R2_model	R2_val	RMSE_model	RMSEP_val	Rel_RMSE_model	Rel_RMSEP_val
2D	no	0.967	0.828	0.42	0.92	0.0346	0.0763
2D	yes	0.964	0.853	0.44	0.85	0.0363	0.0704
2D+3D	no	0.967	0.835	0.41	0.90	0.0342	0.0747
2D+3D	yes	0.964	0.857	0.43	0.83	0.0361	0.0694
(b) In-house LC data (effective 39 min separation): average of 240 models							
2D/3D	Mod.	R2_model	R2_val	RMSE_model	RMSEP_val	Rel_RMSE_model	Rel_RMSEP_val
2D	no	0.978	0.851	1.01	2.63	0.0260	0.0675
2D	yes	0.977	0.868	1.03	2.48	0.0263	0.0635
2D+3D	no	0.979	0.851	0.98	2.63	0.0251	0.0674
2D+3D	yes	0.978	0.867	1.01	2.48	0.0259	0.0636
(c) External LC data (effective 11 min separation): average of 240 models							
2D/3D	Mod.	R2_model	R2_val	RMSE_model	RMSEP_val	Rel_RMSE_model	Rel_RMSEP_val
2D	no	0.963	0.831	0.48	1.00	0.0436	0.0909
2D	yes	0.962	0.822	0.48	1.03	0.0436	0.0936
2D+3D	no	0.963	0.843	0.48	0.97	0.0436	0.0882
2D+3D	yes	0.962	0.836	0.49	0.99	0.0445	0.0897

compounds eluting in the linear temperature gradient were selected, the beginning of the gradient being adjusted to be $t = 0$ min. RTs and SMILES of all compounds are given in Supplementary Material S2.

In-House LC:

The retentions of 291 compounds were obtained by injecting standards on a Dionex (Thermo Scientific) UHPLC system running a linear gradient separation at a constant flow rate of 0.2 mL/min. The system consisted of a vacuum degasser, a high-pressure binary pump, an autosampler with a temperature-controlled sample tray set at 7 °C, and a column oven set at 30 °C. Chromatographic separation was performed using a 100 × 2.1 mm, 1.8-µm Zorbax Eclipse Plus C18 column (Agilent Technologies). The mobile phase consisted of 5-mM ammonium formate in 0.02% formic acid (solvent A) and a 90:10 mixture of acetonitrile–water (*v/v*) containing 5-mM ammonium formate and 0.01% formic acid (solvent B). A gradient elution program started at $t = 0$ with solvent B at 0% and then increased linearly to 100 % within 40 min, where it was held for 5 min before returning to 0% within 1 min. The injection volume was 5 mL. Peak identities were confirmed by MS (MS method: see Supplementary Material S1). Only compounds eluting during the slope of the gradient were used for modelling; RTs and SMILES of all used compounds are given in Supplementary Material S2.

External LC Data:

Reference 3 is—to our knowledge—the only recent study that includes more than 200 compounds measured with a (more or less) linear gradient separation and provides a ready-to-use matrix-containing compound identification, RT, and SMILES format notation. This data was used as an external data source to test and benchmark the software. 511 of the available 528 compounds, that is, all compounds from the linear gradient part of the separation, were used.

Obtaining SMILES for the Compounds: Structural information in SMILES format for nearly all known compounds was obtained from PubChem; care has been taken to use ISOMERIC SMILES (PubChem definition) if the compound conformation requires that (*cis/trans;* alpha/beta). The developed workflow allows direct manual entry of SMILES as well as automatic

retrieval of SMILES using the PubChem compound identification number (CID). For the latter a converted version of PubChem is needed. The publicly available PubChem can be downloaded and subsequently converted using a published PubChem converter (23) following the procedure described in Supplementary Material S3. If SMILES are not available through PubChem they have to be drawn and exported in SMILES format using an external software package (https://cactus.nci.nih.gov/translate/) or obtained through manual internet searches.

Automated Software Events: A detailed description is available in "Details on the procedures done in automation by the software.docx" in Supplementary Material S1. In brief, CalcRetModel uses balloon (24) in-line to transform SMILES to three-dimensional (3D) (in structure-data file format) SDF files. Consecutively, PaDEL (25) is used in-line to calculate molecular descriptors from the SDF files. At this point there is an option to transform a subset of the descriptor values by a natural log (GC) or by a natural exponential (LC) (See "Details on descriptor modification.docx" in Supplementary Material S1). The data set is sorted and split three ways (Examples in Supplementary Material S4). Two parts are sorted and used for descriptor selection and for generating models using Support Vector Machine Regression (SVM-R) (26). The remaining part is solely used for validation, which is performed after modelling. The developed software automatically selects descriptors and generates slightly differing models. In practice, a high number of models are generated and their prediction outcome averaged to obtain final predicted values. A second program, PredictRet, is also provided in which any new SMILES can be run through the CalcRetModel-generated models to give an averaged RT.

Programming and Hardware: Both of these software programmes are written in Microsoft Visual C++ 2010 and compiled to Windows executables. The compiled binaries are available in the Supplementary Material S5. Calculations have been performed on a hyper-threaded 16-core PC (32 virtual cores; 2.9 GHz; 64 Gb RAM) equipped with a solid-state disc and operated under a Windows (7 or 10) 64 bit operating system. Typically, calculation of 240 models takes about 1–2 days of calculations using 32 cores. In practice, predicting a RT for verification of a new candidate compound using SMILES format takes only a few minutes (Powerpoint manuals of modelling and prediction software modules are supplied in Supplementary Material S5).

Results and Discussion

Modelling and Prediction Results: Three different chromatographic separations were used to assess the performance of this retention time modelling and prediction software. Two of these—GC and LC data—were acquired in our laboratory; one LC data set was taken from the literature (3). The results are given in Table 1 and Figure 2. For all three separations the models were validated with a third of the original data set; these validation compounds (red triangles in Figure 2) were not used in modelling and are totally independent.

Modelling and prediction were performed with and without descriptor modification and 3D descriptors (see Table 1) for all three data sets. The RMSEP_val (root mean square error of prediction) values were divided by the effective separation time for the different data sets to obtain a relative value (that is, Rel_RMSEP_val). All three data sets have similar Rel_RMSEP_val values, which indicates that their relative precision of RT prediction is also similar. For in-house data sets (GC and LC), a slight improvement was obtained by modifying descriptors prior to modelling and prediction (See also "Details on descriptor modification.docx" in Supplementary Material S1). Adding 3D descriptors may have a small effect on the external LC data set. Since descriptor modification and adding 3D descriptors may be beneficial, these options were included in the calculations and software.

Averaging RT Output from Multiple Models: Descriptor selection is automated (see "Details on the procedures done in automation by the software.docx" in Supplementary Material S1) for each of the 240 models generated for each data set. Each model is (slightly) different and may perform better for some compounds than for others. Selecting the best performing models may be a biased choice towards those compounds used. It was therefore decided to use all generated models and average the predicted RTs afterwards to remain as independent as possible. If the Rel_RMSEP_val (with descriptor modification and using 3D descriptors) is taken from each individual model (240 total) and averaged and the standard deviations calculated, for the in-house GC, in-house LC, and the external LC data, respectively, an averaged Rel_RMSEP_val (standard deviation) of 0.0825 (0.0058), 0.0751 (0.0074), and 0.1009 (0.0055) is obtained. Comparison to the values in Table 1 (0.0694, 0.0636, and 0.0897, respectively) shows that an approach in which multiple models are used after which the predicted RTs are averaged decreases prediction

errors. Large numbers of models were generated, since this is automatic and just needs computing time.

Benchmarking Using the External LC Data Set: For the external LC data it was reported in the literature that the best $R^2 = 0.887$ and the best RMSEP = 0.941 (3) after trying various methods. With this newly developed software approach, an $R^2 = 0.836$ and RMSEP_val = 0.99 was obtained.

Comparing Descriptor Lists Between Different Chromatographic Separations: In Supplementary Material 4 a list of descriptors ("Used_descriptors.xlsx") and the number of times they occur in 240 models is compiled.

In-House GC Data:

All GC models included the descriptors piPC1 (PaDEL Path Count: Conventional bond order ID number of order 1 [ln(1+x)[27]) and MLFER_L (theoretically estimated solute gas-hexadecane partition coefficient [28]). piPC1 is related to ln(nBonds) (See "Details on descriptor modification. docx" in Supplementary Material S1); nBonds (Number of bonds [excluding bonds with hydrogen] [25]) is present in 228 models. Therefore both size, bond order, and theoretical partitioning constant are represented in the models.

In-House LC Data:

All models for the in-house (one-slope) linear acetonitrile gradient LC data contain CrippenLogP (Crippens theoretical logarithm of the octanol–water partitioning coefficient based on 68 types of atomic contributions [29]) and SpMax2_Bhv (Largest absolute eigenvalue of Burden modified matrix - n 2 / weighted by relative van der Waals volume [3]). The log of the octanol–water partitioning coefficient (LogP) has long been known to contribute to RT modelling of C18-type separations such as those observed here (13). Burden descriptors have been used in prediction of LogP (30). SpMax2_Bhv contains information on atom connections as well as van der Waals volume.

