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Plant Metabolomic Workflows Using Reversed-Phase LC and HILIC with ESI-TOF-MS

In the field of metabolomics, researchers seek to acquire almost complete information about the metabolic composition of a sample to provide fundamental information about the cellular state of organisms. In metabolomics analysis today, typically reversed-phase (RP) liquid chromatography (LC) is coupled with specific, sensitive, and robust mass spectrometry (MS). That approach, however, misses many moderately polar, and all very polar, compounds; this situation is a problem in plant metabolomics, because plant metabolites are mainly water-soluble species and thus very polar. Here, we describe new developments in polarity-extended separations using the serial coupling of reversed-phase LC and hydrophilic-interaction chromatography (HILIC) separation steps, in combination with electrospray ionization-time-of-flight-mass spectrometry (ESI-TOF-MS), and the application of this approach to plant metabolomics. The resulting retention time versus mass plots are molecular fingerprints, as well as sources of further molecular descriptors. Extraction methods, molecular analysis, and data evaluation have to be adapted to the matrix under consideration. Representative strategies using this polarity extending approach, following so-called *suspects* and *nontargeted screening* approaches, are presented.

Rofida Wahman, Johanna Grassmann, Peter Schröder, and Thomas Letzel

Plant metabolomics, which is a comparatively young field, aims to provide almost complete molecular coverage of plant metabolites, to establish fundamental information about the plant under investigation based on changes in its metabolism (1,2). Plant metabolites can be roughly divided into primary and secondary metabolites. Secondary metabolites, although not used during plant growth and development, are important, because of their usage in plant defense, food, and medicine. Moreover, secondary metabolites play a substantial role in the adaptation of plants to environmental stress (3). Plants respond to exogenous factors through signaling pathways that induce downstream stress responses, including the modulation of gene expression and the regulation of a wide range of biochemical processes, ending with a remodeling of the metabolism (4).

Lemna minor (duckweed) is sometimes used as a model plant in plant metabolomics studies. It has also been proposed for phytoremediation of heavy metals in a glass house experiment (5). Additionally, *L. minor* has medical importance (6). Compared to other plant species, duckweed has a simple structure and rapid growth. Furthermore, *L. minor* is easy to harvest.

Targeted, suspects, and nontargeted screening strategies can be used for plant metabolite analysis (7). Targeted screening, formerly known as *quantitative analysis*, observes analytes using a reference substance. Compound identification and quantification has to be validated with isotopically labeled reference substances using mass spectrometry (MS) detection. Suspects screening typically is performed with accurate-mass and high-resolution mass spectrometry (HRMS) to observe the empirical formula of each formula present, or with tandem MS to observe specific fragment spectra. Subsequently, the empirical formula can be compared with chemical databases, such as Chemspider (http://www.chemspider.com), Chemicalize (https://chemicalize.com), or STOFF-IDENT (SI; https://www.lfu.bayern.de/stoffident), and with mass-spectrometric databases containing analytically observed mass spectra (such as MassBank, https://massbank.eu/MassBank/), or local databases in laboratories. If these

databases lead to clear hits, one then has to validate the observation by an equivalent reference substance. Provided that the identity is proven, one can use it further in targeted screening analysis applying isotopically labeled standards. As stated before, this approach may require HRMS to generate the empirical formula of expected substances.

HRMS instrumentation is strictly needed when nontargeted screening is used as an analytical strategy. This screening strategy is split into screening for so-called *hidden targets* (also referred to as *known unknowns*) and *unknown targets* (also referred to as *unknown unknowns*). The hidden-target screening approach is similar to suspect screening, but starts without a list of metabolites or analytical databases. However, nontargeted screening data frequently can be compared with data in chemical and analytical databases (especially in retrospective analyses). For this purpose, analytical platform solutions are needed to handle nontargeted screening data. FOR-IDENT (FI; https://water.for-ident.org) is an open-access example of such a platform; it enables data evaluation by retention time index, accurate mass, and other important features of the molecule analyzed. The core of FOR-IDENT is a compound database called STOFF-IDENT, which is filled with anthropogenic compounds relevant in the aqueous environment. Another compound database under development is PLANT-IDENT, which contains general plant metabolites and includes exact masses, as well as other analytical results, and makes use of a retention index tool; this database is expected to encourage nontargeted screening in plant metabolomics.

Detailed metabolomics strategies (also for so-called *unknown unknowns*) are described in a 2014 paper (8). The nontargeted screening approach is encouraged by recent advances in HRMS that provide increased mass resolution and accuracy, enabling the identification of metabolites often simply by their accurate mass determinations, and by developments in tandem MS that enable the determination of accurate fragment spectra (containing additional structural information) (9). Gas chromatography–mass spectrometry (GC–MS) and reversed-phase liquid chromatography–mass spectrometry (LC–MS) are the methods of choice for qualitative and quantitative metabolomics analysis. The ultimate aim of nontargeted screening is to be able to accurately detect, monitor, and (eventually) identify every relevant metabolite in plant extracts, which cannot be achieved by any single existing analytical method. The methodology has to be thoughtful, from initial solvent extraction through chromatography via ionization and MS to data evaluation. Recently, plant metabolites analysis has been carried out through direct examination of crude extracts or after reversed-phase LC separation coupled with quadrupole MS, time-of-flight MS (TOF-MS), or

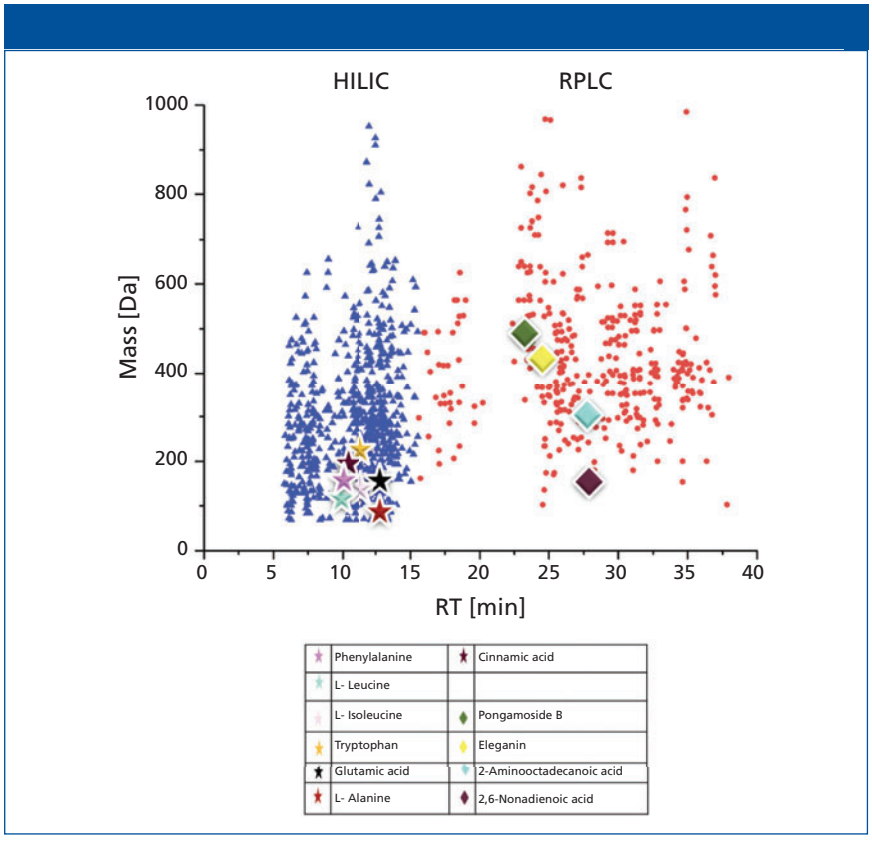

Figure 1: Retention time versus mass plot of *Lemna minor* extract (of an untreated sample). Blue triangles represent hydrophilic interaction liquid chromatography (HILIC)–retained molecules eluted from 0 to 15 min; red circles represent reversed-phase LC–retained molecules eluted from 16 to 38 min. Different colored and shaped dots represent examples of identified (stars) and expected (diamonds) compounds, as indicated in the legend.

other MS techniques, like ultrahigh-resolution Fourier transform ion cyclotron MS (FT–MS) (1).

The monitoring and identification of (very) polar compounds is important, because these compounds play a vital role in plant metabolic pathway changes. The use of hydrophilic interaction liquid chromatography (HILIC) columns in plant metabolomics analysis has facilitated the retention of polar and very polar compounds in a chromatographic column prior to elution into the mass spectrometer (10). The demand for a new analytical method that can identify a plant's metabolites in a single run has been answered previously (for red wine analysis [11]). The polarity-extended serial coupling of reversed-phase LC and HILIC, in combination with HRMS (12), allows for the robust and repeatable analysis of a large variety of compounds in a single run (13,14), and has been applied in several disciplines (11,15–18). In such polarity-extended studies, a new extraction method for sample preparation was confirmed that it is applicable to polarity-extended chromatography analysis (19). The current study focuses on the use of serial coupling of reversed-phase LC and HILIC, coupled to high-resolution TOF-MS and general data evaluation strategies for plant metabolomics, applied to the analysis of *L. minor* extracts. Therefore, samples were used before and after plant incubation with an exemplary (model) pharmaceutical that can influence the metabolism of the treated plants. Accordingly, new insights are provided, using accurate-mass MS and HRMS as well as novel data evaluation workflows.

Experimental

Reagents and Chemicals

Methanol and water were obtained in LC–MS grade from VWR (Darmstadt, Germany). *L. minor* was kindly provided by the German Research Center for Environmental Health, Plant Microbe Interactions, Helmholtz Centrum of Munich. Amino acids standards were obtained from Sigma (Missouri, United States).

Sample Preparation

First, 500 mg of freeze-dried and milled *L. minor* (incubated with and without 10 µM of diclofenac, respectively) were extracted with 5 mL of methanol. Extraction solvent was sonicated at 35 KHz (Sonorex super RK 106, Bandelin, Germany) with plant material for 10 min at 4 °C. Afterwards, the samples were centrifuged at 1500 rpm for 20 min, and the supernatant was transferred to a test tube. The extraction method was triplicated under exactly the same experimental conditions. Finally, the extracts were evaporated to dryness (using SpeedVac Fischer Scientific, Sweden), and redissolved in (50:50) methanol:water. The standard solutions and the solvent extract were injected three times, respectively.

Reversed-Phase LC and HILIC combined with ESI-TOF-MS

The polarity-extended serial coupling or reversed-phase LC and HILIC was connected via a Jet Stream ESI interface to an Agilent 6230 TOF-MS instrument (both Agilent Technologies, Santa Clara, California, United States), as described recently (11). Column effluent was coupled via a T-piece to an isocratic pump (Agilent Technologies, Waldbronn, Germany), which provides a constant flow of reference solution for MS calibration prior entering the ion source of the MS. The separation system consisted of an autosampler, a column oven, two columns, two binary pumps, and an ultraviolet (UV) detector (all Agilent Technologies, Waldbronn, Germany). The initial binary pump was connected to a non-polar 120 EC-C18 Poroshell column (Agilent Technologies, Waldbronn, Germany). The outlet of this column

was connected to a ZIC-HILIC column (Merck, Darmstadt, Germany), and to a second binary pump via a T-piece (Upchurch Scientific, IDEX, Illinois, USA). Ions were detected in positive ionization mode, with a mass range of 50–2100 Da. The instrument resolution was greater than 10,000 at *m/z* 922. The parameters were as follows: 325 °C gas temperature, 10 L/min drying gas flow, 325 °C sheath gas temperature, 7.5 L/min sheath gas flow, 45-psi nebulizer operating pressure and 100 V fragmentor voltage.

Data Processing

The data were evaluated with Agilent MassHunter Profinder B.08.00, Mass Profiler, and Mass Profiler Professional (MPP) 13.1.1 software. Profinder parameters applied for the automation of compound retention times (t_R), and extraction and molecular weights, were set to a peak filter of 300 counts peak height, ion species to "positive ions" with H^+, Na^+ and K^+, "charge state" to 1, and the "expected retention time" to ±3.00 min. The extracted ion chromatograms (EICs) were smoothed with a Gaussian function, using 9 data points width and 5000 points Gaussian width. Then, the "features" from the blanks dataset were deleted from sample datasets. These parameters limit the result for 2000 compound groups. The data set was exported to MPP. In MPP, the compounds were aligned. Subsequently, the retention times and masses were corrected, and C, H, O, N, and S atoms were used to compute the empirical formula of the compounds. The compounds' intensity logarithmic fold changes between the two samples are calculated and subsequently are drawn as a scatter plot. The calculated logD at pH 7.4 was based on the retention time/logD (pH 7) calibration curve of twelve different standards (18,20). The logD (pH) values were predicated from ChemAxon software (https://disco.chemaxon.com/apps/demos/logd/) and then exported to Windows Excel 2016 for further data evaluation. For hidden-target screening, the amino acids standards were organized, and injected three times. The mean of each of the standard masses (S) and t_R values (S) were calculated in daltons and in minutes, respectively. The variation between the extract and the standard mean isotopic masses (Δ ppm) was calculated according to following equation:

$$\Delta ppm = \frac{(mean\ of\ Standard\ masses - mean\ of\ Sample\ masses)}{mean\ of\ Sample\ masses} \times 10^6 \quad [1]$$

Moreover, the standard deviation (SD) of t_R and relative standard deviation (RSD) were calculated.

Percent of RSD = SD of compound t_R values in different injection divided by the mean of compound t_R values.

The nontargeted measurements were evaluated through FOR-IDENT (FI; https://water.for-ident.org) and Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=batch_search) databases to search for expected and hidden targets ("known unknowns"). The workflow was conducted in the steps described below.

First, the list of accurate masses was uploaded into the database. The matching features were downloaded with their physiochemical properties. For FOR-IDENT results, the feature hits below a retention time of 15 min were filtered by excluding positive logD values. The amino acids were confirmed by reference standard injection (category 1) (21,22). However, cinnamic acid and other hits from the Metlin database remained as suspects (category 2) (21,22).

