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Frontiers in Food Analysis

The RAFA 2022 organizers reveal what the 10th International Symposium on Recent Advances in Food Analysis (RAFA 2022) has to offer chromatographers in this special supplement compiled in collaboration with LCGC Europe.

The 10th International Symposium on Recent Advances in Food Analysis (RAFA 2022) will take place in Prague, Czech Republic, on 6–9 September 2022.

The RAFA organizers, the University of Chemistry and Technology, Prague (UCT Prague, Czech Republic), and Wageningen Food Safety Research (WFSR), part of Wageningen University & Research (The Netherlands), would like to invite food scientists from academia, together with representatives of industry, national and international agencies, control authorities, and governmental and commercial laboratories to attend this event.

The RAFA 2022 symposium will provide an overview of contemporary trends in analytical and bioanalytical strategies in food quality and safety control, and will discuss challenges and novel approaches in food and natural products analysis, including: multi-omics in food analysis; food fraud; forensics and authenticity; organic crops and foodstuffs; bioactivity determination and identification; micro- and nano-plastics in food; portable on-site food analysis; QA/QC, chemometrics, and big data handling; mycotoxins, marine, and plant toxins in a changing climate; process-induced contaminants; pesticide and veterinary drug residues; food legislation and food control; human biomonitoring and exposure assessment; healthy nutrients and vitamins; environmental contaminants; emerging alternative proteins; allergens and other issues; metals, metalloids, and speciation; cannabinoids in food and supplements; and flavours in food engineering.

Vendor seminars and exhibition will be organized by leading companies to introduce recent instrumentation and analytical strategies for advanced food quality and safety control.

Opportunities for Young Scientists
As always, RAFA will offer a platform for young scientists to present their scientific work, with 20% of the contributed oral presentations typically assigned to the next generation. There are opportunities to take advantage of the RAFA 2022 “Student Travel Grants”, and the best poster presentation(s) by a young scientist(s) will be rewarded with the prestigious RAFA Poster Award and sponsored poster award(s).

In summary, RAFA 2022 offers a high-quality scientific programme, with top-quality presentations, stimulating discussions, a series of satellite events, state-of-the-art exhibition, and an attractive social programme. More details can be found on the RAFA 2022 website at www.rafa2022.eu

RAFA 2022 Companion: Hot Topics
To highlight the innovation that is taking place in food analysis and at RAFA 2022, we have collaborated with LCGC to compile a series of articles highlighting hot topics in food analysis, as well as a compilation of interviews with presenters who are speaking at this year’s event. We hope you find this information useful and look forward to meeting you in person in Prague.
Chlorinated paraffins (CPs) are an emerging and ubiquitous group of environmental pollutants associated with adverse effects on human health, including endocrine disruption and possible carcinogenicity. In this study, supercritical fluid chromatography (SFC) and ultrahigh-performance liquid chromatography (UHPLC)—both coupled with high-resolution mass spectrometry (HRMS)—methods for the analysis of short-, medium-, and long-chain CPs in fish oil-based dietary supplements were developed and validated at concentration levels of 0.6 and 3.0 µg/g lipid weight (lw). The recoveries were in the range of 80–96% and repeatabilities, expressed as relative standard deviations, were <19%. The limits of detection for the UHPLC–HRMS method (from 0.03 to 0.05 µg/g lw) were 5 to 10 times lower than those obtained by SFC–HRMS (from 0.13 to 0.50 µg/lw).

Chlorinated paraffins (CPs) are widespread environmental pollutants, and are used predominantly as additives (such as plasticizers and flame retardants) in plastic materials (1,2), high temperature and pressure-resistant lubricants, metal-working fluids, and flame retardants in adhesives and textiles (3). CPs are divided into three main groups by their carbon chain lengths: (i) short-: (SCCPs; C_{10}–C_{13}), (ii) medium-: (MCCPs; C_{14}–C_{17}), and (iii) long-chain chlorinated paraffins (LCCPs: C_{18}–C_{30}) (4).

SCCPs are endocrine disruptors (5) and possible carcinogens to humans (6), therefore, they were added to the Stockholm Convention (Annex A) in 2017 as persistent organic pollutants (POPs) (7). The data on both MCCPs and LCCPs adverse effects on human health are very limited, and so they are not currently regulated (8). On the other hand, MCCPs are under evaluation for listing in the Stockholm Convention, and the risk assessment and risk profile are being performed (7).