External LC Data:

All models for the external (one-slope) linear methanol gradient LC data contain CrippenLogP (29), XlogP (another LogP atom-additive approximation method [31,32])

and LipoaffinityIndex (33) (a theoretical modelled measure for hydrophobicity). In contrast to the acetonitrile gradient for in-house LC-data, hydrophobicity fits better as a factor in this methanol gradient.

Comparing LC Descriptors:

Both LC data sets have a high number of compounds and a broad chemical diversity. Both LC data sets have a separation based on C18 columns. Yet the LC data sets show different numbers for how many times descriptors are used in the 240 models. Examining descriptors in both LC data sets already shows differences ("used_descriptors.xlsx"). This may be the result of differences in polarity and solvation by the organic phase in the elution solvents (that is, acetonitrile in in-house LC vs. methanol in the external LC) (34).

Practical Implications of this Approach for GC–MS and LC–MS: To create models for GC–MS and LC–MS, the SMILES and RTs of hundreds of standards are needed. The models obtained are characteristic of a specific standardized chromatography system. If a totally different stationary phase with different chromatographic characteristics is used then the models obtained are no longer applicable and require redevelopment. When using columns with similar separation characteristics, but perhaps different gradients or only different dimensions, it may be an option to calculate RTs using models from this study and then reference their experimental RTs to compounds measured in this study. Predicting RTs (with PredictRet) can be used to help narrow down the number of candidate compounds in identification questions and exclude false positives (3,7–21). For the validation sets of the in-house GC, in-house LC, and external LC data, a retention window of predicted RT ± 2xRMSEP_val will include approximately 93%, 94%, and 94% of the 169, 97, and 170 validation compounds, respectively. A retention window of predicted RT ±3xRMSEP_val will include more than 99% for all three separations. Previously, Berendsen *et al.* (35,36) modelled the probability of the occurrence of a RT in LC–MS/MS with QSRR and showed that inclusion of chromatographic retention may add significantly to the certainty of identification of unknown and confirmation of known compounds in both tandem- and high-resolution mass spectrometry. Considering that the prediction error with the new software presented here is better, an improvement in identification would certainty be expected.

Conclusion

Open source software was developed for the modelling and prediction of RTs for GC and LC separations. The software runs on a Windows platform and can use multiple processors in parallel. The use of this software is simple and the process is completely automated. This makes it easy to update models if more compounds are added later on. All that is needed as input is enough compounds—described by SMILES and RT—distributed over the standardized chromatographic separation. For in-house GC, pesticides and food contaminants were used. For in-house LC data, compounds listed as doping compounds were used. The external LC data—the benchmark data for which comparable prediction results were achieved—consisted of emerging contaminants. The compounds used in all three separations may be considered to be chemically highly diverse in terms of polarity and retention behaviour and thus quite challenging for modelling and prediction. It is envisaged that the developed software will be useful to identify unknown contaminants in food and the environment, designer drugs in sports doping, and any applications where accurate mass and tandem mass spectrometry data alone are inconclusive for the assignment of postulated molecular structures.

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Supplementary Material

All Supplementary Material mentioned in the text is available at: https://edepot.wur.nl/501020. For further information contact arjen.lommen@wur.nl

References

1) WADA Technical Document – TD2015IDCR (2015): https://www.wada-ama.org/en/resources/science-medicine/td2015idcr
2) Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. SANTE/11813/2017 (2017): http://www.eurl-pesticides.eu/docs/public/tmplt_article.asp?CntID=727
3) R. Aalizadeh, N.S. Thomaidis, A.A. Bletsou, and P. Gago-Ferrero, *Chem. Inf. Model.* **56**, 1384–1398 (2016).
4) R. Kaliszan, *Quantitative Structure-Chromatographic Retention Relationships* (John Wiley and Sons, New York, USA, 1987).
5) R. Kaliszan, *Structure and Retention in Chromatography - A Chemometric Approach* (Harwood, Amsterdam, 1997).
6) K. Heberger, *J. Chromatogr. A* **1158**, 273–305 (2007).
7) H.F. Chen, *Anal. Chim. Acta* **609**, 24–36 (2008).
8) A.G. Fragkaki, A. Tsantili-Kakoulidou, Y.S. Angelis, M. Koupparis, and C. Georgakopoulos, *J. Chromatogr. A* **1216**, 8404–8420 (2009).
9) D.J. Creek, A. Jankevics, R. Breitling, D.G. Watson, M.P. Barrett, and K.E.V. Burgess, *Anal. Chem.* **83**, 8703–8710 (2011).
10) K. Gorynski, B. Bojko, A. Nowaczyk, A.

Bucinski, J. Pawliszyn, and R. Kaliszan, *Anal. Chim. Acta* **797**, 13–19 (2013).
11) T.H. Miller, A. Musenga, D.A. Cowan, and L.P. Barron, *Anal. Chem*. **85**, 10330–10337 (2013).
12) E. Alladio, V. Pirro, A. Salomone, M. Vincenti, and R. Leardi, *Anal. Chim. Acta* **878**, 78–86 (2015).
13) R. Bade, L. Bijlsma, J.V. Sancho, and F. Hernández, *Talanta* **139**, 143–149 (2015).
14) K. Munro, T.H. Miller, C.P.B. Martins, A.M. Edge, D.A. Cowan, and L.P. Barron, *J. Chromatogr. A* **1396**, 34–44 (2015).
15) C.D. Broeckling, A. Ganna, M. Layer, K. Brown, B. Sutton, E. Ingelsson, G. Peers, and J.E. Prenni, *Anal. Chem*. **88**, 9226–9234 (2016).
16) F. Falchi, S.M. Bertozzi, G. Ottonello, G.F. Ruda, G. Colombano, C. Fiorelli, C. Martucci, R. Bertorelli, R. Scarpelli. A. Cavalli, T. Bandiera, and A. Armirotti, *Anal. Chem*. **88**, 9510–9517 (2016).
17) A.M. Wolfer, S. Lozano, T. Umbdenstock, V. Croixmarie, A. Arrault, and P. Vayer, *Metabolomics* **12**(8), 1–13 (2015).
18) R.I.J. Amos, E. Tyteca, M. Talebi, P.R. Haddad, R. Szucs, J.W. Dolan, and C.A. Pohl, *J. Chem. Inf. Model*. **57**, 2754–2762 (2017).
19) C. Zisi, I. Sampsonidis, S. Fasoula, K. Papachristos, M. Witting, H.G. Gika, P. Nikitas, and A. Pappa-Louisi, *Metabolites* **7**, 7 (2017).
20) Y. Marrero-Ponce, S.J. Barigye, M.E. Jorge-Rodríguez, and T. Tran-Thi-Thu, *Chem. Pap*. **72**, 57–69 (2018).
21) M. Taraji, P.R. Haddad, R.I.J. Amos, M. Talebi, R. Szucs, J.W. Dolan, and C.A. Pohl, *Anal. Chim. Acta* **1000**, 20–40 (2018).
22) A. Lommen, H.J. van der Kamp, H.J. Kools, M.K. van der Lee, G. van der Weg, and H.G. Mol, *J. Chromatogr. A* **1263**, 169–178 (2012).
23) A. Lommen, *Anal. Chem*. **86**, 5463–5469 (2014).
24) M.J. Vainio and M.S. Johnson, *J. Chem. Inf. Model* **47**, 2462–2474 (2007).
25) C.W. Yap, *J. Comput. Chem*. **32**, 1466–1474 (2011).
26) C.-C. Chang and C.-J. Lin, *ACM Trans. Intell. Syst. Technol*. **2**, 1–27 (2011).
27) R. Todeschini and V. Consonni, in *Handbook of Molecular Descriptors* (Wiley-VCH Verlag GmbH, 2008).
28) J.A. Platts, D. Butina, M.H. Abraham, and A. Hersey, *J. Chem. Inf. Comput. Sci*. **39**, 835–845 (1999).
29) S.A. Wildman and G.M. Crippen, *J. Chem. Inf. Comput. Sci*. **39**, 868–873 (1999).
30) F.R. Burden, *Quant. Stuct-Act. Relat*. **16**, 309–314 (1997).
31) R. Wang, Y. Fu, and L. Lai, *J. Chem. Inf. Comput. Sci*. **37**, 615–621 (1997).
32) R. Wang, Y. Gao, and L. Lai, *Perspectives in Drug Discovery and Design* **19**, 47–66 (2000).
33) R. Liu, H. Sun, and S.S. So, *J. Chem. Inf. Comput. Sci*. **41**, 1623–1632 (2001).
34) B.P. Johnson, M.G. Khaledi, and J.G. Dorsey, *Anal. Chem*. **58**, 2354–2365 (1986).
35) B.J.A. Berendsen, A.A.M. Stolker, and M.W.F. Nielen, *J. Am. Soc. Mass Spectrom*. **24**, 154–163 (2013).
36) B.J.A. Berendsen, R.S. Wegh, Th. Meijer, and M.W.F. Nielen, *J. Am. Soc. Mass Spectrom*. **26**, 337–346 (2015).