The log2-normalized data were used in statistical analysis to improve normality and group 2724 metabolites. Differences between the two samples were considered significant when the *P* value (calculated using a Student's *t*-test) cut-off was 0.05. The principal component analysis (PCA) was done using the multivariate analysis of OriginPro 2017. The heatmap was developed using MPP of the same data set as used in the PCA analysis.

Results and Discussion

Formerly, the serial coupling of reversed-phase LC and HILIC with ESI-TOF-MS was established for trace organic compound analysis in wine samples (10), water samples (15), and oxidative (17) as well as enzymatic conversion screening samples (18). More recently, this coupling is also being applied in plant metabolomic studies, such as to study metabolic changes caused by stress or by external compounds. *L. minor* is a good model plant for such studies and can easily be incubated, extracted, and analyzed.

The TOF-MS technique, and its specificity, allow accurate compound detection over a broad mass range (in different matrices). The so-called *nontargeted screening strategy* was performed in this study in positive ionization mode and with a mass range of 50–2100 Da. Concerning a typical methanol extract analysis of untreated *L. minor* samples with the serial coupling of reversed-phase LC and HILIC with ESI-TOF-MS, feature extraction was possible as shown in Figure 1. For such a feature, the retention time (t_R), the accurate mass, and the signal intensity were extracted from a total ion chromatogram (TIC), each reflecting an independent (but still unknown) molecule. More than half of the isolated compounds (686) were retained and separated on the (very) polar HILIC column (blue colored triangular compounds in Figure 1; they are located with a retention time between 5 and 15 min, and have a logarithm of distribution coefficient logD [pH 7] < 0). In addition, 383 compounds were separated by reversed-phase LC (nonpolar) (red-colored circles compounds in Figure 1; they were eluted later than 16 min, and have logD values [pH 7] > 0), respectively. The empirical formula could be predicted for most features. The data sets were evaluated via nontargeted screening strategies as described above in the introduction and references therein.

First, the nontargeted screening data were evaluated by exporting results to the open-access platform FOR-IDENT. There, the features were uploaded and the normalized retention time (t_R) and accurate mass were compared with the compound database STOFF-IDENT (containing anthropogenic compounds expected in the aqueous environment). This search yielded several hits of suggested compounds with respective empirical formula and logD values. Applying this strategy resulted in hits and suggested compounds containing amino acids and organic acids (see Figure 1; specifically, molecules labeled with stars and included in the table). For ex-

Table I: List of example amino acids found in *Lemna minor* extract with the mean monoisotopic mass of reference standard (S), extract (M), the variation between them; mean retention time (t_R) of reference standard (S) and the extract (M) and the variation between them.

Compound Name	Mean Monoisotopic Mass (Da) (S)	Mean Monoisotopic Mass (Da) (M)	Δ ppm	Mean t_R (Min) (S)	Mean t_R (Min) (M)	Δ t_R
L-Isoleucine	131.0944	131.0947	-2.29	10.82	10.83	-0.01
L-Leucine	131.0941	131.0945	-3.05	10.79	10.83	-0.04
Phenylalanine	165.0787	165.0789	0.081	10.71	10.72	0.00
Tryptophan	204.0898	204.0899	-0.26	11.45	11.61	-0.15
Glutamic acid	147.0529	147.0525	2.49	12.63	12.71	-0.08
L-Alanine	89.0477	89.0476	1.12	12.84	12.93	-0.09

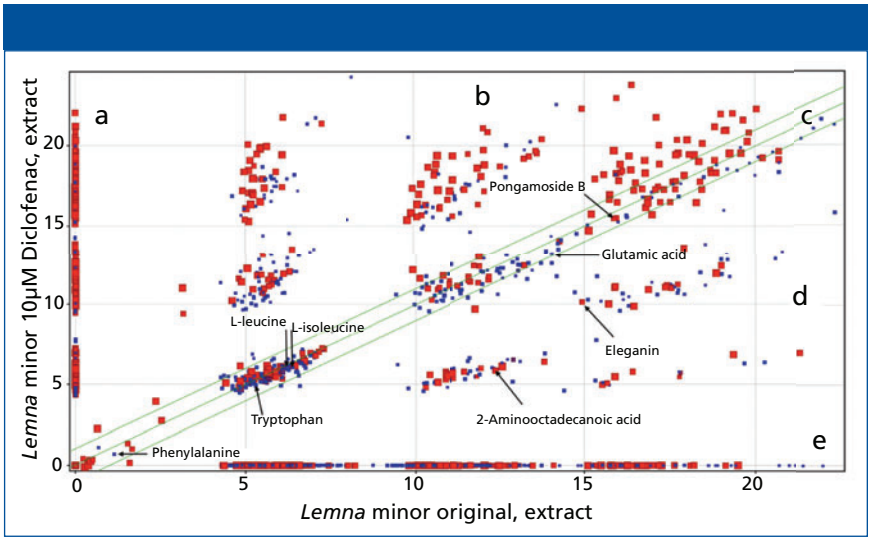

Figure 2: Feature (signal intensity) comparison plot of an extract using original untreated *Lemna minor* sample versus an extract of *L. minor* sample treated with 10 µM of diclofenac. Examples of found amino acids and expected compounds are found in both samples. Small blue squares represent retention time (t_R) (0–15 min) and big red squares represent the t_R (16–38 min) of (a) compounds only found in the *Lemna* sample treated with 10-µM diclofenac extract; (b) compounds found in both samples with increased feature intensity in treated compared to untreated *Lemna* samples; (c) compounds found in both samples; (d) compounds found in both samples with decreased feature intensity in treated compared to the untreated *Lemna* sample; and (e) compounds only found in the untreated *Lemna* sample extract.

ample, glutamic acid and cinnamic acid were the only hits in STOFF-IDENT for 147.0532 Da and 148.052 Da, respectively. Thus, one can confirm the identity of both very quickly and easily by measuring the respective reference substance for each compound.

However, the process is not always that simple. For example, the mass of 165.079 gives 10 matches, of which nine have a positive logD value, and one a negative value. Thus, the nine matches could be deleted, because the feature eluted in the negative logD region was retained by HILIC. The remaining hit (after deletion) was the amino acid phenylalanine and could easily be evaluated further by comparing to reference material.

Sometimes the use of compound databases does not lead to such unequivocal results. For example, a feature at 89.048 Da resulted in five hits. After excluding the two positive logD values, three hits were remaining with negative logD values. L-alanine was confirmed by a reference standard also eluting in the HILIC region. In general, this strategy leads to explicit and filtered suggestions for compound identities.

Furthermore, tryptophan, leucine, and isoleucine (as shown in Figure 1) were identified similarly. After excluding the positive logD results, the remaining hits with negative logD values were either the corresponding amino acid, or various synthetic compounds. Later hits are typically anthropogenic (brought into the environment), and cannot be originated in the plant, thus they were neglected in this study. Reference standards for the remaining amino acids were injected to prove the identity and presence in the *L. minor* extracts, which also were reported previously in the literature (24). Finally, for the successful application of the hidden-target screening strategy (7) using the compound database STOFF-IDENT, amino acid standards were available, directly confirming the hits of expected amino acids in *L. minor* methanol extracts. The amino acids identified are phenylalanine, L-leucine, L-isoleucine, tryptophan, glutamic acid, and L-alanine, as labeled in Figure 1. The amino acid standards were injected in triplicate, and the mean of each standard mass (S) and retention time were calculated. The identification was done through comparing the differences between the mean retention times of the standard and extract, which was in the range of 0 min (phenylalanine) to 0.15 min (tryptophan). Moreover, the mass variation between the standard mass (S) and the extract mass (M) is less than 5 ppm. In Table I, the six identified amino acids were listed with their mean isotopic mass and mean retention times. In addition, standards mean isotopic mass and retention times were calculated for the three injections.

Table II: Examples of compounds found in *Lemna minor* extract using a hidden-target screening strategy, showing the monoisotopic mass in literature (L), mean monoisotopic in extract (M), the variation between them, the mean retention time (t_R) of the extract (M), standard deviation, relative standard deviation, logD in predicted (P), and logD experimental (E).

Compound Name	Monoisotopic Mass (Da) (L)	Mean Monoisotopic Mass (Da) (M)	Δ ppm	Mean t_R (Min) (M)	SD of t_R (Min)	RSD	Log D (P) pH 7.4	Log D (E) pH 7
Cinnamic acid	148.0524	148.052	2	10.72	0.033	0.003	-0.81	-0.32
Pongamoside B	470.1213	470.122	1	23.29	0.084	0.004	0.40	0.97
Eleganin	434.1577	434.159	3	24.34	0.029	0.001	0.76	1.3
2-Aminoocta-decanoic acid	299.2824	299.282	1	27.61	0.034	0.001	3.91	2.31
2,6-Nonadienoic acid	154.0994	154.0995	2	27.00	0.016	0.001	1.3	2.12

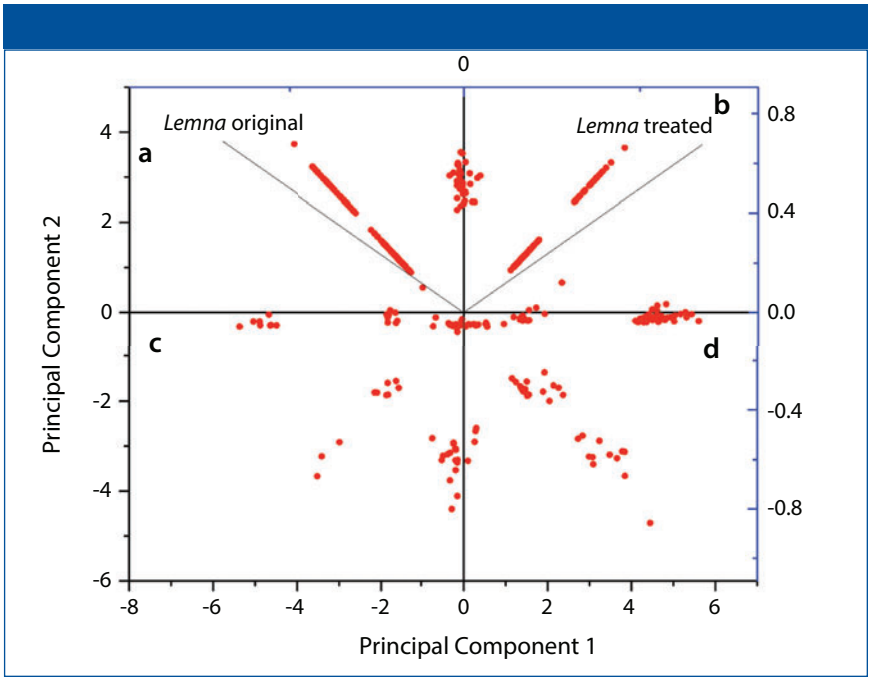

Figure 3: Principal component analysis (PCA) plot of features occurring in untreated *Lemna minor* sample extract versus *L. minor* sample extract treated with 10-µM diclofenac: (a) Dots represent the compounds related positively to untreated sample and negatively to treated sample; (b) dots represent compounds that are positively related to both samples; (c) dots represent compounds that are negatively related to both samples; (d) dots represent compounds related positively to treated sample and negatively to untreated sample.

The identification was done using the following workflow. The differences in t_R (Δt_R) between the standard and the extract were in the range of ≈0–0.15 min. The variations between the extract and the standard mean isotopic masses (Δ ppm) were calculated in the six amino acids. The Δ ppm values were in the range of –3.05 for L-leucine to +0.08 for phenylalanine. All the amino acids were in the same retention time and mass range as formerly reported for the serial coupling of HILIC and reversed-phase LC in an aqueous environment (15). Also, without using tandem MS (and its significant fragment spectra) the hits resulted in category 1 of the identification scheme published earlier (21,22).