The instrumental analysis of CPs in food and biological samples (usually at trace levels) (9,10) is a demanding task. In recent years, several gas chromatography (GC) or liquid chromatography (LC)-based methods have been published (9–12). Nevertheless, CPs cannot be completely separated to individual compounds, which has led (together with the lack of well-characterized standard mixtures) to the need for unconventional quantification approaches (13–15). The LCCP standards have the least available options on the market.

The main goal of this study was to develop and validate supercritical fluid chromatography (SFC) and ultrahigh-performance liquid chromatography (UHPLC)—
both coupled with high-resolution mass spectrometry (HRMS) with electrospray ionization operated in negative mode (ESI−)—methods for the determination of CPs (particularly LCCPs as those cannot be analyzed by GC–HRMS due to low vapour pressure). Fish oil dietary supplements were selected as the sample matrix, as an example of an adequately complex type of sample. The validated methods are necessary for future studies to assess the CPs levels in food and the environment, particularly for LCCPs, which are the least studied among the major CP groups.

**Materials and Methods**

**Standard Solutions and Chemicals:** Thirty-one standard mixtures of SCCPs, MCCPs, and LCCPs of 10 µg/mL or 100 µg/mL cyclohexane with various chlorine contents (from 36.0% to 65.3%) were obtained from LGC Standards. The detailed list is documented in our previous paper (16). Isotopically labelled internal standard of β-hexabromocyclododecane ($^{13}$C$_{12}$β-HBCD; 50 µg/mL toluene) was purchased from Wellington Laboratories.

Acetonitrile, methanol, n-hexane, ethyl acetate, and isopropanol were obtained from Honeywell. Dichloromethane, isoctane, sulfuric acid, ammonium formate, and silica gel 60 (particle size 0.063–0.200 mm) were purchased from Merck, while DIC (Hach–Ner) was bought from Lach–Ner. Deionized water was made by a water purification system Milli-Q by Merck. Technical gases (carbon dioxide 4.8 and nitrogen 4.0) were supplied by SIAD.

**Sample Preparation Procedure:** In this study, a sample of fish oil-based dietary supplement with no measurable CPs contamination found in a previous study (16) was chosen for method development and validation. Four other samples (with higher levels of CPs contamination previously determined by GC–HRMS) were analyzed to compare the new methods.

A multilayer solid-phase extraction (SPE) was used in this study for the sample preparation. Briefly, the CPs were isolated from fish oil on an SPE column (78 × 12 mm with Luer tip) (LCTech GmbH) filled with (from bottom to top) silica gel (0.5 g; deactivated by 2% deionized water, w/w), sodium sulfate (1 g; anhydrous baked for 4 h at 600 °C), and sulphuric acid-modified silica gel (1 g; 40% of H$_2$SO$_4$, w/w). Each column was washed with 3:1 (v/v) n-hexane–dichloromethane and then conditioned with n-hexane. A 100-mg measure of the sample diluted in n-hexane was loaded onto the SPE column and the analytes were eluted with 3:1 v/v n-hexane–dichloromethane. The sample was subsequently concentrated by a rotary vacuum evaporator followed by drying in a gentle stream of nitrogen. It was then dissolved in 500 µL of n-hexane, and the residual lipids were subsequently mineralized by a few drops of concentrated sulfuric acid. After one hour, an aliquot of 250 µL was evaporated and redissolved in 250 µL of the syringe standard (50 ng/mL $^{13}$C$_{12}$β-HBCD) in acetonitrile.

**Instrumental Analysis:**

**UHPLC–ESI(−)–HRMS Analysis:** The LC–ESI(−)–HRMS analysis of CPs was performed by Dionex UlItiMate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a TripleTOF 6600 HRMS system (Sciex) with electrospray ionization operated in a negative mode. The method was developed from initial conditions published elsewhere (17). In this study, the target analytes (injection volume 5 µL acetonitrile) were separated on a 100 × 2.1 mm, 1.7-µm Acuity UPLC BEH C18 (Waters) column maintained at 40 °C. Methanol (A) and a mixture of 65:30:5 (v/v/v) isopropanol–methanol–water (B) were used as mobile phases. The initial conditions were 10% B for 1 min followed by a gradient with the following steps: to 30% B at 1.5 min, to 60% B at 2 min, to 80% at 3 min, to 90% B at 3.5 min, to 100% B at 4 min (3.5 min isocratic hold; the total run time was 11 min including the return to the initial state and equilibration). The mobile phase flow rate was 0.2 mL/min.

Regarding MS source conditions, desolvation temperature was set to 450 °C and the capillary voltage was -4.5 kV. The acquisition speed was 2 spectra/s and the mass range 100 to 1500 m/z. The [M+Cl]$^-$ ions were monitored.