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Comprehensive Identification of Migrating Compounds from Plastic Food Packaging Materials Using LC–HRAMS

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In order to ensure the continued use of plastic packaging in food, the packaging used must be safe for contact with food products, and not cause contact contamination. Of rising concern is the role of non-intentionally added substances (NIAS). Therefore, this study assesses whether two commonly used plastic packaging materials, polyethylene and low-density polyethylene plus nylon, are safe for use. Their potential migrants are analyzed using data-independent acquisition (DIA) and liquid chromatography (LC) coupled with high-resolution accurate mass spectrometry (HRAMS). Using automatic detection capabilities and filtering procedures, MS and tandem mass spectrometry (MS/MS) data were processed to find chemical formulae and structures and toxicity information for the most abundant analytes in a given sample. Through this method, 26 migrating compounds, mainly cyclic oligomers, were identified. Using the toxicity rules set out by Cramer, 19 of these 26 compounds were identified as having moderate or high toxicity.

Food packaging is a major market segment in the global food industry, often compared in size to that of the pharmaceutical industry (1). Over one third of all food packaging comprises materials made from plastic (1,2), which over the past several decades has extended to different plastic materials, copolymers, and additional ingredients.

Packaging itself is essential to our modern society. As our food supply chain has grown to become more global, plastic packaging has been instrumental in ensuring that the food reaching consumers is safe to eat. Packaging helps to preserve food by protecting it from light, humidity, oxygen, foreign compounds, mechanical influences, and microbial contamination, all of which help to improve shelf life (1). Two polymers used frequently in food packaging include polypropylene and polyethylene, with the latter being used in various forms including low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), and high-density polyethylene (HDPE) (3).

However, while food packaging protects from external contaminant introduction, there is increasing concern about the potential of chemical contamination from the plastic packaging itself. In fact, food contact migration is reported as one of the largest sources of food contamination, and is approximately 100–1000 times higher than that caused by pesticide residues (3,4). This migration can occur from multiple sources, including from contact with the internal face of the packaging, from diffusion and partition processes within multi-layered plastics, or even from adhesives.

The potential health implications that plastic contamination poses to the consumer have resulted in the control and supervision of plastic materials in multiple countries. For instance, the European Union has imposed Regulation EU 10/2011, which has established specific rules for what plastic materials can be applied safely to food packaging, and lists specific compounds that are authorized for use in plastic formulations and manufacturing (5). But, this regulation does not take into account unintentional substances, often referred to as *non-intentionally added substances* (NIAS), meaning they frequently do not appear in lists of permitted ingredients. NIAS may be introduced through a variety of processes: a result of interaction between ingredients, degradation of material components, or from impurities within the raw material (6). As a result, the migration of non-authorized substances must not exceed a level of 0.01 mg/kg of food or simulant.

TABLE 1: Source parameters in positive polarity

Ion source gas 1	40 psi
Ion source gas 2	50 psi
Curtain gas	25 psi
CAD gas	7 psi
Temperature	450 °C
Spray voltage	5500 V
Resolution power of the TOF system	32,000 FWHM (for *m/z* 200)

TABLE 2: Data-independent acquisition parameters used in the full scan mode

Accumulation time	0.2 s
Declustering potential	80 V
TOF start mass	100
TOF stop mass	950

To meet these strict criteria, highly sensitive and advanced analytical instruments and techniques are needed to test samples for the presence of NIAS. For nonvolatile chemicals in particular, liquid chromatography coupled to high-resolution accurate mass spectrometry (LC–HRAMS) has a proven history identifying nontarget compounds (7). In addition, nontargeted screening approaches assist in identifying potentially unknown contaminants compared to other acquisition methods. This technology has already been successfully applied to proteomics and metabolomics (8), as well as clinical and forensic toxicology (9).

The present study investigates the migration of compounds from two different multilayer plastic packaging materials commonly used in fruit puree and juices. Using an LC–QTOF instrument, coupled with data-independent acquisition, nontargeted peaks were identified and concentrations were compared against those dictated by EU regulation. Following the successful identification of molecular formulae and structures, the toxicity of these structures was assessed to determine the potential harm such NIAS pose to consumers.

TABLE 3: MS/MS mode parameters

Accumulation time	0.2 s
TOF start mass	50
TOF stop mass	950
Generic collision energy	35 ±15 V
Isolation windows in Q1	10, divided as follows: 100–185, 184–270, 269–355, 354–440, 439–525, 524–610, 609–695, 694–780, 779–865, and 864–950
Total cycle time	0.78 s

Experimental

Method and Apparatus: Two commercially available multilayer plastic materials, used for the packaging of purees and juices from fruits and vegetables, were purchased over the internet for testing. The two multilayered materials used were: a polyethylene based material, and an LDPE plus nylon material.

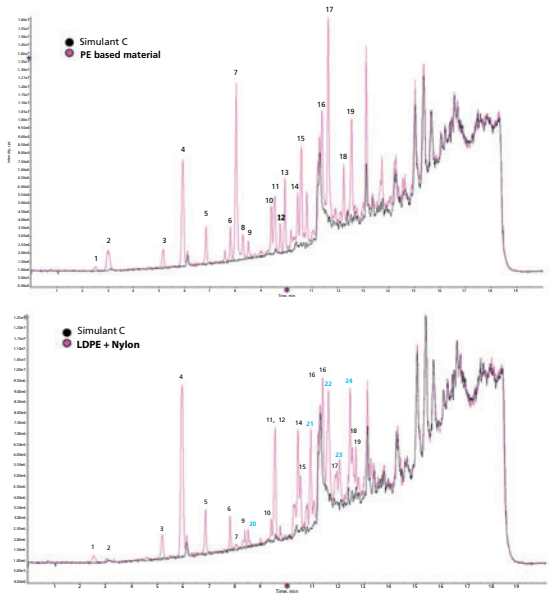
The migration test was performed according to the procedures established by EU Regulation 10/2011. The standardized test conditions, which simulate the long-term storage of food at or below room temperature, involve subjecting the material to 10 days of contact time with a contact temperature of 40 °C. Migration tests were performed using 1 dm^3 surface area. This surface area was chosen as the regulation has previously established that materials can now be manufactured in such a way that the material is not releasing more than 10 mg per 1 dm^3 of the plastic material.

Two simulants were used to measure the impact of material contact: simulant B, which consists of a solution of 3% acetic acid in water (*w/v*), and simulant C, which is a solution of 20% ethanol in water (*v/v*). For each material, 10 × 10 cm of plastic material was added to a 50 mL PTFE tube with 35 mL of the relevant simulant, in which carbendazim-d3 and malathion-d10 were added to the simulants as internal standards for quality control purposes. Three replicates and one control were performed. Each tube was placed in an incubator for 10 days at 40 °C. Afterwards, one aliquot of each sample was directly injected and analyzed by LC–HRAMS along with a calibration curve of ε-caprolactam.

The liquid chromatography separation was carried out using an Exion LC (Sciex). Mobile phase A was made up from 98% water and 2% methanol, and mobile phase B was made up from 98% methanol and 2% water. Both phases contained 5 mM of ammonium formate and 0.1% formic acid. Separation was carried out using a 100 mm × 2.1 mm, 1.8-µm Zorbax Eclipse Plus C8 column (Agilent). The column was thermostatted at 35 °C. The mobile phase gradient started from 80% of mobile phase A, which was maintained for 2 min. From 2 to 15 min, the amount of mobile phase B increased linearly to 100%, which was then maintained for 2 min. The mobile phase was then changed to 80% A, maintained for 3 min for re-equilibration, leading to a total running time of 20 min. An X500R (Sciex) mass spectrometer equipped with Turbo V Source with a Twin Sprayer probe was used for acquisition. The data-independent acquisition tool SWATH Acquisition was used to analyze the data. A mixture containing 10 compounds with masses in the range of 132.9049–2034.6255 g/mol was used for calibration. Sciex OS 1.3 was used for qualitative and quantitative analysis. The source parameters for the mass spectrometer are reported in Tables 1–3.

Control samples were run in similar conditions, which are left to stand for 10 days at 40 °C in the PTFE tube. Control samples were then compared with the three samples and used to filter out chemical peaks where the

FIGURE 1: Spectra of the two materials obtained using simulant C. The most abundant peaks are identified using compound ID numbers, the identities of which are reported in Table 4.



difference in intensity of the peaks was deemed to be negligible.

Results and Discussion

Identifying Migrant Compounds: A data-independent nontargeted acquisition mode was used to analyze the analytes. This approach segments the full scanned mass range into smaller segments for simpler MS/MS analysis, reducing the complexity of the fragmentation spectra. This allows for a retrospective analysis of the data, while ensuring that overlapping analyte peaks are not missed. This analysis proved vital in this study, owing to the complex overlap of spectra.

Using the strategy outlined in the experimental section, a total of 3149 spectral features were identified in the polyethylene-based material. Using the filtering strategy, the number of elucidating ions decreased from 3149 to 57 (which is characteristic of polyethylene packaging material), which then further decreased to only 21 ions after focusing on the most abundant compounds (those with a signal intensity above 1×10^6). However, to date, there is no library of NIAS substances to screen against the identified compounds, and none of the relevant ions were present in the commercial MS/MS spectral library used (Sciex). MS/MS libraries are constantly evolving but still only cover a limited number of analytes. For those compounds whose MS and MS/MS had the lowest associated error, probable structures were constructed using ChemSpider and then modelled *in silico* to compare fragmentation patterns against those observed. Owing to the numerous possible structures, chemical intuition and prior knowledge of the substance was essential to rule out many database structures.