Generally, nontargeted screening data from plant extracts can be compared with an available metabolomics compound database like Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=batch_search) to find expected molecular hits using the hidden-target strategy described previously. A disadvantage compared to the FOR-IDENT platform is that other databases like Metlin have no automated comparison functionality. In applying the Metlin database, four compounds were identified by manually comparing the t_R and logD of the compounds with the literature; these compounds were pongamoside B, eleganin, 2-aminooctadeconic acid, and 2,6-nonadienoic acid (Figure 1). These compounds and cinnamic acid (additionally observed in FOR-IDENT) were not yet confirmed by standards; thus, these compounds remain in category 2 as suggested by references (21,22). However, the four compounds presumably identified through Metlin and cinnamic acid by the same workflow are listed in Table II. The variation between measured monoisotopic mass and the mass found in the literature is less than ±4 ppm. Moreover, the standard deviation (SD) of t_R and relative standard deviation (RSD) were calculated. The all-compounds RSD values were <0.1% for the three injections, which indicates the reproducibility of the LC method, and as formerly reported for the serial coupling of HILIC and reversed-phase LC (15). Consequently, the t_R for each compound could be used in the standards t_R indices calibration curve to calculate the logD (pH 7) (20,25). The lower polarity limit of polar compounds was set to a logD (pH 7) value of zero because of the reversed-phase LC column used; therefore, it can likely retain compounds above this polarity. The polarity region below logD zero in this study is restricted to HILIC (15). Pongamoside B, eleganin, 2-aminooctadeconic acid, and 2,6-nonadienoic acid were retained by reversed-phase LC, thus their t_R values were longer than 15 min. The experimental logD values of suspect compounds were calculated

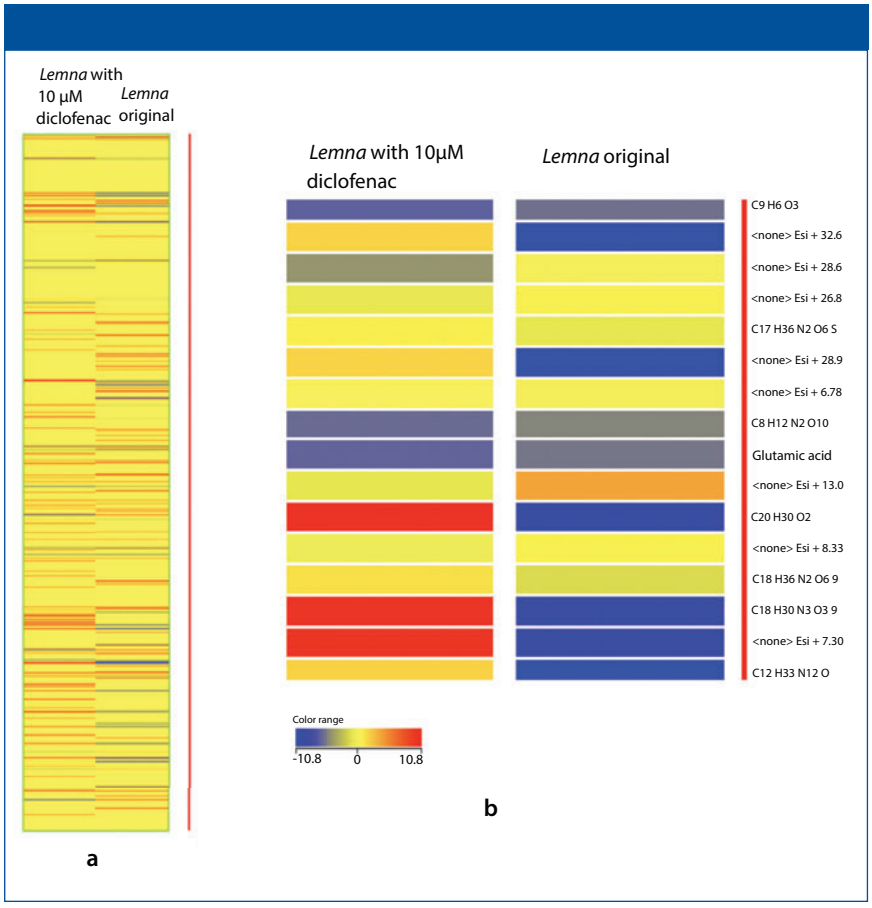

Figure 4: (a) Heatmap plot of different compound intensities in untreated *Lemna minor* sample extract versus *Lemna minor* sample extract treated with 10 µM of diclofenac (with glutamic acid as an concrete example). (b) Magnified view of a part of the heatmap. Red color indicates higher intensity of compound in the sample. The compound intensity is ≤10.8. However, the blue color indicates lower intensity, which means intensity is ≥ −10.8. The yellow color indicates the absence of compound in the sample, which means the intensity is equal to zero.

from the 12 standards calibration curve of logD (pH7) and retention time index. The difference between experimental and predicated logD (pH 7) values was in the range of 0.5 (cinnamic acid) to 1.6 (2-aminooctadecanoic acid). Subsequently, the calculated logD values for all compounds are compatible with the literature logD values. In consideration of supporting parameters, such as variation between masses, t_R, and logD values of cinnamic acid, pongamoside B, eleganin, 2-aminooctadeconic acid, and 2,6-nonadienoic acid were presumably identified through the Metlin database using the hidden-target screening strategy.

However, identifying molecules and "calling them by name" often is not the goal of nontargeted screening measurements or workflows. Moreover, nontargeted screening of the total sample may inform researchers about recent changes in the metabolome in combination with statistical tools and graphical visualization. For instance, direct feature comparison (19), PCA (26), heat plots (27), and clustering (28) were used to reduce and visualize the complex metabolomics datasets (23).

A study in the laboratory using the model plant *L. minor* was performed by incubation with 10 µM of the pharmaceutical diclofenac, which may be enriched in the plant, metabolized in the plant, or change the plant's metabolic pathways. The latter can be monitored in principle by nontargeted screening measurement and subsequent application of statistical tools like the above stated direct sample comparisons or PCA.

In sample comparisons, features were found reflecting *L. minor* samples with and without pharmaceutical incubation, which were located on the *y*-axis in Figure 2a, and on the *x*-axis in Figure 2e, respectively. Other compounds were not affected by incubation, and had the same signal intensity in the untreated as well as treated samples, thus located in the middle part of the comparison plot (see Figure 2c). However, some compound intensities were increased due to incubation, and are located in the upper part in Figure 2b. Others were decreased due to incubation, and are found in the lower part of Figure 2d.

The category 1 identified phenylalanine, tryptophan, L-leucine, glutamic acid, L-isoleucine, and category 2 identified pongamoside B were found in the two samples with the same intensity (see Figure 2c). Consequently, incubation with diclofenac or its degradation products might not affect their biosynthetic pathways. However, the incubation of *L. minor* with diclofenac caused decreases in the intensity of some compounds. Category 2 identified eleganin and 2-aminooctadececanoic acid have a higher intensity in the original samples, indicating that diclofenac or its degradation products affected their biosynthetic pathways. Accordingly, incubation of *L. minor* with 10-µM diclofenac causes changes in its metabolome by altering the intensity or disappearance of the compounds.

PCA of the samples showed that samples were grouped into two groups (Figure 3). *L. minor* with 10-µM diclofenac was related to principal component 1 (PC1), whereas the original untreated sample was related to principal component 2 (PC2). The dots (features) located in the upper left part (the positive part of principal component 2) represent the compounds related to the untreated sample (see Figure 3a). Also, the compounds related positively to both samples are located in the upper right part of Figure 3b. Furthermore, the compounds related negatively to both samples are located in lower left part of Figure 3c. However, the compounds related to treated samples are drawn in the positive region of PC2 (see Figure 3d).

In addition, the heat map of the *L. minor* with 10-µM of diclofenac and the original samples presented the compounds intensities in the two samples.

The blue color indicates that the compounds have low intensity and the red color indicates that the compounds have higher intensity. As an example, glutamic acid was found with low intensity –5.81 and –6.62 in *L. minor* original untreated and treated with 10 µM of diclofenac, respectively (Figures 4a and 4b).

Conclusion

Advances in MS are driving nontargeted screening by generating an accurate empirical formula, using tandem MS with structural information observed by molecule fragmentation (not in this study), and normalizing retention times and correlating the latter with logD values. Thus, the application of data evaluation platforms and compound databases can be helpful in identifying compounds that lack reference standards, by searching for the exact masses in a hidden-target screening approach, and to analyze suspect metabolites in nontargeted screening. A new database resembling FOR-IDENT, that will focus on plant metabolites, will be launched. The new database, PLANT-IDENT, will have the same concept and advantages of the FOR-IDENT platform. Moreover, the new database will decrease the number of hits compared to chemical databases like Chemspider.com, and thus will avoid many false positive results. The launch of this database will help researchers who use highly sensitive mass spectrometers in identification of plant metabolomics through nontargeted screening.

In this study, extracts containing untreated or pharmaceutical-incubated *L. minor* plants were investigated with nontargeted screening strategies like hidden-target screening. The applied workflow in *L. minor* metabolite analysis will be a touchstone for subsequent research. Even when using different mass spectrometers, researchers can apply this workflow with or without reference materials to identify suspect and hidden targets. The statistical nontargeted screening workflow was conducted through different statistical tests to monitor metabolite differences between different samples. In addition, it allows the monitoring of metabolites changes. Studies that are more complex might require more complex or combined statistical tools.

Acknowledgments

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A Robust and Sensitive Method for Detecting Glyphosate and Other Polar Pesticides in Food and Water: Multiple Analytes in a Single Injection without Derivatization

Monitoring a highly polar, small organic pesticide such as glyphosate in food and water sources presents a significant challenge. Polar pesticides are not amenable to standard extraction procedures, are frequently poor ionizers, and do not separate well. Current analysis methods rely on labor-intensive derivatization and cleanup steps. This study provides details of a robust high-throughput liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for the detection of glyphosate and its metabolites in complex food and water matrices, without the need for derivatization of samples prior to analysis. Polar pesticides are water soluble, and the extraction was therefore aqueous with methanol and formic acid added to improve efficiency. The large variety of interfering particulates in environmental and drinking water samples requires further sample preparation, and a simple filtration step was performed here before the MS/MS analysis. The food analytes were well separated from matrix interferences in most of the foods tested, and all water sample analytes were well retained after removal of interferences. This new method largely eliminated matrix effects and achieved unambiguous identification, reproducible retention times, and sensitivity; and attains the targeted limits of detection.

Wim Broer, Ugo Chiuminato, Jianru Stahl-Zeng, Daniel McMillan, and Phil Taylor

Despite its association with various health risks, glyphosate remains the most commonly used herbicide worldwide. Glyphosate-based pesticide formulations are used as a simple solution to improve yields by reducing weed growth around glyphosate resistant crops. Almost inevitably, some level of glyphosate residue ends up in the food chain, and it has been detected in the urine of a range of animal species, including humans (1). Polar pesticides are frequently poor ionizers, may suffer from low extraction from the sample matrix, and demonstrate poor chromatographic separation. These pesticides, therefore, have historically required complex single residue methods to make them amenable to analysis, involving time-consuming derivatization steps and considerable clean up procedures.

The analysis of glyphosate using high performance liquid chromatography with fluorescence detection (HPLC–FLD) in water is well known, with or without derivatization. Usually, glyphosate undergoes derivatization by the reaction of the native glyphosate with fluorenylmethyloxycarbonyl chloride (FMOC-Cl) before separation on a reversed-phase column. If the separation is performed on a polar chromatographic column, post-column derivatization before detection is required. The tedious derivatization step complicates the analysis, and the reproducibility is poor. Thus, there is a growing need for a method that can detect not only glyphosate and its major metabolite aminomethylphosphonic acid (AMPA), but also glufosinate and similar highly polar

compounds, in their underivatized states.

Modern triple quadrupole mass spectrometers that allow fragmentation analysis offer very low detection limits and high detection selectivity. This approach enables laboratories to rapidly screen samples for a variety of regulated pesticides using the European Union Reference Laboratories' (EURL) quick, easy, cheap, efficient, rugged, and safe (QuEChERS) sample extraction and cleanup method. Owing to this, multi-residue liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses have become the minimum standard for the quantification of many pesticides in food and water samples. Nevertheless, glyphosate and other highly polar pesticides are not suitable for this type of analysis, and remain challenging for routine screening.

The EURL quick polar pesticides (QuPPe) method allows the simultaneous analysis of a number of highly polar pesticides that are not amenable to common multiresidue methods. However, in practice, the separation is not robust, and requires intense system maintenance. The method involves extraction with acidified methanol and LC–MS/MS measurement, using isotope labeled internal standards for accurate quantification. Ion chromatography is the preferred separation technique for polar ionic analytes, such as anions, cations or small polar analytes (metabolites), and sugars. To use an ion chromatographic approach for the analysis of polar pesticides offers the ability to include multiple analytes in a single injection, without derivatization or the use of an ion suppressor. Food testing laboratories would benefit greatly, in terms of both time and cost, from a methodology that avoids these complex analysis procedures. Here, we present a robust and sensitive method for the direct analysis of polar pesticides in food and environmental samples without derivatization (2).

Experimental

Extraction

Food Samples

The EURL QuPPe method for extraction of polar pesticides from food samples of plant and animal origin is well developed (3). In this experiment, the QuPPe extraction was adjusted by using a little less methanol and more formic acid. Because polar pesticides are water soluble, the extraction was aqueous with methanol and formic acid added to improve efficiency. The addition of internal standards to matrices is considered essential in order to compensate for matrix effects and the shifting retention times observed in many chromatographic separation procedures. Dispersive solid-phase extraction (SPE) cleanup using C18 was also carried out as described in the QuPPe-AO3 method. A push-through method was finalized using two sorbents and SPE filters, because the extracts obtained from the QuPPe method require extensive cleanup for more stable chromatography and less pollution of the source.

The eluents' incompatibility with electrospray ionization sources requires the use of a suppressor, so as to lower the ion load for the electrospray ionization (ESI) source, and gain sensitivity. By employing a polyvinyl alcohol based column with quaternary ammonium groups, and using an ammonium bi-

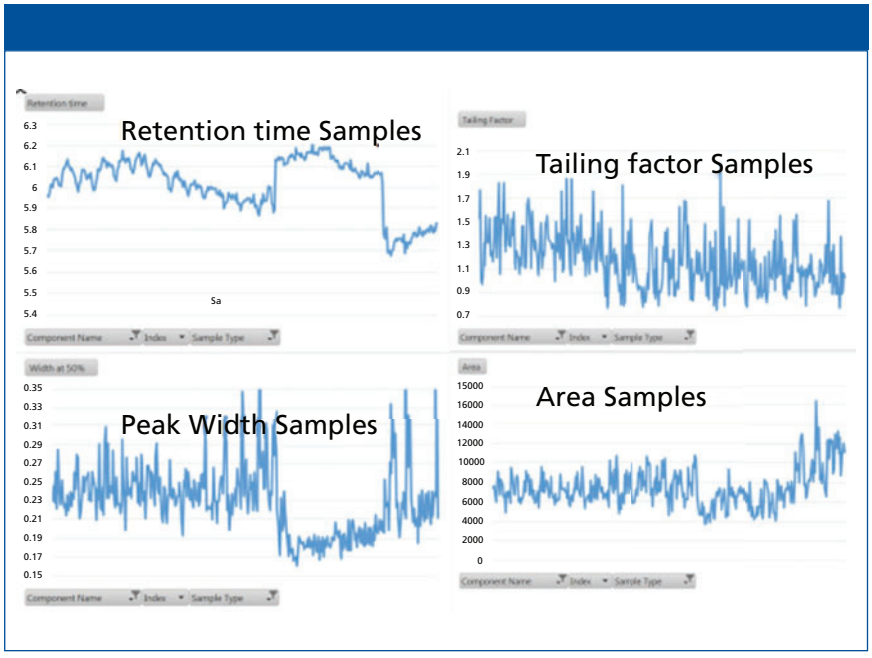

Figure 1: Reproducibility data for glyphosate internal standard (IS). The modified method for food samples tested over one hundred injections of extracts from the variety of food matrices presented in Table I.

carbonate buffer prior to detection by a highly sensitive quadrupole MS system, the need for a suppressor was removed.

Water Samples

Environmental and drinking water samples vary widely in the degree of particle content, causing difficulties for LC injection and the reproducibility. SPE-type cleanup would add significant time, as well as financial cost, in a high throughput laboratory situation where minimal sample preparation is desirable. To overcome these challenges, and to be able to swiftly change between food and environmental samples, the same setup for food samples (except the 500 µL injection volume) was carried out. A simple filtration step, using Chromacol 17-SF-02 (RC) from 17-mm syringe filters, was performed when transferring the samples to the LC vials. Internal standards to a final concentration of 1 ppb were added to samples and standard solutions, and QC samples in tap water were prepared in a similar fashion. To minimize matrix interferences, standard additions to the samples were used.

Separation

Separation was achieved using a Shimadzu Nexera ultrahigh-pressure liquid chromatography (UHPLC) system comprising LC-30AD pumps, a SIL-30AC autosampler fitted with a 500 µL loop, and a CTO-20A column oven. For chromatography, a polyvinyl alcohol column, with quaternary ammonium groups using a bicarbonate eluent between pH 8 and 10, was used.

Food Samples

The finalized chromatographic method used 10 µL injections onto a 150 x 4 mm column, employing a 20 mm guard column of the same material, and a 0.5 µm filter, with a flow rate of 0.6 mL/min. Both columns were replaced every 250 samples to maintain performance, and to keep the MS source clean. Performance was shown to be robust and reproducible on a variety of food matrices used for method verification laid out in Table I, all subject to the cleanup method as described above.

Water Samples

An injection volume of 500 µL was employed in a chromatographic method similar to that used for the food samples.

MS/MS Analysis

Analyses were performed using a triple quadrupole linear ion trap mass spectrometer (Sciex QTRAP 6500+) in negative electrospray ionization mode (Table II, source parameters).

At least two multiple reaction monitoring (MRM) transitions were optimized for each analyte, in order to quantify and confirm their concentration in all samples (Table III, analytes with MRM transitions employed).

Data were acquired using Analyst 1.6.3 (Sciex) and processed for quantification and confirmation with reference to internal standards (IS), using MultiQuant 3.0.2 software (Sciex) (Table IV, summary for limits of detection).

Table I: List of the food matrices used for method verification

Lists of Validated Commodities	
A	Fruit and vegetables
B	Seeds
C	Vegetable oil, fat and fatty acids
D	Grain
E	Herbs and spices
F	Meat and seafood
G	Animal oil, fat and fatty acids
H	Eggs and egg products
I	Milk and milk products
J	Fatty acids

Table II: Source parameters for the mass spectrometer system

Source Parameters	
Curtain gas (CUR)	30 psi
Collison gas (CAD)	9 psi
IonSpray voltage (IS)	–3000 V
Temperature (TEM)	500 °C
Ion source gas (GS1)	55 psi
Ion source gas (GS2)	65 psi

Results and Discussion

Results

The stability and reproducibility of the method, in terms of retention time, peak width, peak area, and tailing factor, were found to be excellent, and over one thousand food samples from a variety of commodities were

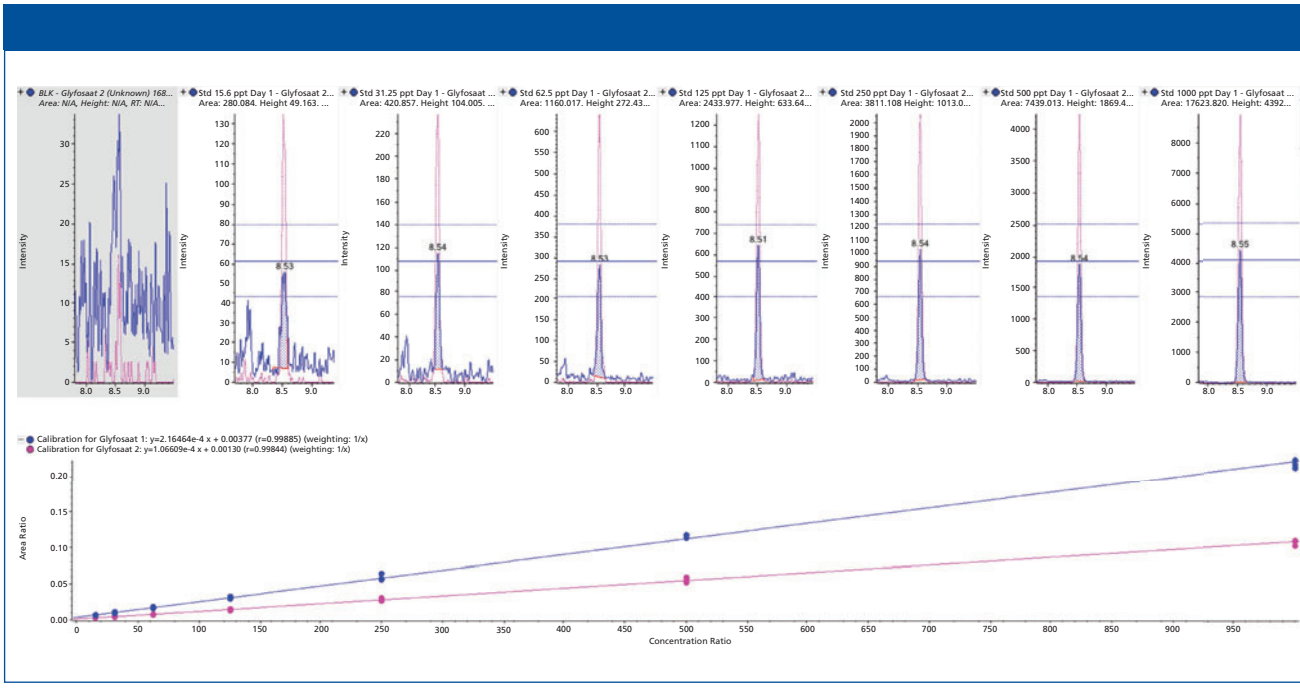

Figure 2: Method sensitivity and linearity of glyphosate. Several measures of reproducibility based on the glyphosate internal standard (IS). Calibration standards were achieved using the modified method for water samples. Ion ratios were all within the specified ±20% tolerance.

Table III: List of analytes with MRM transitions employed. Internal standards (IS) are crucial to this method and must be used

Analyte	Q1 *m/z*	Q3 *m/z*
Glyphosate 1	167.9	150.0
Glyphosate 2	167.9	78.8
Glyphosate 3	167.9	62.8
Ethephon 1	142.9	106.8
Ethephon 2	142.9	79.0
N-ac glufosinate 1	222.0	136.0
N-ac glufosinate 2	222.0	62.8
N-ac glufosinate 3	222.0	59.1
AMPA 1	110.0	81.2
AMPA 2	110.0	79.1
AMPA 3	110.0	62.9
Glufosinate 1	180.0	136.0
Glufosinate 2	180.0	95.0
Glufosinate 3	180.0	85.0
Glufosinate 4	180.0	63.1
3-MPPA 1	151.0	132.9
3-MPPA 2	151.0	107.0
3-MPPA 3	151.0	63.1
Phosphonic acid 1	81.0	62.9
Phosphonic acid 2	81.0	79.0

analyzed without system maintenance (Figure 1, reproducibility data for glyphosate internal standard [IS]).

The sensitivity of the method was demonstrated by considerably lower limit of detection (LOD) for every analyte and commodity combination than is required in most cases (Figure 3, example chromatography from surface water samples). Regulations are not expected to change significantly within the lifetime of the current generation of the most sensitive MS instruments. The linearity of the calibration curve was established in the concentration range of 15.6 to 1000 ng/L of glyphosate. By plotting the ratio of the peak area of the standard to that of the IS against the ratio of their concentrations at seven calibration levels, calibration curves were obtained (Figure 2, method sensitivity and linearity of glyphosate).

Discussion

The method described is highly robust and sensitive, achieving the target limits of detection required to meet current and proposed regulations. The separation has also been found to minimize any matrix interferences in all but the most complex samples. Through incorporation of

Table IV: Summary of limits of detection (LODs) achieved in various food matrices using the modified method. In the second column, the regulatory limits for each commodity are shown as European Union (EU) maximum residue limits (MRLs).

Product	Glufosinate sum			Fosetyl sum			Glyphosate			Chlorate			Ethephon		
	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL	LOD	MRL	%RSD at MRL
Fruit and vegetables	16	30	11%	25	2000	13%	5	100	15%	8	10	15%	18	50	11%
Seeds	12	30	12%	90	2000	15%	8	100	15%	3	10	10%	6	50	14%
Vegetable oil, fat, and fatty acids	15	30	19%	40	2000	12%	7	100	22%	2	10	6%	3	50	7%
Grain	18	30	12%	71	2000	14%	8	100	7%	7	10	14%	9	50	6%
Herbs and spices	25	100	8%	87	2000	13%	23	100	6%	8	10	15%	8	100	16%
Meat and seafood	19	30	15%	23	100	12%	9	50	23%	4	10	8%	4	50	10%
Animal oil, fat, and fatty acids	14	30	20%	51	100	11%	9	50	25%	10	10	16%	7	50	12%
Eggs and egg products	18	30	12%	33	100	11%	4	50	13%	12	10	9%	6	50	17%
Milk and milk products	17	30	9%	20	100	6%	8	50	22%	5	10	12%	5	50	13%
Fatty acids	21	100	14%	70	1000	14%	3	100	18%	4	10	9%	3	100	10%

this high throughput method into the workflows of food testing laboratories, complex sample preparation can be eliminated, running costs can be kept low, and valuable time can be saved.

Chromatographic performance using these modified methods for food and water samples achieved good separation between the analytes and from matrix interferences, and excellent repeatability in terms of peak profile and retention time. This cleanup is performed on all samples, not only for food from animal origin. The ion source was clean even after more than one hundred injections.

In order to maintain peak shape and retention times, various chromatographic methods were investigated. Ion chromatography gave the most promising results, but the resulting eluents were not compatible with electrospray ionization sources and required a suppressor, which is detrimental to peak width. The need for a suppressor was removed by the use of ammonium buffers, a deviation from traditional LC buffers that enables detection of polar pesticides by MS/MS. The setup in this study allowed switching between polar and normal pesticide (reverse phase) suites without changing inlet, giving sharper, more intense peaks, and shorter run times. In busy food testing labs that primarily work with reversed-phase LC, this setup saves valuable time, eliminating the need to change inlet systems between analyses.

Food samples analyzed using the modified method for QuPPe extracts achieved unambiguous separation between the analytes as well as excellent repeatability in terms of peak profile and retention times. Analytes were also well separated from matrix interferences in most of the foods tested. The EU maximum residue limits (MRLs) for pesticides in food commodities vary widely, depending on the pesticide and commodity in question. Although some matrix interference was present in the most complex food samples, LODs in both food and water samples were well below the concentration limits laid out in EU regulation. Over one thousand food samples from a variety of commodities were analyzed without maintenance of the system, and the stability in terms of retention time, peak width, peak area, and tailing factor was found to be excellent.

This method largely eliminated matrix interferences in food sample extracts, further enhancing the reproducibility and robustness of the method. However, in the most complex food matrices, MRM ion ratios were observed outside of the standard ±20% tolerance. In future work, the QTRAP will be used to confirm positive results by their full scan MS/MS spectra, and the sample cleanup procedure will be further developed in order to remove background interferences.

It is commonly known that LC–MS/MS, especially when working in ESI mode, is susceptible to matrix effects, affecting quantitative accuracy. This new methodology overcomes the necessity of pesticide derivatization. This was achieved by combining an ion chromatography separation with the high sensitivity of the mass spectrometer for analyte

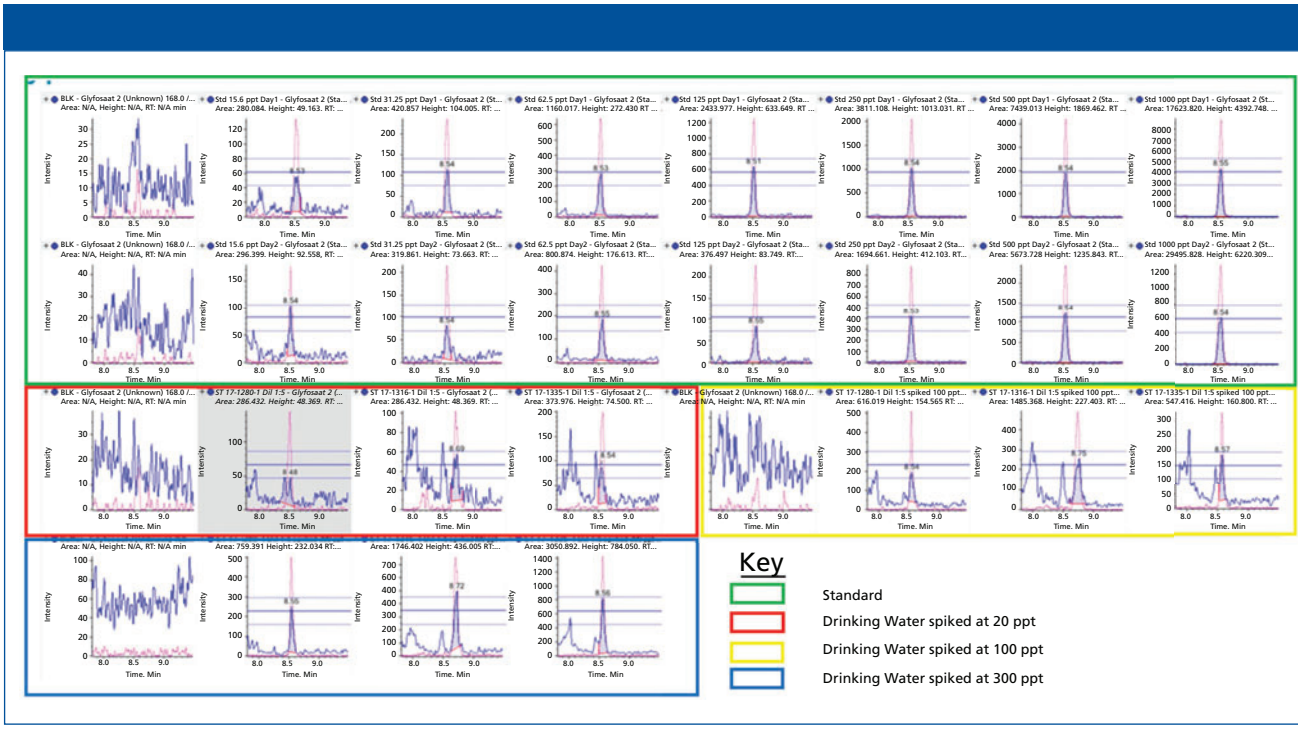

Figure 3: Example chromatography from surface water samples using the modified water method. The graphs highlight that even at concentrations five times (5x) lower than the European Union (EU) water regulations requirement, the presence of glyphosate in real environmental samples was detected and confirmed by the ion ratio between primary and secondary multiple reaction monitoring (MRM) transitions.

detection. Analytes are successfully separated using an LC column in a method switching reversed-phase system, with MS-amenable mobile phases at around pH 9. All analytes are well retained with reproducible retention times, allowing detection of the majority of background components. The method meets the Directorate-General for Health and Food Safety (DG SANTE) requirements of reproducibility (<20%) and recovery (80 to 110%), and the limit of detection (LOD) of the method is below 0.01 mg/kg. Excellent long-term stability and robustness were achieved throughout the validation of this method for food samples extracted by the QuPPe procedure.