From the two packaging materials, a total of 26 migrating compounds were identified from the screening method. Of these, 21 were assigned a plausible structure, which was later confirmed by fragmentation and bibliography agreement. A further three migrants were assigned a plausible structure based on fragmentation data, but these could not be confirmed. The final two structures could only be assigned molecular formulae, with not enough information collected to accurately assign structures. Spectra for the two materials using simulant C are reported in Figure 1. The summarized identity of those compounds identified is summarized in Table 4.

Most migrants were identified in both materials. The only compound listed in Regulation EU 10/2011 was caprolactam, a monomer of nylon (compound 2). It was found that caprolactam was present at 42 mg/kg in the polyethylene material and 9.33 mg/kg in the LDPE + nylon material. As European legislation permits concentrations of only 15 mg/kg, the migrant compound caprolactam is present at more than twofold higher concentrations in the polyethylene-based material (5). In addition, four cyclic oligomers of caprolactam were detected in both materials corresponding to the dimer, trimer, tetramer, and pentamer (compounds 4, 3, 5, and 6, respectively). This phenomenon is not surprising as oligomers up to the octamer have previously been identified in other migration studies (3,9,10).

Two other substances were identified: the plasticizer bis (2-methoxyethyl) adipate (compound 9) and diethyl 5-({[(2,4,5-trimethoxybenzoyl)oxy] acetyl}amino) isophthalate (compound 15). These were the results of the best matches based on identified chemical structures and fragmentation patterns.

All other compounds identified were classified as NIAS, with the majority being oligomers. The main migrants were oligomers from polyurethane adhesives. Cyclic ester oligomers were found to be made up of the monomers adipic acid (AA), phthalic acid (PA), diethylene glycol (DEG), monoethylene glycol (MEG), and neopentilglycol (NPG) in the combination 1:1 (AA-DEG, PA-DEG), 1:2 (PA-DEG-DEG), 2:2 (AA-MEG-AA-MEG, AA-DEG-AA-DEG, PA-DEG-PA-DEG), 1:1:2 (AA-MEG-AA-DEG, AA-DEG-PA-DEG) or 1:1:1:1 (PA-MEG-AA-DEG, PA-DEG-AA-NPG). However, once again most of the oligomers

were not present in any library database consulted, and therefore identification was made based on a bibliographic search of their molecular masses. All oligomers were identified in the literature except for 3,6,9,12,15-Pentaoxabicyclo(15.3.1) henicosa-1(21),17,19-triene-2,16-dione, an ester whose appearance was attributed to the conceivable combination of monomers present in the materials.

Only four substances were present only in the LDPE and nylon material. These are labelled as peaks 20–24 in Figure 1. Compounds 21, 22, and 24 had not been fully described in the literature, and there is currently no thorough hypothesis or explanation for the formation of these compounds in the present study. However, the peaks displayed similar characteristics to other oligomers already identified in the simulants. Peak 20 was identified as 1/6-dioxacyclodecane-7,12-dione and is attributed in the literature to the presence of polyurethane adhesives used to laminate packaging multilayer materials (11). Compound 23 was identified as 1,6,13,18-tetraoxacyclotetracosane-2,5,14,17-tetrone, also found in compostable adhesives (12).

The two peaks that were not identified are those labelled as 17 and 19 in Figure 1. It was only possible to assign the most probable chemical formula, identified as $C_{19}H_{33}NO_8$ and $C_{21}H_{29}NO_8$. Due to the number of potential structures in the databases (22 and 83 possibilities, respectively), it was not possible to identify a specific structure.

The chromatographs for the migration of compounds for simulants B and C are overall very similar. The overlapping chromatographs are shown in Figure 2. Only two peaks were identified as exclusively present in simulant B, labelled as compounds 25 and 26. Based on individual masses, these molecules were identified as the cyclic oligomers AA-DEG and AA-DEG-PA-DEG with a molecule of water.

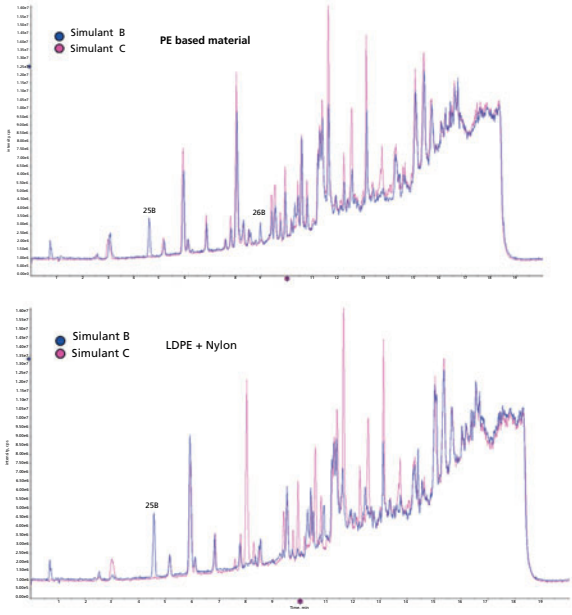
TABLE 4: Compounds identified in the polyethylene and LDPE materials using simulants B and C

ID	Elemental Composition	Mass / g mol-1	Compound	TC
1	$C_{12}H_{22}N_2O_2$	227.1754	1,8-diazacyclotetradecane-2,9-dione	I
2	$C_6H_{11}O$	114.0913	Caprolactam Total Specific Migration Limit: 15 mg/kg (RD 10/2011)	III
3	$C_{18}H_{33}N_3O_3$	340.25947	Caprolactam trimer 1,8,15-Triazacyclohenicosane-2,9,16-trione	III
4	$C_{10}H_{16}O_5$	217.10705	Caprolactam dimer 1,4,7-Trioxacyclotridecane-8,13-dione	III
5	$C_{24}H_{44}N_4O_4$	453.34353	Caprolactam tetramer 1,8,15,22-Tetraazacyclo-octacosane-2,9,16,23-tetrone	III
6	$C_{30}H_{55}N_5O_5$	566.4276	Caprolactam pentamer 1,8,15,22,29-Pentaazacyclopentatriacontane-2,9,16,23,30-pentone	III
7	$C_{12}H_{12}O_5$	237.07575	3,4,6,7-Tetrahydro-2,5,8-benzotrioxacycloundecin-1,9-dione	I
8	$C_{16}H_{20}O_7$	325.12818	3,6,9,12,15-Pentaoxabicyclo(15.3.1)henicosa-1(21),17,19-triene-2,16-dione	III
9	$C_{12}H_{22}O_6$	263.14891	Bis(2-methoxyethyl) adipate	I
10	$C_{16}H_{24}O_8$	345.15439	1,6,11,16-tetraoxacycloicosane-2,5,12,15-tetrone	I
11	$C_{18}H_{28}O_9$	389.18061	AA-MEG-AA-DEG	III
12	$C_{20}H_{32}O_{10}$	433.20682	AA-DEG-AA-DEG	III
13	$C_{22}H_{28}O_{10}$	453.17552	AA-DEG-PA-DEG	III
14	$C_{20}H_{24}O_9$	409.14931	PA-MEG-AA-DEG	III
15	$C_{20}H_{27}NO_{10}$	490.17077	Diethyl 5-({[(2,4,5-trimethoxybenzoyl)oxy]acetyl} amino) isophthalate	III
16	$C_{24}H_{24}O_{10}$	473.14422	PA-DEG-PA-DEG	III
17	$C_{19}H_{33}O_8$	404.22789	22 database possibilities	N/A
18	$C_{23}H_{30}O_9$	451.19626	PA-DEG-AA-NPG	III
19	$C_{21}H_{29}NO_8$	424.19659	83 database possibilities	N/A
20	$C_{10}H_{16}O_4$	201.11214	1,6-dioxacyclodecane-7,12-dione	I
21	$C_{20}H_{32}O_9$	417.21191	AA-DEG-AA-DEG -O	III
22	$C_{22}H_{28}O_9$	437.18061	PA-DEG-AA-DEG - O	III
23	$C_{20}H_{32}O_8$	401.21699	1,6,13,18-Tetraoxacyclotetracosane-2,5,14,17-tetrone	I
24	$C_{24}H_{24}O_9$	457.14931	PA-DEG-PA-DEG - O	III
25	$C_{10}H_{18}O_6$	235.11761	AA-DEG + H_2O	I
26	$C_{22}H_{30}O_{11}$	471.18609	PA-DEG-AA-DEG + H_2O	III

Packaging Material Toxicity

The toxicity of individual NIAS was then assessed to determine whether those compounds identified in the analysis are hazardous to public health. A bibliographic search for their toxicity found that the majority have not been registered in EU regulation. Thus, a theoretical assessment was performed using the Threshold of Toxicological Concern (TTC) approach (13–15). TTC is based on Cramer rules, which assigns the toxicity of compounds based on the molecular structure into the following categories: low (class I), moderate (class

FIGURE 2: Overlapping spectra of the two materials using simulants B and C. Compounds 20–24 are only observed in simulant B and are identified in Table 4.