Conclusion

This modified LC–MS/MS analysis offers the possibility to analyze multiple polar pesticides in a single injection without derivatization. The ion chromatography based approach uses ammonium buffers, a deviation from traditional LC buffers that enables detection of polar pesticides by MS/MS. A sufficiently sensitive mass spectrometer allows the analysis to be performed without the need for an ion suppressor using a standard reversed-phase LC based system. This eliminates the need to change inlets between typical pesticide analyses.

By removing the need for derivatization, sample preparation is greatly simplified, and the time required to analyze polar pesticides is reduced. As an additional advantage, the column lifetime is well over one thousand matrix samples, allowing high-throughput polar pesticide analysis with less system maintenance. This method was found to be considerably more robust and sensitive than other approaches, and has achieved the target limit of detection required to meet existing and proposed future regulations of glyphosate and similar polar pesticides.

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GC×GC–MS for Forensic Analysis

Gas chromatography–mass spectrometry (GC–MS) is considered the gold standard in forensic trace evidence analysis, because of its ability to chromatographically separate and analyze components in mixtures. Although two-dimensional GC–MS (GC×GC–MS) has been used extensively in the oil and petroleum and flavor and fragrance industries, it has not been fully explored in the forensic sector. However, forensic scientists often encounter highly complex samples that would benefit from the capabilities of GC×GC–MS, such as sexual lubricants, automobile paints, and tires. GC×GC–MS analysis can allow for the deconvolution of coeluted components, while providing increased sensitivity of minor components to help benefit any forensic laboratory.

Candice M. Bridge, Mark Maric, and Kaitlin Jones

Gas chromatography–mass spectrometry (GC–MS) is a "go-to" analytical technique, primarily because of its versatility for isolating and analyzing different components in unknown mixtures, without requiring substantial method development for each new sample. This is the primary reason why GC–MS is the gold standard in the forensic analysis of trace evidence, such as ignitable liquids and drugs. However, there are limitations in using GC–MS for all unknown mixtures, because of the complexity of some of these mixtures. The primary limitation is coelution of the compounds in a mixture. This is where multidimensional gas chromatography (MDGC) can increase component separation with the potential to increase the sensitivity of compounds that may not meet the limit of detection in GC–MS. There are a few types of multidimensional gas chromatography configurations, all of which can be coupled to a mass spectrometer: comprehensive (GC×GC–MS) or heart-cut (GC–GC–MS). There are three types of commercially available MDGC–MS configurations: thermal modulation (TM), Deans switch (DS), and differential flow modulation (DFM). Discussions of TM and DS can be found in the literature (1,2). This article will discuss the use of the latter modulator for forensic trace evidence analysis to rapidly differentiate complex mixtures by observing the unique chromatographic "fingerprint" (3). These "fingerprints" are similar to a topography chart, which shows the trends of compounds that are chemically related; that is, normal alkanes and isoparaffins. As a result of increased sensitivity, this "fingerprint" shows both major components, as well as those minor components that may have been hidden as a result of coelution.

GC×GC–MS systems have been used in the edible oil industry to investigate minor compounds (3,4), as well as the petroleum and biodiesel industries for rapid determination of the chemical formulation (5). However, the technique has yet to be evaluated for complex forensic evidence. This article discusses the use of GC×GC–MS for several forensic samples.

Experimental

Both GC–MS and GC×GC–MS analysis of the trace evidence samples were performed on the same GC–MS system, using the same column configuration. The GC system was a 7890B gas chromatograph, equipped with a split–splitless injector coupled to a 5977 quadrupole mass spectrometer (Agilent) (6).

The pyrolysis analysis of automobile paint and tires used the same GC×GC–MS method from the lubricant analysis. However, to conduct pyrolysis of the sample, a Pryoprobe 4000 (CDS Analytical LLC) was used. The flash pyroprobe profile was started at 50 °C for 2 s, and then was ramped to 750 °C at 50 °C/s ,and held for 2 s. All samples were analyzed in their natural, unmodified state.

Forensic Lubricant Analysis

A recent survey conducted by the National Center for Injury Prevention and Control revealed that approximately 1 in 5 women, and 1 in 71 men, will be sexually assaulted in their lifetime (7). Despite this staggering statistic, most criminal investigators primarily rely on DNA evidence to solve these crimes—from semen, skin cells underneath fingernails, or any other biological evidence. However, the use of condoms by sexual perpetrators has increased, primarily because they think that it will mitigate the deposition of semen at the crime scene or on the victim, thus preventing their identification based on DNA. A study by Nancy Ritter demonstrated that approximately 30% of sexual assault kits do not contain any probative DNA profiles for the perpetrator (8). This is where the forensic analysis of sexual lubricants can support the current analysis of sexual assaults. In the absence of DNA, lubricant analysis can provide another link between the perpetrator and the victim or crime

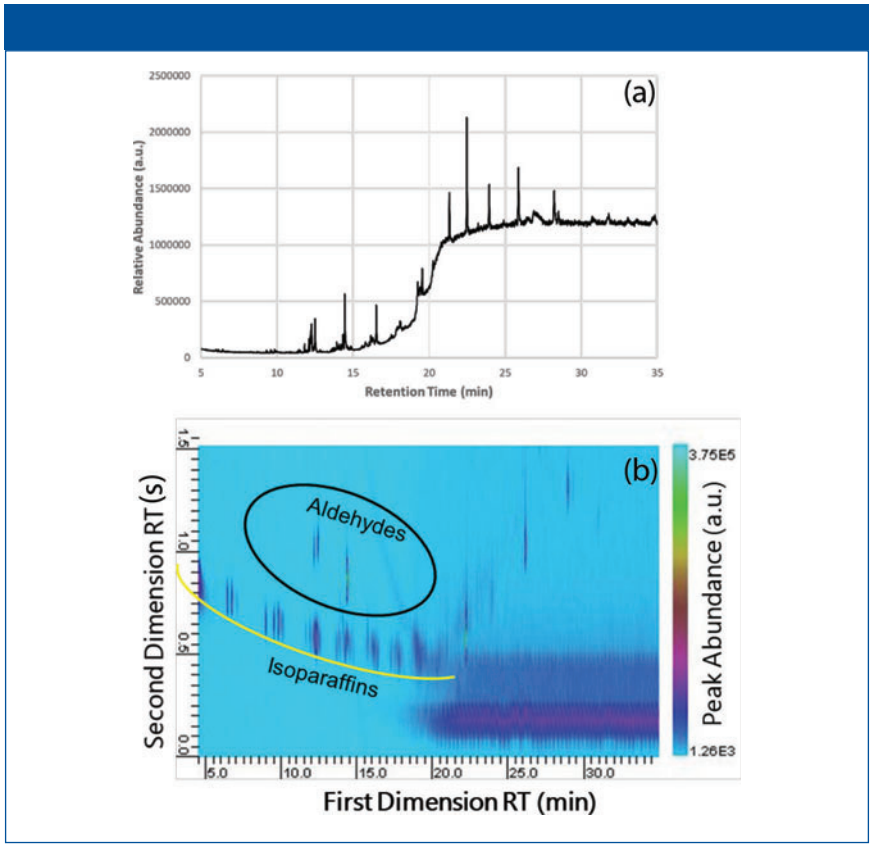

Figure 1: (a) GC–MS and (b) GC×GC–MS of an oil-based lubricant.

scene. However, many lubricants are made from natural oils, which are comprised of many compounds that may be difficult to differentiate using traditional GC–MS.

An example of a typical oil-based organic personal lubricant is one comprised of several organic butters (cocoa and shea), as well as vitamin E oil, beeswax, sweet almond oil, and even sunflower oil. Each of these oils and butters are comprised of many different oils and components themselves. Lubricant samples were prepared by hexane solvent extraction. Despite the fact that the oil-based lubricant only has six labeled ingredients, GC–MS analysis shows that there were more than the six labeled components, but there was a substantial amount of coelution between retention times (t_Rs) of 7 and 20 min (Figure 1a). However, using GC×GC–MS analysis, more than 25 different components were readily observed. Between the 10 and 15 min first dimension retention times (FDRTs), several components were separated in the second dimension

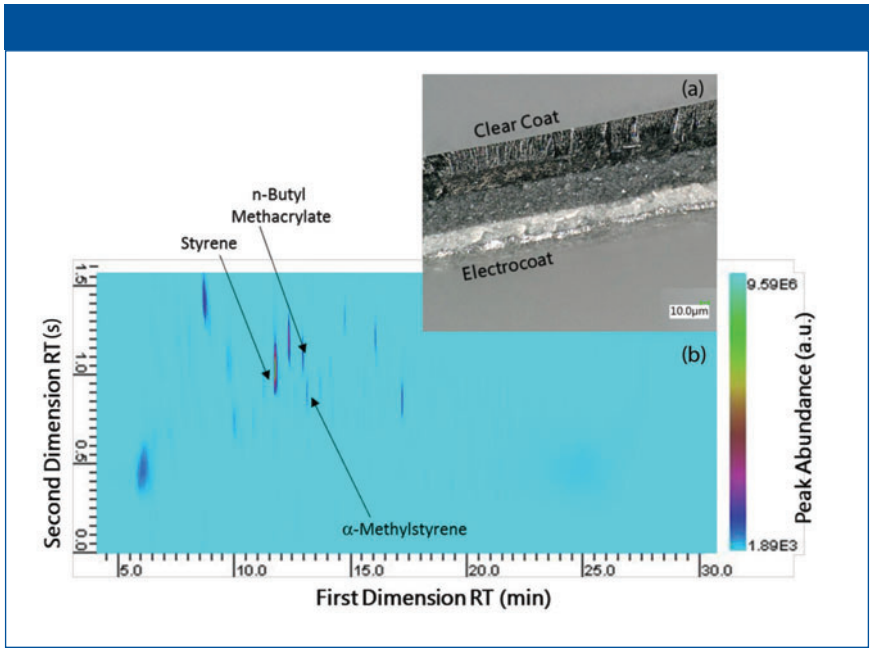

Figure 2: (a) Cross-section of automotive paint system, (b) Py-GC×GC-MS profile of the clear coat.

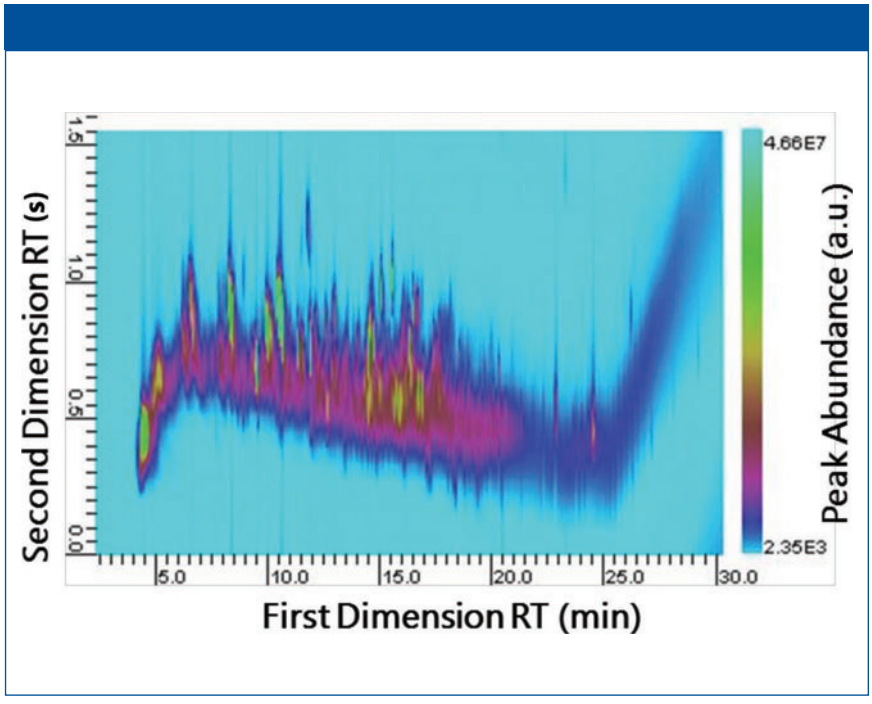

Figure 3: Py-GC×GC–MS profile of a tire sample.

that were coeluted during GC–MS analysis (Figure 1a).

When compared to other natural oil-based or plant-based lubricants, the overall chromatographic profile is similar, but the differences are readily observed between the FDRTs of 7 and 17 min. Isoparaffinic compounds make up the lower arc of the early GC×GC profile (underlined in yellow) and the aldehydes are above (circled in black). Many of the heavier oils elute later on the first-dimension column, such as vitamin E oil. This oil is not readily observed in this sample, primarily because of the low concentration in the sample. Based on the analysis of other natural lubricants and lotions, vitamin E (also known as a-tocopherol) elutes off the second column adjacent to the column bleed located at the lower right-hand corner of the chromatographic plane. What is also immediately noticeable is the increased intensity.

It was not immediately clear why there was a background shadow observed between first dimension retention times 20 to 35 min (lower right hand of Figure 1b). It is possible that this "shadow" was a result of either a column bleed from either the first-dimension or second-dimension column, considering the elevated oven temperatures at the end of the chromatograph run (280 °C).

Automotive Paint Analysis

Automotive paint is a type of forensic evidence collected at car accidents, hit-and-runs, and any other crime involving a vehicle. This type of evidence is encountered frequently, and thus it is critical to improve current analytical techniques, as well as evaluate new options that could provide more information than current techniques can provide.

Automotive paint is chemically complex because it is a multilayer system and different components are present in each layer. The four main components that make up automotive paint are pigments, additives, binders, and solvent. When automotive paint coatings are applied by the original equipment manufacturer (OEM), they are added in the following order: electrocoat, primer surfacer, basecoat, and clear coat. Each of the coatings have a different purpose with regards to the car's appearance. The electrocoat is used to prevent corrosion and the primer surfacer provides the car with a smooth surface. The basecoat determines the color of the vehicle, and the glossy finish is provided by the clear coat, which contains hindered amine light stabilizers and UV absorbers to protect the underlying paint layers from weathering and environmental effects (9).

Currently, there are three techniques used to analyze automotive paint: microscopy, infrared (IR) spectroscopy, and pyrolysis (py)-GC–MS. Py-GC–MS has the most discriminating power among these three techniques, and can differentiate between samples with similar binders and pigments, not typ-

ically achievable with IR spectroscopy (10). The ability of py-GC–MS to discriminate between similar samples is significant, yet there is still room for improvement. Pyrograms of automotive clear coat samples analyzed using py-GC–MS have indicated that coelution occurs with certain compounds of interest; that is, toluene and 1,2-propandial, which can limit the ability to differentiate clear coats (10).

To overcome the obstacle of coelution, py-GC×GC–MS was used to analyze automotive clear coats. To our knowledge, there is no literature published on the analysis of paints using py-GC×GC–MS. Increased separation of paint components is demonstrated using py-GC×GC–MS, especially for peaks that typically coelute in GC–MS. The two peaks around FDRT 11.6 min (Figure 2b) illustrate the improved separation that is achieved in py-GC×GC–MS. α-methylstyrene (11.776 min FDRT) and n-butyl methacrylate (11.600 min FDRT) would normally coelute in the first column. However, the second column allows the two peaks to be distinguished from one another. With additional method development, we aim to increase the separation of clear coat peaks.

Tire Analysis

Much like automotive paint, traces of tire rubber are often encountered on road surfaces or on the victim of automotive-related incidents, such as hit-and-run accidents. The forensic analysis of tire evidence is useful to investigators, specifically when attempting to reconstruct vehicle trajectories, velocities, and dynamics in incidents (11). Tire impressions from a crime scene are routinely compared to the tread pattern of tires from the suspect vehicle. However, in many instances the impression may be of poor quality, which is when the chemical analysis of the rubber traces may help to provide investigative leads. The physiochemical complexity of trace tire particulates makes the characterization of this evidence challenging and time-consuming. Py-GC–MS is the technique primarily used by forensic scientists for the chemical analysis of tire evidence (12,13). The pyrograms from rubber traces obtained from the tire impressions can then be compared to the tire from a suspect vehicle. Tires are extremely chemically complex, often containing over 200 components, including natural and synthetic rubber, oils, plasticizers, antioxidants, antiozonants, accelerators, vulcanizing agents, and curing systems (14). This chemical complexity can result in coelution of components, which may prevent a correct match and lead to significant errors.

A flash pyrolysis method was used to pyrolyze a small portion (~50 µg) of the main tread of a Firestone Destination LE tire. Multidimensional separation of the pyrolysates was performed, and the resultant GC×GC plot is presented in Figure 3. The complexity of tire samples makes identification of the individual components difficult using one-dimensional py-GC–MS. Py-GC×GC–MS was able to differentiate many components in the second dimension, which is beneficial to eliminate the ambiguity in making comparisons, and improves match determinations and reduces errors, which is imperative in forensic investigations.

Conclusions

With complex mixtures commonly encountered in forensic trace analysis, it is necessary to start evaluating techniques other than GC–MS. The use of GC×GC–MS or py-GC×GC–MS provide the forensic community with a new methodology that can achieve such separation. This could be the next frontier for increasing the actionable intelligence that forensic laboratories provide the criminal investigation system.

Acknowledgments

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The Benefits of Data-Independent Acquisition in Metabolomics

Interest in data-independent acquisition (DIA) in mass spectrometry is growing in many fields, including in metabolomics. The approach makes it possible to re-interrogate data from earlier analyses, such as when a researcher wishes to determine if newly identified compounds appeared in a sample analyzed previously. In this interview, Craig Wheelock, the head of the Integrative Molecular Phenotyping Laboratory at the Karolinska Institute (Sweden), discusses the use of DIA in metabolomics.

Alasdair Matheson

Q. What aspects of metabolomics is your group focusing on?

A: The analytical work in our group has two distinct focuses. We initially developed targeted methods for metabolic profiling of lipid mediators, such as eicosanoids in respiratory disease. A few years ago, we naively decided to expand into metabolomics, but quickly realized that there are numerous analytical challenges in performing a metabolomics experiment. Therefore, we decided to start from scratch, and construct our own workflow, similar to many other laboratories. The primary aim of our method was to acquire as much high quality data in a single experiment as possible.

The goal was to then use these metabolite data for omics integration models in systems medicine studies. We wanted a long list of accurately identified metabolites, while simultaneously reducing the use of putative identifications. We were concerned that incorrect metabolite identifications would be problematic for our integrative modeling efforts, and lead to inaccurate interpretation of the observed biology. The last few years have been spent on developing our metabolomics method, which was finally published last year (1). Our focus on metabolite annotation has led us to be strong proponents of the efforts of the Metabolomics Standards Initiative to provide clear criteria on the accuracy of metabolite identification (2).

Q. What are the main challenges for chromatographers engaged in metabolomics at the moment?

A: We are currently using liquid chromatography (LC) for our metabolomics work in biofluids, primarily urine and blood. When developing an LC method for metabolomics, it is clearly a trade-off between separation and throughput. Ideally, we would have three hour long gradients using two-dimensional (2D) nanoflow methods. This would optimize our metabolite separation, which would greatly help in unequivocal metabolite identification. Run times of this length are not feasible for our applications, and there is a question of the long-term robustness and retention time stability of such an approach. As with most methods, our current approach represents a compromise. The field appears to have currently coalesced around 15–20 min gradients. However, there is a strong case to be made for short, fast methods, in the order of 2–3 min, using microbore technologies. An additional obstacle is the variability associated with hydrophilic-interaction chromatography (HILIC). It has been challenging to obtain reproducible retention times for HILIC chromatography, especially for larger studies. However, while this has historically been an issue, new HILIC columns have greatly improved in performance. We have successfully used HILIC chromatography for the analysis of more than one thousand samples in a single study. In many ways, gas chromatography (GC) offers several advantages over LC approaches, including extremely consistent retention times, robustness, reduced ion suppression, and excellent spectral libraries. Conversely, analysis of samples using GC generally requires a derivatization step, and the compounds need to be thermally stable. In order to capture the structural diversity of a metabolome, multiple methods are necessary. There is, unfortunately, no single analytical platform capable of simultaneously acquiring a metabolome. I therefore see the current optimal solution to involve multiple methods, including both LC– and GC– based approaches.

Q. You recently developed a method using liquid chromatography high resolution mass spectrometry (LC–HRMS) for metabolite identification using a data-independent acquisition (DIA) approach? What was the aim of this research?

A: The aim of this research (1,3) was to develop a method suitable for our laboratory goals, as I noted previously. We wanted to be able to accurately identify as many metabolites as possible in a single acquisition. Using DIA provided us with fragmentation from three different collision energies (0, 10 eV, and 30 eV), which greatly helps with compound identification.

We are able to identify metabolites based upon our in-house

library of standards. However, the DIA approach enables the simultaneous acquisition of targeted and nontargeted data. We can then go through our nontargeted data, and mine it for interesting metabolites that are not included in our database. If we want to conclusively identify a new metabolite, we can acquire the standard, add it to our database, and reprocess the data to characterize the compound. One of the primary challenges for us with the analysis of the nontargeted data is the ability to perform metabolite identification and annotation across thousands of samples in a single study.

Q. What is DIA? What benefits does it offer?
A: To improve metabolite identification, and reduce the requirement for multiple analytical runs for structural confirmation, two different tandem mass spectrometry (MS/MS) strategies have been implemented: with selection of the precursor ion (data-dependent acquisition [DDA]), and without selection of the precursor ion (DIA). DIA-based MS generates MS/MS spectra containing a mixed population of product ions together with their precursor ions, and the extracted ion chromatogram (EIC) of each product ion needs to be mapped to its parent compound.

DIA approaches have been successfully used to conduct multiple fragmentation experiments in a single acquisition. One useful application of the DIA approach is to identify coeluting isobaric compounds, where DIA data are combined with software deconvolution algorithms that merge precursor ions from low-energy experiments and product ions from high-energy experiments. An advantage of the current DIA approach is the concurrent collection of full scan data, enabling identification of metabolites not included in the database. Our data acquisition strategy enables a simultaneous mixture of database-dependent targeted and nontargeted metabolomics in combination with improved accuracy in metabolite identification, increasing the quality of the biological information acquired in a metabolomics experiment.

Q. What were the main analytical challenges you had to overcome in this project, and how did you overcome them?
A: A lot of the work performed for this study was time-consuming, and rather repetitive. The characterization of more than four hundred standards to create the in-house library and measure the ion ratios took a very long time. The development of custom standard libraries is not efficient, and it does not make sense for laboratories to do this independently. Happily, there are now multiple metabolite libraries available that have the advantage of being well organized, and all metabolites have known solubilities.

Q. What is novel about your approach?
A: The aim of our work was to establish a comprehensive analytical workflow for the application of LC–HRMS to nontargeted metabolomics with a high level of accuracy in metabolite identification. Our application of DIA mode includes three sequential full scans, at 0, 10 eV, and 30 eV collision energies. In the subsequent data analysis, EIC from any precursor or associated product ions of interest can be extracted from the low- or high-energy scan data. One EIC is chosen for relative quantification (the quantifier ion) of the metabolite, and further product ions from the same compound are used as qualifier ions. This approach enables us to potentially distinguish coeluting isobaric pairs (provided that unique fragments can be identified). In addition, we have added an ion ratio approach, which means that the ratios of qualifier–quantifier ion intensities are established from analytical standards, and should therefore be preserved when measured in a biological sample, increasing the accuracy of the identification. The same acquired data (0 eV) can be used in parallel for a global metabolite profiling workflow, enabling a combined database-dependent targeted and nontargeted metabolomics experiment. The combination of the DIA-based data acquisition with the ion ratio confirmation and deconvoluted coeluting isobaric pairs provides a useful method for increasing the accuracy of metabolite identification in a metabolomics experiment.

Q. Can you describe a practical example to illustrate how this approach would benefit the analyst in practice?
A: The strength of the current method was demonstrated in urine, using the homoserine and threonine isobaric pair as an example. We analyzed a clinical cohort of urine samples from asthmatics using our published metabolomics methods. The homoserine and threonine peak areas were then each integrated separately using their specific product ion as a quantifier ion (homoserine, *m/z* 55.0189 at 30 eV; threonine, *m/z* 74.0248 at 10 eV), and their combined peak area (homoserine + threonine, *m/z* 118.0509 at 0 eV) was also integrated. The samples were then stratified by the abundance of the threonine value (obtained from threonine-specific peak integration) and the homoserine value (obtained from homoserine-specific peak integration). Following stratification, the 25% and 75% quantile of each sample set were selected in an extreme value approach to test for significance. The combined homoserine + threonine integrated peak was not significant ($p = 0.2$), but the threonine-specific peak was significantly different between the 25% and 75% quantile ($p < 0.0001$). In addition, the percentage relative standard deviations (RSDs) decreased in the homoserine- and threonine-specific integrated peaks between the two quantiles relative to the combined peak integration, increasing the precision of the measurement. This metabolite-dependent deconvolution example demonstrates the strength of the current method for increasing the accuracy of metabolite annotation by targeted ion selection, which can have a significant effect upon the observed biological shifts.

Q. What is the aim of the Metabolomics Standards Initiative?
A: The field of metabolomics is growing rapidly, and it is exciting to see the recognition of the importance of metabolomics in integrative omics studies. However, with this recognition comes a need for increased standardization in the field. The work by the Metabolomics Standards Initiative is an important step in this direction. In addition, repositories, such as MetaboLights (4), are vital for the science. I think that the field would benefit from a set of standardized recommendations on how to perform a metabolomics experiment. An example can be taken from the development of microarray methods. It is now considered as essentially obligatory that the Minimum Information About a Microarray Experiment (MIAME) (5,6) standards are followed in an experiment, and that the data are deposited in a database such as GEO. It would be

(Continued on page 34)

Fully Automated Determination of 3-MCPD and Glycidol in Edible Oils by GC–MS Based on the Commonly Used Methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10)

Dominik Lucas, Andreas Hoffmann, and Carlos Gil, Gerstel GmbH & Co. KG

The process contaminants 3-MCPD, glycidol, and their fatty acid esters are formed when certain edible oils and fats are refined or otherwise heated in the presence of chloride. At least some of these are classified as potential human carcinogens, and tolerable daily intake values as well as maximum levels in edible oils have been introduced.