II), and high (class III). Cramer has also devised a maximum recommended intake value for each compound class of 1.8, 0.54, and 0.09 mg/person/day, respectively. Using these rules, only seven of the identified compounds were identified as low toxicity, with the results for all compounds summarized in Table 4.

Due to the potential toxicity these compounds pose, their presence in plastic materials intended for food could generate a risk for consumers. Given the peak intensity, and the high presence of caprolactam, the polyethylene-based material could pose a higher risk to consumers compared to the LDPE material.

Conclusions

This study sought to identify the potential migrating substances from food packaging contact. Two packaging materials were tested, polyethylene and low-density polyethylene plus nylon. A total of 26 substances were identified in this study, combining those identified in the LDPE and polyethylene materials: 21 have been assigned a confirmed structure, three have a tentative assigned structure, and the final two only have assigned molecular formulae. The majority of the compounds are considered by Cramer's rules to be of moderate or high toxicity meaning their presence should be restricted to low concentrations. Only one compound identified is listed in the Regulation (EU) 10/2011: caprolactam, which was found to be above suggested concentrations in the sample of polyethylene tested. This work has shown the relevance and importance of evaluating NIAS in food contact materials to ensure consumer and food safety.

References

1) J. Muncke, *Sci. Total Environ.* **407**, 4549 (2009). https://doi.org/10.1016/j.scitotenv.2009.05.006.
2) B. Athenstädt, M. Fünfrocken, and T.C. Schmidt, *Rapid Commun. Mass Spectrom.* **26**, 1810 (2012). https://doi.org/10.1002/rcm.6286.
3) M. Hoppe, P. de Voogt, and R. Franz, *Trends Food Sci. Technol.* **50**, 118 (2016). https://doi.org/10.1016/j.tifs.2016.01.018.
4) K. Grob, *Food Control* **46**, 312 (2014). https://doi.org/10.1016/j.foodcont.2014.05.044.
5) Commission Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food, European Commission, The Official Journal of the European Union, 2011, 15.1.2011. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R0010&from=EN.
6) C. Bignardi, A. Cavazza, C. Laganà, P. Salvadeo, and C. Corradini, *Food Control* **71**, 329 (2017). https://doi.org/10.1016/j.foodcont.2016.07.013.
7) A.E. Lewandowska, K. Macur, P. Czaplewska, J. Liss, K. Lukaszuk, and S. Oldziej, *J. Proteome Res.* **16**, 3053 (2017). https://doi.org/10.1021/acs.jproteome.7b00366.
8) M.P. Elmiger, M. Poetzsch, A.E. Steuer, and T. Kraemer, *Anal. Bioanal. Chem.* **409**, 6495 (2017). https://doi.org/10.1007/s00216-017-0594-1.
9) D. Jenke, M. Poss, S. Sadain, J. Story, W. Smith, and D. Reiber, *J. Appl. Polym. Sci.* **95**, 1262 (2005). https://doi.org/10.1002/app.21341.
10) S. Úbeda, M. Aznar, P. Vera, C. Nerín, L. Henríquez, L. Taborda, and C. Restrepo, *Food Addit. and Contam., Part A* **34**, 1784 (2017). https://doi.org/10.1080/19440049.2017.1346390.
11) J.S. Félix, F. Isella, O. Bosetti, and C. Nerín, *Anal. Bioanal. Chem.* **403**, 2869 (2012). https://doi.org/10.1007/s00216-012-5965-z.
12) E. Canellas, P. Vera, and C. Nerín, *Anal. Bioanal. Chem.* **407,** 6781 (2015). https://doi.org/10.1007/s00216-015-8848-2.
13) G.M. Cramer, R.A. Ford, and R.L. Hall, *Food Cosmet. Toxicol.* **16**, 255 (1976). https://doi.org/10.1016/S0015-6264(76)80522-6.
14) I.C. Munro, A.G. Renwick, and B. Danielewska-Nikiel, *Toxicol. Lett.* **180**, 151 (2008). https://doi.org/10.1016/j.toxlet.2008.05.006.
15) C. Nerin, P. Alfaro, M. Aznar, and C. Domeño, *Anal. Chim. Acta* **775**, 14 (2013). https://doi.org/10.1016/j.aca.2013.02.028.

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A Multi-Analyte LC–ESI-MS/MS Method to Analyze BPA, BADGE, and Related Analytes

LCGC Europe **interviewed Stefan van Leeuwen from Wageningen Food Safety Research (WFSR), in Wageningen, The Netherlands, on his novel multi-analyte approach to investigate bisphenol A (BPA), bisphenol A diglycidyl ether (BADGE), and their analogues using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS).**

Interview by Alasdair Matheson, Editor-in-Chief, *LCGC Europe*

Stefan van Leeuwen is a senior scientist at Wageningen Food Safety Research (WFSR, Wageningen, The Netherlands). During his Ph.D. on environmental analytical chemistry at the VU University in Amsterdam, he worked on method development for new persistent organic pollutants (POPs), such as brominated flame retardants and perfluoroalkyl substances (PFASs). Over the years he has worked at NIZO food research, Wageningen Marine Research, VU University Amsterdam, and in 2011 he joined RIKILT (current name Wageningen Food Safety Research, WFSR). Research on new environmental contaminants has been the main thread throughout his career, focusing on method development and food analysis. In recent years he has started to work on processing contaminants formed when food is heated.

Q. You recently developed a technique to analyze bisphenol A (BPA), bisphenol A diglycidyl ether (BADGE), and their analogues in food and beverages (1). How did this project come about and why are these analytes being investigated?

A: BPA is used as a building block to create polycarbonate plastics. These plastics are widely used in the automotive industry and many applications, including construction, electronics, CDs and DVDs, packaging material and thermal paper, but there is an intensive ongoing debate about the safety of BPA between scientists, national authorities, industries, and food safety authorities.

We have learned from other cases, such as perfluorooctane sulfonate (PFOS) or perfluoroctanoate (PFOA), that when a substance is under discussion, chemical manufacturers may move to produce alternative substances that have similar chemical structures. There are numerous examples of these so-called *BPA-analogues,* including, bisphenol-B (BPB), bisphenol-F (BPF), and bisphenol-S (BPS).

Several studies were published that reported the occurrence of these replacement chemicals in foods and human samples showing that we are being exposed to these chemicals (2,3,4). Our group wanted to study the situation for these chemicals in The Netherlands, and we needed to design a new analytical approach.

Q. You have developed a new multi-analyte method using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). What is novel about this approach?

A: The method needed to be applicable to complex matrices found in foods and beverages. We successfully created a true multi-analyte method to analyze 23 BPA and BADGE analogues, a substantially higher number than in earlier published studies. The method also demonstrated excellent sensitivity because we choose to use alkaline MS ionization conditions rather than acidic conditions.

Q. What were the main analytical challenges you had to overcome?

A: First of all, we wanted to obtain a very sensitive method in the low-ppb range in foods and low-ppt range in beverages. We also wanted to resolve some important

structural isomers. We learned that the structural isomers 4,4'-BPA, 2,2'-BPA, and 2,4'-BPA could not be separated by MS/MS because they fragmented similarly, giving the same product ions. The same was true for 2,2'-BPF and 4,4'-BPF isomers. Therefore we experimented with three different ultrahigh-pressure liquid chromatography (UHPLC) columns with C18 stationary phases, modifiers (acetonitrile and methanol), and ionization aids (ammonium formate and ammonium hydroxide). The acetonitrile–water gradient containing ammonium hydroxide gave the best separation of 2,2-BPF and 4,4-BPF on all three columns investigated, whereas the separation of these compounds in methanol–water gradient was poor. The ionization with the ammonium hydroxide produced the best responses, and we ended up with excellent sensitivity of approx 1–10 pg on-column for most compounds included in our study.

Another difficulty we discovered is that if BADGE was present in an extract of, for example, a canned beverage, the in-source fragmentation leads to the transformation of BADGE into BPA. This BPA entered the mass analyzer and fragmented into the typical BPA fragments. We figured out that this "virtual" BPA (resulting from in-source BADGE fragmentation) eluted closely to the true BPA peak, meaning that potential misidentification was possible if no proper attention was paid to this issue. We did not need to adapt our methods in this case, but caution is needed to prevent misidentification.

Finally, all laboratories that work in the area of omnipresent environmental contaminants, such as perfluoroalkyl substances (PFASs), flame retardants, mineral oil saturated hydrocarbons-mineral oil aromatic hydrocarbons (MOSH-MOAH), and chlorinated paraffins are familiar with a major challenge, which is to keep the blanks low. Because of the wide application of these chemicals in many products, they are present everywhere, including in the laboratory environment. Dust particles contaminating your extract during sample preparation may alter the levels of your target analytes substantially, and one should take good care to work clean and avoid contamination of the sample during sample processing and analysis.

The method demonstrated excellent sensitivity because we choose to use alkaline MS ionization conditions rather than acidic conditions.

Dust particles contaminating your extract during sample preparation may alter the levels of your target analytes substantially, and one should take good care to work clean and avoid contamination of the sample during sample processing and analysis.

Q. What were your main findings?
A: Once we had the LC–MS/MS method on track, we wanted to screen a couple of real food and beverage samples. We designed a sample preparation strategy based on acetonitrile extraction (for solid samples), and sequential clean-up by mixed-mode solid-phase extraction (SPE) and silica SPE. The resulting purified extract was analyzed by our LC–MS/MS method. We did a small survey with the purpose of getting a first hint on which BPA analogues and BADGE analogues we would encounter in these food and beverage samples. 4,4'-BPA was detected in several samples, but also BPS, 2,2'-BPF, 4,4'-BPF, BADGE, and some BADGE analogues were detected. This shows that several analogues may be present in food.

It should be noted that detection of these substances does not automatically imply that there is a risk, but I would recommend researchers modify their methods to include more of these analogues. Our small-scale survey findings fit with data reported in other peer-reviewed studies on these analogues (1).

Q. Are you planning to use this multi-analyte approach for other applications?
A: The benefit of multi-analyte approaches is that it saves resources because you get more data out of the same analytical run. We therefore aim to design methods that can accommodate multiple compounds, or compound classes. In the area of environmental contaminants that enter the food chain we also use a multi-analyte approach (with tandem MS) for per- and polyfluoroalkyl substances. We analyze approximately 20 different PFASs in

a single method, and continuously look at expanding this number.

A true multi-analyte approach is the LC-high resolution (HR) MS method we are currently designing for chloroparaffins. This environmental contaminant class consists of thousands of individual homologues and isomers that we would like to capture in a single method. Such complex mixtures are extremely challenging, and so far, no laboratory has been able to design an approach to detect the individual homologues and isomers needed to study the contamination patterns in foods and to support toxicological studies. It really is like finding a needle in a haystack, or worse! We currently use a modified method originally published by Bogdal *et al.* (5) and are now able to analyze different chain lengths and chlorination degree. But even then, we do not know which positional isomers we are looking at, so more development work is highly needed in that area. Also, the MOSH-MOAH mixture originating from, for example, printing ink residues in recycled food packaging have a complex nature.

> **The benefit of multi-analyte approaches is that it saves resources because you get more data out of the same analytical run.**

Q. Have you used hyphenated tandem mass spectrometry for other areas of food and beverage analysis?

A: Most of the food control-related sample analysis in our institute is performed on gas chromatography (GC)–MS/MS and LC–MS/MS systems. These machines routinely run thousands of samples on pesticides, natural toxins, veterinary drugs, environmental contaminants, and many more compound classes. Next to that we employ the magnetic sector HRMS for dioxins and PCBs and we use the orbital ion trap mass spectrometers to analyze more complex matrices, or to work on identification of unknown compounds encountered in food or environmental samples. Hyphenation in our case also means automating sample preparation with on-line introduction of the sample into the GC–MS/MS or LC–MS/MS system.

> **Tandem mass spectrometry combines excellent selectivity and unsurpassed sensitivity and is a very versatile technique.**

Q. Do you have any practical advice for chromatographers who have not used tandem mass spectrometry before?

A: Tandem mass spectrometry combines excellent selectivity and unsurpassed sensitivity and is a very versatile technique. If you are looking for these characteristics when you design your analytical approach for targeted analysis, I would recommend tandem mass spectrometry. It is relatively easy to use and you can obtain data from multiple analytes in a single run.

Q. What other areas of food analysis are you currently investigating?

A: We are investigating a broad suite of environmental contaminants. I already mentioned the PFASs and MOSH-MOAH, and we routinely look at dioxins, PCBs, and brominated flame retardants. In recent years I have also become interested in the field of heat-induced processing contaminants. For processing contaminants (acrylamide, AGEs, 3-MCPD, and furan), we study the effect of heating of foods in relation to the production of these contaminants. Obviously, a reliable analytical approach is instrumental to that aim.

Q. What is the future for the analysis of environmental contaminants?

A: We have seen large developments in detecting and identifying new environmental contaminants since I started working in this field 20 years ago. I look forward to the next 20 years, and I think several challenges are still ahead of us. There are approximately 5000 PFASs compounds that can potentially enter the environment and we need to find ways to resolve complex mixtures, such as chloroparaffins. That will keep me busy for a while!

References

1) S.P.J. van Leeuwen, T.F.H. Bovee, M. Awchi, M.D. Klijnstra, A.R.M. Hamers, R.L.A.P. Hoogenboom, L. Portier, and A. Gerssen, *Chemosphere* **221**, 246–253 (2019).
2) X.-L. Cao and S. Popovic, *J. Food Protect.* **78**,1402–1407 (2015).
3) C. Liao and K. Kannan, *J. Agric. Food Chem.* **61**, 4655–4662 (2013).
4) H.J. Lehmler, B. Liu, M. Gadogbe, and W. Bao, *ACS Omega* **3**, 6523–6532 (2018).
5) C. Bogdal, T. Alsberg, P.S. Diefenbacher, M. MacLeod, and U. Berger, *Analytical Chemistry* **87**(5), 2852–2860 (2015).

Categorizing Olive Oil Using Untargeted GC–MS with a Multivariate Statistical Approach

Carlos Sales and Joaquin Beltrán from The Research Institute for Pesticides and Water at the University Jaume I, in Castellón, Spain, discuss a novel untargeted gas chromatography–mass spectrometry (GC–MS) method, incorporating dynamic headspace (DHS) with thermal desorption and a novel deconvolution approach, to classify olive oil.

Interview by Kate Jones, Managing Editor, *LCGC Europe*

Carlos Sales is a Ph.D. student in sciences at the Research Institute of Pesticides and Water in the University Jaume I, in Castellón, Spain. His research is focused on two main topics: GC–MS combined with a novel APCI source for the quantification of persistent organic pollutants and volatile organic compounds in complex environmental and food samples, and also the development of untargeted GC–MS and GC–HRMS techniques for the quality classification of food products.

Joaquin Beltrán is Full Professor in the Physical and Analytical Chemistry department and member of the Research Institute of Pesticides and Water of the University Jaume I. His research focuses on the use of GC–MS techniques in the lastest generation high-resolution MS instruments.

Q. Your group recently published a study on the development of an untargeted gas chromatography–mass spectrometry (GC–MS) method and statistical approach to classify olive oil quality (1). What led your group to investigate this approach?

Carlos Sales and Joaquin Beltrán: In 2012 our group started a collaboration with the Interprofesional del Aceite de Oliva Español looking for a method for virgin olive oil quality classification complementary to the official classification method, known as a *panel test*. A panel test is a human-based approach composed of a team of specialists that give a numerical value to many characteristics about flavours, synthesizing a sensory analysis. The oils are classified according to two main properties: defects (negative factors) and positive attributes (positive factors). The major sensory defects are rancid, fusty/muddy sediment,musty/humid/earthy, acetone, burnt/heated, frozen/wet wood, and winey/vinegary, and the positive attributes can be fruity (specifying green attribute), bitter, and spicy. From the beginning, we decided to develop a method based on the volatile fraction of the oil because the volatiles present in olive oil are considered to be predominantly responsible for flavour, and therefore directly related to oil classification as extra virgin or virgin. Using this perspective and taking into account the high number of volatile compounds present in olive oil (2), which can vary depending on the geographical region of the olive cultivars, the type of olives, and climatic factors, we focused on the development of a nontargeted method based on the capabilities that a novel GC–atmospheric-pressure chemical ionization (APCI) in GC–MS offers for the detection of the molecular ion for every analyte (3). Data produced with this analytical approach were treated with automatic open source MzMine 2.0 deconvolution software (http://mzmine.github.io/) to extract the relevant chemical information and then treated with multivariate statistics to define which volatiles were responsible for each olive oil class (extra, virgin, and lampante) or defect (including "rancid", "fusty", "musty', "acetone", "burnt", "frozen", and "winey").

Though the results obtained were quite promising for the quality classification of olive oils, the method had three main drawbacks.

First, the equipment used, GC–APCI-quadrupole time-of-flight (QTOF)-high resolution mass spectrometry (HRMS), is complex and not available in many routine laboratories that would find this approach useful for olive oil quality control. Second, the extraction used, based on an in-house purge-and-trap method (4) with solvent elution, is time-consuming and requires solvent evaporation prior to injection, which can discriminate some of the most volatile components. Finally, GC–APCI–HRMS lacks spectral libraries to compare the experimental spectra, which makes elucidation a complex and time-consuming step when analyzing unknown compounds. With that in mind, efforts were devoted to the development of a GC–EI-MS metabolomic approach method with a single quadrupole as analyzer, making use of dynamic headspace entrainment followed by thermal desorption to achieve the same purpose with an easier sample treatment and using a more affordable and less complex instrument (1).

Q. What are the benefits of this method over other existing methods for olive oil classification?
CS and JB: When classifying olive oils, most analytical methods rely on target analyses to distinguish between olive oils with different attributes (5). These methods focus on a determined number of predominant compounds, but miss all the chemical information not predefined (targeted) in the acquisition method. With recent advances in data treatment, nontargeted analysis has been used for the classification of olive oil, mainly by variety or by cultivar region (6). For olive oil quality classification, the preferred methods are based on headspace as the sampling technique and GC–MS or GC–FID focusing on selected compounds. The technique developed in our group relies on dynamic headspace (DHS) with thermal desorption, which allows preconcentration of the sample prior to the injection without discrimination of the low boiling point compounds.