This application note describes an automated solution for the fully automated determination of 3-MCPD and glycidol in edible oils based on the reliable indirect method DGF C-VI 18 (10), similar to the ISO 18363-1 and AOCS Cd 29c-13 methods.

Experimental

The sample is divided into two parts, which are saponified using sodium-hydroxymethanol. In assay A, free glycidol is converted to 3-MCPD using acidic quenching conditions in the presence of chloride. In assay B, the quenching reagent is an acidic chloride free salt solution, in which free glycidol is not converted into 3-MCPD. Following derivatization, the 3-MCPD amounts in both samples are determined by GC–MS as phenylboronic acid (PBA) esters. Assay B determines the amount of 3-MCPD in the sample; assay A determines the combined amounts of 3-MCPD and glycidol. The amount of glycidol is determined as the difference between the assay A and assay B results.

One manual step is required, followed by the long list of steps shown below prescribed in the unified method DGF C-VI 18 (10). These are all performed automatically by the Gerstel MultiPurpose Sampler (MPS). Depending on the instrument configuration, introduction of the prepared extract to the GC–MS system can be included.

Table I: amount of 3-MCPD found in three different edible oils in mg/kg

	Amount (mg/kg)	
3-MCPD	Reference	Automated
Oil 1	0.77	0.68
Oil 2	0.68	0.63
Oil 3	0.27	0.29

- Weigh a 100 mg sample into a vial.
- Add MTBE to the sample.
- Add ISTD solution and mix, or melt and mix (solids).

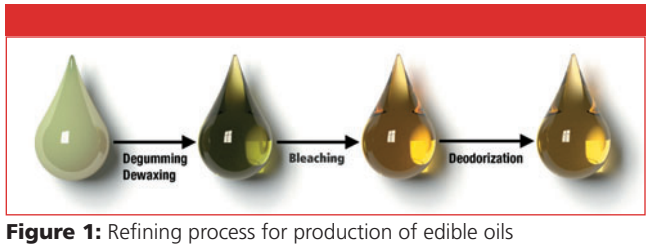


Figure 1: Refining process for production of edible oils

Figure 2: Gerstel MPS workstation used for automated sample preparation of edible oils prior to GC–MS determination of 3-MCPD and glycidol.

- Add CH_3OH/NaOH mixture.
- Agitate and incubate
- Add acidic NaCl solution (Assay A).
- Add acidic NaBr solution (Assay B).
- Add n-hexane for matrix extraction.
- Agitate and incubate.
- Discard hexane phase.
- Repeat extraction with n-hexane twice.
- Perform two analyte extractions using MTBE/ethylacetate 3:2 (v/v): transfer the organic phases to a collection vial.
- Add phenylboronic acid solution.
- Evaporate to dryness and derivatize in the mVAP at 50 °C and subambient pressure.
- Take up the derivatives in isooctane.
- Introduction to GC–MS(/MS) if integrated with sampler.

Table II: Glycidol amounts in three different edible oils

	Amount (mg/kg)	
Glycidol	Reference	Automated
Oil 1	0.14	0.18
Oil 2	0.44	0.48
Oil 3	0.11	0.09

The work presented here involves an automated evaporation step, as prescribed in standard methods. Apart from compliance,, this ensures that, for most matrices, the presently required limits of detection can be reached using a single quadrupole mass spectrometer (MSD). Using a triple quadrupole MS, even much lower limits can be reached. The evaporation step also separates out excess derivatization reagent, which could otherwise build up in the GC–MS system, and thereby helps to improve system stability.

The obtained results show good correlation with reference data.

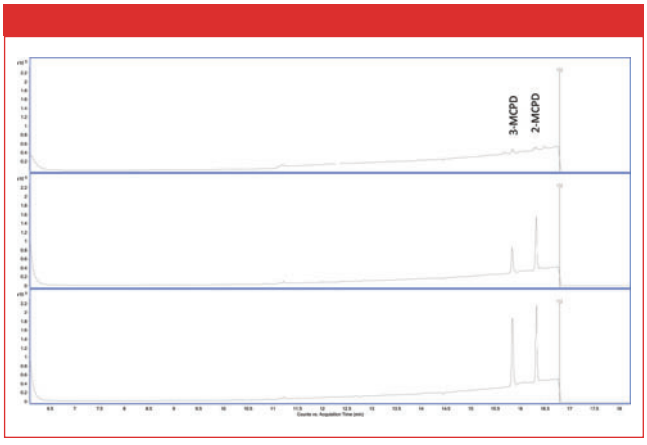


Figure 3: SIM-chromatogram showing *m/z* 198. Top: virgin olive oil used as blank oil. Middle: edible oil sample assay B (3-MCPD). Bottom: edible oil sample assay A (3-MCPD + glycidol).

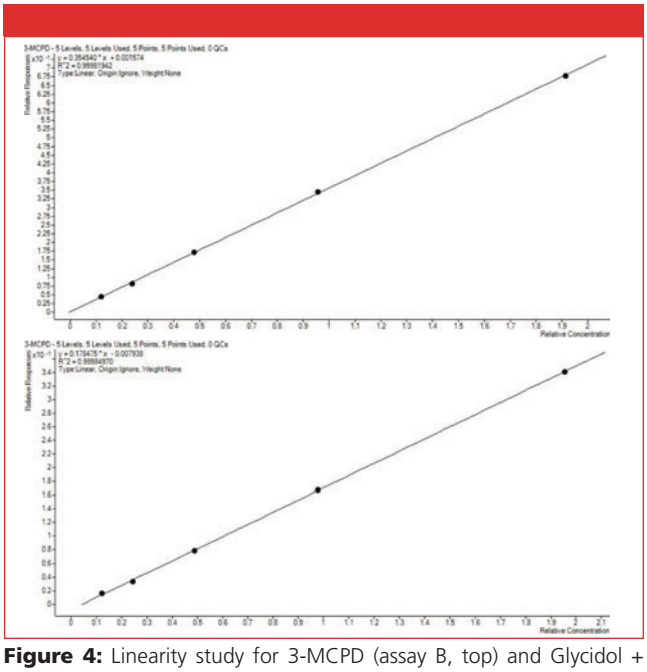


Figure 4: Linearity study for 3-MCPD (assay B, top) and Glycidol + 3-MCPD (Assay A), bottom, 0.12–1.9 mg/kg each.

Excellent standard deviation is achieved for the complete sample preparation and analysis workflow, speaking in favor of automation.

Instrumentation

Automated sample preparation is performed on a Gerstel MultiPurpose Sampler (MPS robotic), DualHead version. The Gerstel QuickMix module performs vigorous shaking for liquid–liquid extraction, and the mVAP module performs evaporative concentration during the derivatization step as specified in methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10). These steps are fully automated. The sample is injected to the GC–MS(/MS) system via a cooled injection system CIS 4, PTV-type inlet (Gerstel), and transferred to the GC column using programmed temperature vaporization. A 7890 GC and a 5977 MS instruments were used (both from Agilent Technologies).

For detailed analysis conditions, please see Gerstel application note 191.

The linearity of the method was verified by analyzing virgin olive oil spiked at five different levels. This was performed for both assays. Figure 4 shows a very good linearity for both assays.

Three different edible vegetable oil samples were analyzed, and the results compared with the provided reference values. These were in the low level range for 3-MCPD and glycidol contamination. Table I shows the results from assay B.

Table III: Repeatability for 3-MCPD and Glycidol (*n*=5)

	Amount (mg/kg)	
	3-MCPD	Glycidol
1	0.72	0.54
2	0.63	0.53
3	0.66	0.49
4	0.69	0.50
5	0.68	0.57
Mean	0.68	0.52
SD	0.03	0.03
RSD %	5.00	6.44

The difference between the results for assays A and B, multiplied by a previously determined conversion factor, is used to calculate the amount of glycidol in the sample. In Table II, the results are listed along with reference values.

Repeatability: Five samples of the same edible oil were individually prepared and analyzed. Table III shows the repeatability for the entire complete process, including GC–MS analysis.

Conclusion

Method ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10) can be automated using the Gerstel MPS for GC–MS(/MS) analysis with good correlation to reference data. The excellent standard deviations achieved speak in favor of automation. The described automation steps have previously been tested for use in derivatization methods like the recently presented 3-in-1 approach, for which it can be adapted with similar performance. The presented method has the advantage of being able to analyze a sample for glycidol, 3-monochloropropanediol (3-MCPD), and additionally 2-monochloropropanediol (2-MCPD). When equipped with the SPE option, the described automation platform can further extract and determine PAHs in edible oils using SPE-GC–MS.

Literature

ISO 18363-1:2015 Animal and vegetable fats and oils—Determination of fatty-acid-bound chloropropanediols (MCPDs) and glycidol by GC/MS—Part 1: Method using fast alkaline transesterification and measurement for 3-MCPD and differential measurement for glycidol.

GERSTEL

Gerstel, Inc.
E-Mail: gerstel@gerstel.com
Website: www.gerstel.com

LC–MS/MS Analysis of Mycotoxins in Peanut Powder in 5.5 Min

Restek Corporation

- Fast analysis for higher sample throughput
- Excellent separation improves accuracy for 12 regulated mycotoxins
- Quick and easy sample preparation (dilute-filter-shoot)

Certain fungi that can grow on agricultural products produce toxic metabolites known as mycotoxins. Modern food processing procedures cannot completely remove these compounds if they are present, so strict monitoring protocols have been established. Although a universal method for the analysis of mycotoxins would allow highly efficient screening, it is very challenging to develop such a method, due to differences in physiochemical properties of mycotoxins, extraction efficiencies, and matrix effects. Zhang and associates published a multi-lab study (1) aimed at providing labs with an analytical procedure that could be broadly applied to the analysis of a variety of mycotoxins in many different matrices. Using that work as inspiration, we developed the following LC–MS/MS method that resolves 12 FDA regulated mycotoxins within the pressure limits of traditional HPLC instruments.

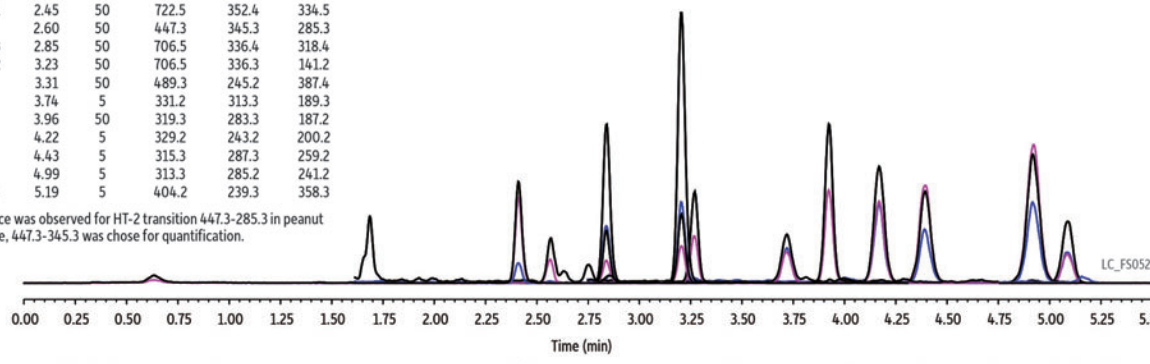
In this example, mycotoxins were analyzed in a peanut powder matrix. The use of a relatively short column format, the selectivity of the Biphenyl stationary phase, and the efficiency of 2.7-µm Raptor superficially porous particles provided excellent separations in a fast 5.5-min analysis (total cycle time of 7 min). A coeluting matrix compound that shared the most abundant MRM transition for mycotoxin HT-2 (447.3-285.3) was observed, so a less abundant transition (447.3-345.3) was selected for quantitation. To increase sensitivity, an ammonium buffer was used to promote better ionization of mycotoxins. The Raptor Biphenyl column worked very well for the 12 mycotoxins studied in the cited work, but for longer compound lists containing isobaric mycotoxins with similar structures, the Raptor FluoroPhenyl phase may be necessary to provide adequate chromatographic resolution. The selectivity of the Raptor FluoroPhenyl column is demonstrated in an analysis of 20 mycotoxins that can be found by visiting www.restek.com and entering LC_FS0511 in the search.

This method showed excellent precision and accuracy for the 12 FDA regulated mycotoxins that were evaluated during a validation study that covered a variety of matrices (including multiple sources of cornmeal and brown rice flour, in addition to the peanut powder example shown here).

Restek would like to thank Dr. Zhang for his technical support during this project.

Peaks	tR (min)	Conc. (ng/g)	Precursor Ion	Product Ion 1	Product Ion 2
1. Deoxynivalenol	0.62	50	297.3	249.3	231.2
2. Fumonisin B1	2.45	50	722.5	352.4	334.5
3. HT-2	2.60	50	447.3	345.3	285.3
4. Fumonisin B3	2.85	50	706.5	336.4	318.4
5. Fumonisin B2	3.23	50	706.5	336.3	141.2
6. T2	3.31	50	489.3	245.2	387.4
7. Aflatoxin G2	3.74	5	331.2	313.3	189.3
8. Zearalenone	3.96	50	319.3	283.3	187.2
9. Aflatoxin G1	4.22	5	329.2	243.2	200.2
10. Aflatoxin B2	4.43	5	315.3	287.3	259.2
11. Aflatoxin B1	4.99	5	313.3	285.2	241.2
12. Ochratoxin A	5.19	5	404.2	239.3	358.3

Matrix interference was observed for HT-2 transition 447.3-285.3 in peanut powder. Therefore, 447.3-345.3 was chose for quantification.



Column: Raptor Biphenyl (cat.# 9309A52); Dimensions: 50 mm x 2.1 mm ID; Particle Size: 2.7 µm; Pore Size: 90 Å; Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252); Temp.: 40 °C; Inj. Vol.: 5 µL; **Mobile Phase:** A: Water, 2 mM ammonium formate, 0.1% formic acid; B: Methanol, 2 mM ammonium formate, 0.1% formic acid; **Gradient (%B):** 0.00 min (30%), 0.6 min (30%); 0.7 min (50%); 3.00 min (70%); 4.5 min (75%); 5.0 min (90%); 5.2 min (90%); 5.21 min (75%); 6.00 min (75%); 6.01 min (30%); 7.00 min (30%); **Flow:** 0.5 mL/min; **Detector:** MS/MS; Ion Mode: ESI+; Mode: MRM; **Instrument:** UHPLC; **Notes:** Weighed 1.00 gram of peanut powder in a 50 mL centrifuge tube and added 2.00 mL of water. Vortexed at 3000 rpm for 5 min followed by the addition of 4.0 mL of extraction solvent (50:50 water:acetonitrile, v/v). The tube was then vortexed at 3000 rpm for 5 min followed by centrifugation for 15 min at 4200 rpm. 475 µL of the supernatant was filtered through a Thomson SINGLE StEP Nano filter vial (0.2 µm, cat.# 25882). The sample was then fortified with 25 µL of a standard solution prepared in water at 1000 ng/mL (100 ng/mL for aflatoxins and ochratoxin A) as part of the matrix-matched calibration curve. Vortexed at 3000 rpm for 1 min prior to analysis.

Raptor Biphenyl LC Columns (USP L11)

Length	2.1 mm cat.#	3.0 mm cat.#	4.6 mm cat.#
1.8 µm Columns			
30 mm	9309232	—	—
50 mm	9309252	930925E	—
100 mm	9309212	930921E	—
150 mm	9309262	—	—
2.7 µm Columns			
30 mm	9309A32	9309A3E	9309A35
50 mm	9309A52	9309A5E	9309A55
100 mm	9309A12	9309A1E	9309A15
150 mm	9309A62	9309A6E	9309A65
5 µm Columns			
30 mm	—	930953E	—
50 mm	9309552	930955E	9309555
100 mm	9309512	930951E	9309515
150 mm	9309562	930956E	9309565
250 mm	—	—	9309575

Reference

(1) K. Zhang, M.R. Schaab, G. Southwood, E.R. Tor, L.S. Aston, W. Song, B. Eitzer, S. Majumdar, T. Lapainis, H. Mai, K. Tran, A. El-Demerdash, V. Vega, Y. Cai, J.W. Wong, A.J. Krynitsky, T.H. Begley, *J Agr Food Chem,* **65**(33) 7138–7152 (2017). https://www.ncbi.nlm.nih.gov/pubmed/27983809.

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Simultaneous Analysis of Ten Water-Soluble Vitamins Using a Polymer-Based Reversed-Phase Column– Shodex™ RSpak DE-413L

Showa Denko America, Inc.

Vitamins are micronutrients essential for the metabolism of living organisms. Because humans cannot produce vitamins, the intake of vitamins must be part of their consumption. There are many commercial foods and drinks supplemented with vitamins for nutrient enhancement purposes, including most processed foods.

Methods using microbiological assays, absorption spectrophotometry, and HPLC have been used to analyze vitamins, creating a long process. A typical HPLC method to separate and quantify vitamins can use an ODS column with an addition of an ion-pair reagent. However, the ion-pair reagent tends to remain on the column and the flow-lines, resulting in an increased background level and lowers the sensitivity.

Therefore, in this application, a simple method to simultaneously analyze various water-soluble vitamins was developed, using the Shodex DE-413L, a polymer-based reversed-phase column, without the use of an ion-pair reagent. We further applied the developed method to quantify vitamins in a commercial multivitamin supplement.

Experimental

Ten vitamins (thiamin HCl, pyridoxine HCl, nicotinamide, ascorbic acid, nicotinic acid, calcium pantothenate, cyanocobalamin, folic acid, riboflavin, and biotin) were used as standards. A 4-mM standard solution was used for biotin and 2-mM standard solutions were used for other vitamins. The other standards were dissolved in 250-mM phosphoric acid. Five levels of multivitamin calibration standards were prepared using standard solutions and 250-mM phosphoric acid. We used 250-mM of phosphoric acid to prevent the oxidation of ascorbic acid.

A Shodex RSpak DE-413L column (4.6 mm i.d. × 250 mm, 4 µm) was used with a PDA detector (190–400 nm). The eluent conditions were as follows: (A) 10 mm H3PO4 aq. / (B) CH³CN, linear gradient (high pressure). Gradient: 0% (0 min) → 30% (5–10 min) → 0% (10.1–20 min). The column was kept at 50 °C, and the flow rate was 1.0 mL/min.

Results and Discussion

Figure 1 shows the UV chromatograms of the standards. Peaks of the 10 vitamins were fully resolved using the developed method. The UV absorbance was measured at 254 nm. However, since the absorbance of pantothenic acid and biotin at 254 nm were low, 210 nm was used for the measurement.

This simple method using phosphoric acid and acetonitrile as the eluents demonstrated a successful simulated analysis of 10 water-soluble vitamins in 20 min, including the column equilibration time.

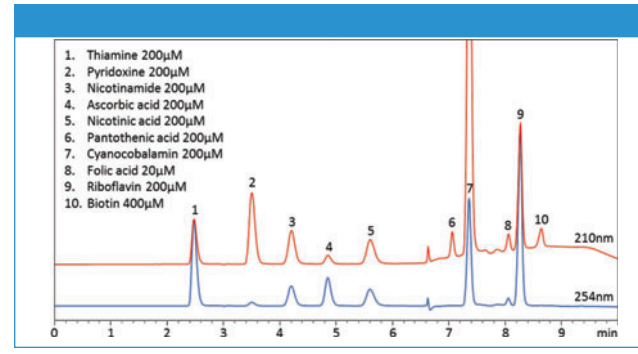

Figure 1: Sample UV chromatograms showing a simultaneous analysis of ten water-soluble vitamins.

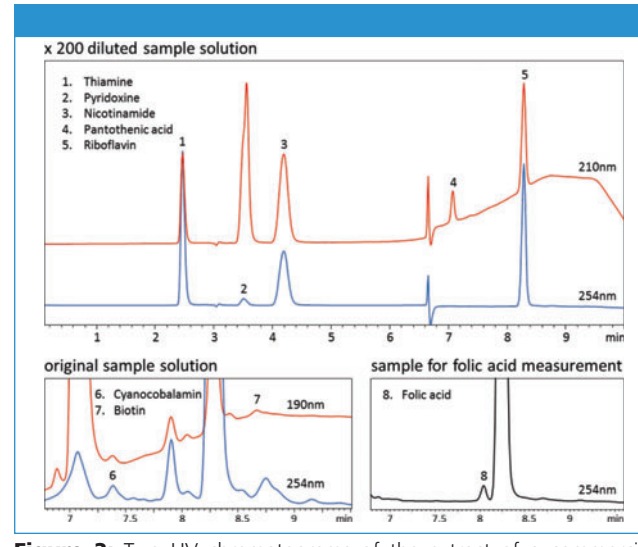

Figure 2: Two UV chromatograms of the extract of a commercial multi-vitamin supplement

We analyzed the extract of a commercial multivitamin supplement. We used a guard column (Shodex RSpak DE-G 4A) in addition to the analytical column during the sample analysis. (Figure 2).

A method for simultaneous analysis of 10 water-soluble vitamins was developed using the Shodex RSpak DE-413L column. The eluents used consisted of a mixture of an acid and acetonitrile. One sample measurement completes in 20 min.

The Shodex RSpak DE series provide a stable analysis even under highly aqueous eluent conditions, without the concern of column deterioration due to the polymer-based packing materials compared to using silica-based material.

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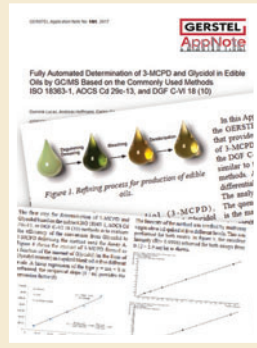
PRODUCTS & RESOURCES

Edible oils application note

An application note from Gerstel describes the determination of 3-MCPD and glycidol inedible oils by gas chromatography–mass spectrometry based on Methods ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10). The note reports that the method meets the requirements of AOCS, ISO, and DGF standard methods.



Gerstel, Inc.,
Linthicum, MD.
www.gerstel.com; www.gerstel.com/pdf/AppNote-191.pdf

HPLC column

The Hamilton PRP-C18 column is designed for reversed-phase separations over an extended column life in nearly any mobile phase or pH. According to the company, the rigid stationary phase has mechanical and thermal stability (> 100 °C), does not shrink or swell, and is inert to most conditions commonly encountered in reversed-phase chromatography.

Hamilton Company, Reno, NV.
www.hamiltoncompany.com

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Wallingford CT.
www.protononsite.com

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Bellefonte, PA.
www.restek.com

SEC columns

The HK-404L polymer-based size-exclusion chromatography column from Shodex is designed for analysis over a broad range of molecular weights. According to the company, the column enables a highly linear calibration curve, and ultra-rapid analysis can be completed on a conventional HPLC instrument.

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www.shodexhplc.com

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Optimize Technologies' UHPLC high-tight EXP trap columns are designed for use at pressures of 20,000+ psi (1400+ bar). According to the company, the expanded trap column line provides packed beds ranging from 2–30 mm in length, with diameters from 1–4.6 mm, and the columns connect directly to any injection valve or in-line with EXP fittings.

Optimize Technologies,
Oregon City, OR.
www.optimizetech.com

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The Shimadzu LCMS-9030 is a research grade mass spectrometer designed to deliver high-resolution, accurate-mass detection with fast data acquisition rates, which, according to the company, allows scientists to identify and quantify compounds with confidence.

Shimadzu Scientific Instruments,
Columbia, MD.
www.ssi.shimadzu.com

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The Mistral EVO self-contained gas generator from VICI is designed to produce greater than 99% pure LC–MS-grade nitrogen gas with pressures up to 116 psig, and with flows up to 40 L/min. According to the company, all gases are produced using a combination of compressors, filtration, and high-performance pressure swing technologies.

VICI DBS Gas Generators,
Houston, TX.
www.vicidbs.com

(Continued from page 27)
preferable if journal editors and reviewers insisted that metabolomics experiments follow this same format.

Another helpful movement in metabolomics is the need for defined reference materials. The National Institute of Standards and Technology (NIST) group has been active in developing these materials, characterizing them in ring trials, and making them available to the research community. This can be quite helpful in benchmarking methods against a known metabolic profile in a well-described biofluid. Defined parameters for metabolite identification, public availability of acquired datasets, and clear experimental descriptions will help to expand the field and the utility of metabolomics as an informative platform for understanding metabolism.

Q. What is your group working on next?
A: We are currently expanding upon the metabolomics method we published last year (1). One of the primary aims is to automate the method as much as possible. We have, therefore, formatted the sample preparation for both urine and plasma using an automated liquid handling platform. We can then prepare all samples in 96-well plates for analysis, which reduces both sample handling and preparation time. As part of these efforts, we are focusing on fully annotating the observed urinary metabolome with our current methodology. These efforts include evaluating shifts in observed metabolites associated with glucuronidase and urease treatment, as well as concentration and fractionation steps.

We envision developing a series of methods enabling us to capture different metabolic fractions of the urinary metabolome depending upon the biological question. One of the weaknesses of metabolomics is that it is less sensitive compared to targeted approaches. There are multiple metabolites, such as lipid mediators and halogenated tyrosine derivatives, that are of extreme interest in respiratory biology, but cannot be detected by our metabolomics methods, because of their low endogenous concentration. We would like to develop a concentration and clean-up step that would provide us with a urinary metabolomics platform to detect these low abundant compounds. The presence of high abundant metabolites, such as creatinine, will be a challenge. We are also working on developing high-throughput metabolomics applications. There are multiple challenges associated with these efforts, but there is the potential to offer extremely fast analysis times. We are not there yet, but the field is making major advances, and it will be exciting to see where we are in another 5–10 years.

References

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(2) L.W. Sumner et al., *Metabolomics* **3**(3), 211–221 (2007).
(3) R. Chaleckis, S. Naz, I. Meister, and C.E. Wheelock, *Methods Mol. Biol.* **1730,** 45–58 (2018).
(4) https://www.ebi.ac.uk/metabolights.
(5) http://fged.org/projects/miame/.
(6) A. Brazma, P. Hingamp, J. Quackenbush et al., *Nat. Genet.* **29**(4), 365–371 (2001). doi:10.1038/ng1201-365.

Craig Wheelock is head of the Integrative Molecular Phenotyping Laboratory at the Karolinska Institute (Sweden) and an associate professor within the Centre for Allergy Research (CfA). He is also a distinguished visiting professor of metabolomics at the Gunma Institute for Advanced Research (GIAR) at Gunma University (Japan). He is currently a member of the European Respiratory Society Scientific Events Working Group and board member of the International Metabolomics Society.

AD INDEX

3-MCPD?
2-MCPD?
Glycidol?

Automated determination of contaminants in edible oil and fat based on ISO 18363-1, AOCS Cd 29c-13, and DGF C-VI 18 (10):

- WorkStation Sample Prep Solution or
- Complete automation incl. GC/MS
- Evaporation step for lowest limits of determination and best system stability
- Analytical support at your service

Samples are automatically prepared and analyzed, including derivatization and evaporation of excess solvent and reagent for best limits of determination and optimal GC/MS system stability.

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