Additionally, the use of the novel open source deconvolution software for GC–MS analysis, PARADISe (http://www.models.life.ku.dk/paradise) (based on the PARAFAC2 algorithm) offers enhanced classification results in terms of sensitivity and accuracy to be obtained. This deconvolution software uses the full spectra enclosed within a defined retention time interval, and is able to distinguish between coeluting compounds or markers. The tentative markers obtained with PARADISe are compounds instead of combinations of mass-to-charge ratio (*m/z*) ions and retention time, which reduces the data matrix and produces more robust results, as interfering ions are not picked as a potential marker. This leads to an enhanced olive oil classification together with a quick identification of most relevant markers.

Q. Can this method be used to classify any other food products?
CS and JB: We believe this method could be effectively applied to any food product where several volatile compounds could be responsible for class characteristics. Indeed, the method comes from the group expertise analyzing tomato and melon volatiles, in which traditional methods were already successful to determine the volatile organic compound (VOC) fingerprint of the different samples (7,8). This method only requires a liquid or solid matrix in which volatile compounds can define the characteristics of a class by themselves, so it could theoretically be applied to almost any vegetable and fruit matrix. It can be extended to other food products and compounds using different analytical approaches (changing both extraction and determination technologies) and thus is not always limited to volatile fraction of the samples.

Q. What were the main analytical challenges you encountered and how did you overcome them?
CS and JB: When applying dynamic headspace entrainment, all the parameters regarding volatile extraction and desorption into the system must be carefully selected. To pick the most suitable sorbent trap, extraction, and desorption conditions, we used a design of experiment (DOE) method based on response surfaces to optimize all these parameters in two days instead of evaluating the results changing one variable at a time. As no automated DHS sampler was available in our laboratory, we adapted an in-house device previously used for the analysis of volatiles in tomato and melon (4,9) to be able to work with thermal desorption traps. This allowed us to cut extraction times, as up to six different samples can be extracted at the same time. However, in routine laboratories this could be escalated (or even automated) as needed, with the chromatographic run-time the only limitation in terms of number of samples analyzed each day.

Q. Are there any problems associated with developing nontargeted methods generally for GC?
CS and JB: When developing nontargeted methods, especially using automated deconvolution

software, some considerations have to be made. First, the method must cover the largest number of compounds possible, as no information about the key compounds is known from the beginning. In our case, we decided to focus on the volatile fraction, but many scientists apply different techniques (both GC–MS and liquid chromatography [LC]–MS) to avoid discriminating against any compound. The same applies to the chromatographic run. A good compromise between chromatographic resolution and analysis time is crucial to obtain a robust method applicable when analyzing a large number of samples. Here, the use of PARADISe also helped because its peak resolution power allows up to eight coeluting peaks (10) to be discriminated. Identification of the markers is still the weakest part of the nontargeted methods and is what slows down transfer of the methods to routine laboratories.

Q. Do you have any comments on the multivariate statistical-based approach you used? Are there any novel aspects or benefits to the approach you used here?

CS and JB: Partial least squares-discriminant analysis (PLS-DA) has been extensively used for classification purposes. Though it is easy to apply and there are a lot of free software options capable of performing PLS-DA, it must be carefully applied to avoid model overfitting. In this case, the novelty is the use of PARADISe as deconvolution software because it compares full spectra within specified retention time windows, giving around a hundred variables (compounds) to work with. Other free deconvolution software, such as the XCMS package of R and MzMine2, can lead to an output matrix ranging from hundreds to thousands of variables because they detect combinations of single ions at a retention time. Such big data matrices then need extensive data treatment to avoid "ghost"and interfering ions. Additionally, a high number of variables tend to model overfitting when applying PLS-DA, which can result in a perfect classification of the samples used to create the model, but a bad classification of blind samples. It must also be noted that PLS-DA, when applied to more than two groups, can lead to misclassification of samples that actually do not belong to any of the modelled classes. In this work, with all the samples provided by official control laboratories and all being certified olive oil samples, there is no chance to misclassify an olive oil as not an olive oil. For other classifications, PLS-DA might not be the best choice, and one class models should be applied to get more reliable results.

Q. Are there any other applications where you think this method could offer the analyst improved results?

CS and JB: In the field of food studies, we have been developing new methods based on the strategy described here that have been successfully applied to the study of the classification of smoked fish depending of the smoking treatment and intensity. In this case, we again used the volatile fraction of the sample as a chemical print related to the taste and conservation of the fish.

Q. What is your group working on next?

CS and JB: We have started to treat metabolomic *in vivo* studies using laboratory organisms to reveal chemical compounds related to the behaviour of animals. These studies have only just started and include not only volatile analytes but also nonvolatile compounds that require the combined use of GC and LC coupled to MS.

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References

1) C. Sales, T. Portolés, L.G. Johnsen, M. Danielsen, and J. Beltran, *Food Chem.* **271**, 488–496 (2019). doi:10.1016/j.foodchem.2018.07.200.
2) F. Angerosa, M. Servili, R. Selvaggini, A. Taticchi, S. Esposto, and G. Montedoro, *J. Chromatogr. A* **1054**, 17–31 (2004). doi:10.1016/j.chroma.2004.07.093.
3) C. Sales, M.I. Cervera, R. Gil, T. Portolés, E. Pitarch, and J. Beltran, *Food Chem.* **216**, 365–373 (2017). doi:10.1016/j.foodchem.2016.08.033.
4) J. Beltran, E. Serrano, F.J. López, A. Peruga, M. Valcarcel, and S. Rosello, *Anal. Bioanal. Chem.* **385**, 1255–1264 (2006). doi:10.1007/s00216-006-0410-9.
5) M.T. Morales, G. Luna, and R. Aparicio, *Food Chem.* **91**, 293–301 (2005). doi:10.1016/j.foodchem.2004.06.011.
6) N.P. Kalogiouri, R. Aalizadeh, and N.S. Thomaidis, *Food Chem.* **256**, 53–61 (2018). doi:10.1016/j.foodchem.2018.02.101.
7) A. Fredes, C. Sales, M. Barreda, M. Valcárcel, S. Roselló, and J. Beltrán, *Food Chemistry* **190**, 689–700 (2016). doi:10.1016/j.foodchem.2015.06.011.
8) J. Beltran, E. Serrano, F.J. López, *et al.*, *Anal. Bioanal. Chem.* **385**, 1255 (2006). doi:10.1007/s00216-006-0410-9
9) A. Fredes, C. Sales, M. Barreda, M. Valcárcel, S. Roselló, and J. Beltrán, *Food Chem.* **190**, 689–700 (2016). doi:10.1016/j.foodchem.2015.06.011.
10) L.G. Johnsen, P.B. Skou, B. Khakimov, and R. Bro, *J. Chromatogr. A* **1503**, 57–64 (2017). doi:10.1016/j.chroma.2017.04.052.

Determination of Pyrrolizidine Alkaloids in Plant Material Using SFC–MS/MS

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Pyrrolizidine alkaloids (PAs) are heterocyclic secondary plant metabolites and many of them have been demonstrated to exhibit hepatotoxic and cancerogenic activity. Estimates indicate that about 3% of the world's flowering plants—about 6000 species—produce pyrrolizidine alkaloids, making them one of the most important classes of naturally occurring toxins. Long-term exposure to PAs in food, beverages, or phytopharmaceuticals is a possible concern for human health and, according to the German Federal Institute for Risk Assessment (BfR), can lead to severe hepatitic damage. Therefore, the Panel on Contaminants in the Food Chain (CONTAM) have proposed a list of PAs to be monitored in foodstuffs (1,2). PAs display a wide structural diversity. Several hundreds have already been identified with novel structures continuously being discovered. Pyrrolizidin alkaloids consist of a necine base, typically including pyrrolizidine, esterified with a necic acid. When they also carry a 1,2-double bond as well as an esterified side chain, they exhibit hepatotoxic activity (3).

Since some of the analytes are isomers that cannot be distinguished by different mass, they have to be separated chromatographically. LC–MS/MS is the standard method for determination of PAs. However, separation of these compounds often poses a challenge. SFC offers complementary chromatographic selectivity to reversed-phase LC and an advantage for separation of stereoisomers, shown here in the development of a separation method for determination of 34 PAs including five lycopsamin and two senecionin isomers in tea samples.

System Configuration

The method of choice is SFC using the Nexera UC system coupled with a tandem mass spectrometer LCMS-8060 (Shimadzu Corporation) (Figure 1), which has been optimized to obtain baseline separation of all isomers in the mixture in just 8 min using a 100 × 3.0 mm, 3-µm Chiralpak IG-3 column and a mobile phase modifier consisting of 50 mM ammonium formate and methanol. For hyphenation to MS from SFC, a direct transfer with a heated backpressure regulator (BPR) is most beneficial in terms of stable spray formation, sensitivity, and robustness. However, this is only possible when the BPR volume is low enough to avoid causing any peak dispersion in the flow line.

Sample Preparation: The tea samples were extracted twice with 0.05 M sulfuric acid by sonication, followed by centrifugation. The pH of the combined extracts was neutralized with ammonium hydroxide before the samples were subjected to SPE (4). After elution the extracts were dried in a stream of nitrogen at 50 °C for about 60 min. The samples were reconstituted with 1 mL methanol, mixed thoroughly, and centrifuged for 10 min before transfer to SFC–MS analysis.

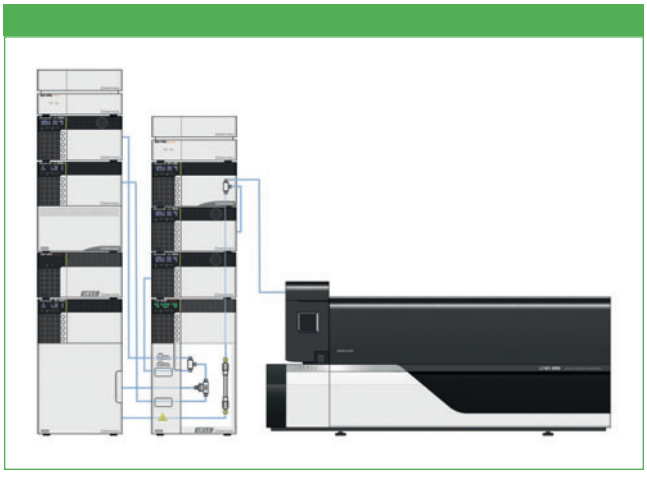

Figure 1: Setup of the SFC–MS/MS analytical system.

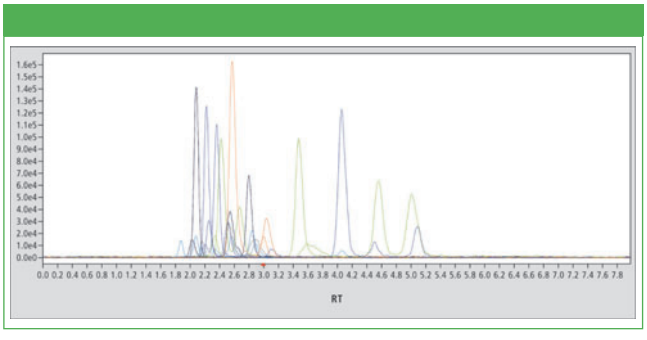

Figure 2: Typical chromatogram of the SFC–MS analysis of 34 pyrrolizidine alkaloids.

Analytical Conditions:

SFC Method

Instrument: Nexera UC (Shimadzu)
Analytical column: 0.3 × 10 cm, 3-µm SFC CHIRALPAK®, IG-3, Daicel
Mobile phase A: CO_2
Mobile phase B: 50 mM ammonium formate in methanol
Mobile phase C: methanol
Mobile phase D: 0.1% formic acid in methanol (make-up flow)
Flow rate: 1 mL/min
Column temperature: 35 °C
BPR pressure: 100 bar
BPR temperature: 50 °C
Time program: 8 min ternary gradient, optimized for separation of PA isomers

MS Conditions:

Instrument: LCMS-8060 (Shimadzu)
Ionization: HESI (positive)
Nebulizing gas flow: 3.0 L/min (N_2)
Drying gas flow: 3.0 L/min (N_2)
Heating gas flow: 17.0 L/min (Air)
CID gas: 270 kPa
Interface voltage: 1 kV
Interface temperature: 400 °C
DL temperature: 250 °C
Heat block temperature: 500 °C

SFC-MS/MS Method Development

The main mobile phase used for SFC is supercritical carbon dioxide, to which polar organic solvents (modifiers) are added for control of solubility and polarity. Ionic additives in aqueous or organic solution can also be used to adjust selectivity. Method scouting for the 34 different PAs was performed by testing 32 combinations of stationary and mobile phases (a set of four different columns from the Daicel CHIRALPAK® series and eight different modifiers). After identification of the most suitable combination for separation of the 34 PAs, gradient conditions, flow rate, and column temperature were optimized to achieve baseline separation of all isomers in a minimized overall analysis time (Figure 2).

Quantitative Analysis of Tea Samples

Using the reported instrument set-up, quantification of 18 PAs and 16 of their related N-Oxides could be achieved. Calibration curves in black tea matrix (determined in duplicate) showed good precision and accuracy. Even in a complex matrix like tea, it was possible to quantify the PAs easily in the range of at least 2 to 200 µg/kg. For all analytes, weighted regression resulting in $r^2 > 0.99$ were obtained, with $S/N > 10$ for LLOQ levels. Exemplary calibration curves are displayed in Figure 3.

In an application example, a total of 10 commercially available tea samples were analyzed. In four of the 10 samples, one or more pyrrolizidine alkaloids could be detected above their LLOQ. Europine, heliotrine, lasiocarpine, and their related N-oxides were present in one sample, while lycopsamine, echinatin and their related N-oxides were present in three samples.

Conclusion

An SFC–MS/MS method for high-sensitivity analysis of 34 PAs in plant material was developed that achieves baseline separation of five lycopsamin and two senecionin stereoisomers in 8 min. Applicability to food samples could be established by determination of PA content in commercial tea samples. The SFC–MS method detected a number of PAs at lower levels than an optimized UHPLC–MS assay, due to increased MS sensitivity.

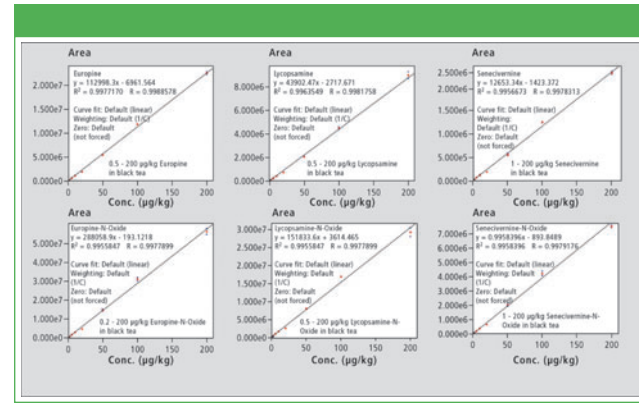

Figure 3: Exemplary calibration curves in black tea matrix.

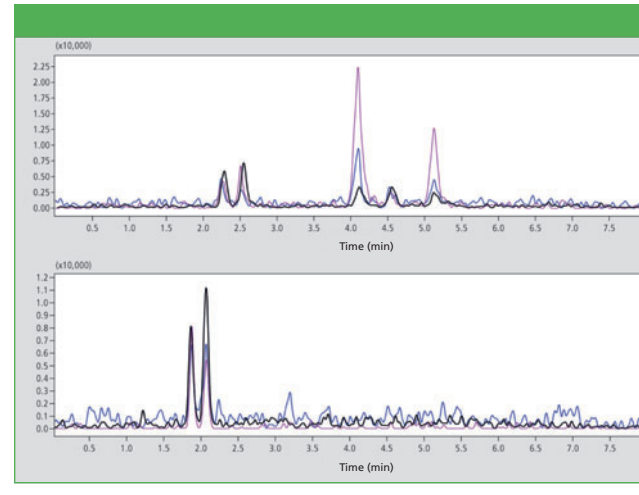

Figure 4 Chromatograms of the separation of Lycopsamin and Senecionine isomers.

References

(1) BfR Opinion 018/2013: Pyrrolizidin alkaloids in herbal teas and teas: https://mobil.bfr.bund.de/cm/349/pyrrolizidine-alkaloids-in-herbal-teas-and-teas.pdf

(2) *EFSA Journal* **15**(7), 4908 (2017).

(3) S. Schramm, N. Köhler, and W. Rozhon, *Molecules* **24**, 498 (2019). DOI:10.3390 / molecules24030498

(4) Method description BfR-PA-Tee-2.0/2014; Determination of pyrrolizidine alkaloids (PA) in plant material using SPE-LC-MS/MS

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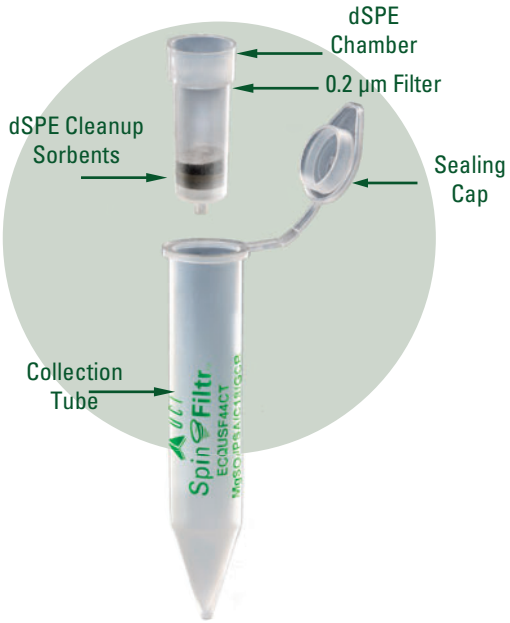

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