**SFC–ESI(−)–HRMS Analysis:** A previously published SFC-based method (16) was used in this study with several modifications (gradient of mobile phases, composition of mobile phase B, and make-up solvent were changed). The supercritical fluid chromatograph Acuity UPC$^2$ coupled with a Synapt G2 Si high-resolution mass spectrometer (both Waters) with electrospray ionization operated in a negative mode was employed. The target analytes (injection volume 3 µL acetonitrile) were separated on a 100 × 3.0 mm, 1.8-µm Viridis HSS C18 SB (Waters) column maintained at 70 °C. Supercritical CO$_2$ was used as mobile phase A and 5 mM...
ammonium formate in 99:1 (v/v) methanol–water was employed as mobile phase B. The initial conditions were 100% A for 0.5 min followed by a gradient to 35% B at 5 min (1 min isocratic hold; the total run time was 8 min including the return to the initial state and equilibration). The mobile phase flow rate was 1.8 mL/min. After the separation under supercritical conditions, the CO$_2$ evaporated and had to be substituted by another mobile phase—a make-up solvent—which leads the analytes into the ion source. In this study, the make-up solvent was a mixture of 45:45:10 (v/v/v) methanol–ethyl acetate–dichloromethane (flow rate 0.5 mL/min).

In the MS system, the desolvation gas temperature was 250 °C and the capillary voltage was set to -3 kV. The acquisition speed was 2 spectra/s and the mass range 250 to 1500 m/z. The [M+Cl]$^-$ ions were monitored.

Method Validation: The analytical method for the determination of SCCPs, MCCPs, and LCCPs was validated by the analysis of artificially contaminated samples (at two different concentration levels, each level prepared in six parallels). The selected spike levels were 0.6 and 3 µg/g lw for each of the CP groups. The standard mixtures used were C$_{10}$–C$_{13}$ 63.0% Cl; C$_{14}$–C$_{17}$ 57.0% Cl, and C$_{18}$–C$_{20}$ 49.0% Cl. The limits of detection (LODs) were determined as the lowest standard level at which any CP congener group was integrable (with S/N ≥ 10).

Evaluation of Matrix Effects: The matrix effects were evaluated by comparing matrix standards (prepared in duplicate) with solvent standards at a level of 0.6 µg/g lw. The equation used for the evaluation was described elsewhere (12). The sample preparation was performed as described in the “Methods and Materials” section. For a comparison, matrix effects of the extract with residual lipids (without sulfuric acid treatment) were evaluated as well.

Results and Discussion

Comparison of Method Performance Characteristics of SFC and UHPLC-Based Methods for the Analysis of CPs in Fish Oil-Based Dietary Supplements: The method performance characteristics of SFC–HRMS and UHPLC–HRMS methods for analysis of SCCPs, MCCPs, and LCCPs are shown in Table 1. There are no officially recommended reference values for recovery and repeatability in the analysis of CPs, therefore the acceptable values (recovery from 60 to 120% and repeatability <20%) were adopted from Commission Regulation (EU) 2017/644 (18) concerning sampling and analysis of polychlorinated dibenzodioxins/furans and polychlorinated biphenyls (other chlorinated POPs) in foodstuffs. The recoveries varied from 84 to 95% (SFC-based instrumental method) and from 80 to 96% (UHPLC-based instrumental method), respectively. The repeatabilities (expressed as relative standard deviations, RSDs) were <19%. Both methods were therefore successfully validated on levels of 0.6 and 3 µg/g lw, with no significant differences regarding recovery and repeatability. The UHPLC instrumentation proved to yield lower LODs (5 to 10 times lower, see Table 1) than the SFC method (probably due to the presence of splitter on the interface of SFC and MS). On the other hand, the SFC proved to be a robust method regarding injection solvent, that is, the use of acetonitrile in this study and isooctane used elsewhere (16), where the samples prepared for GC–HRMS analysis (in isooctane) were also measured by SFC–HRMS (as a complementary method for screening of LCCPs).

Matrix Effects Evaluation: The matrix effects are illustrated in Figure 1. The positive effect of a sulfuric acid treatment is documented there. In samples without the sulfuric acid treatment, there can be seen a strong signal suppression, which was more

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Recoveries at Concentration Level 0.6 µg/g lw (recovery; n = 6)</th>
<th>Recoveries at Concentration Level 3 µg/g lw (recovery; n = 6)</th>
<th>LODs (µg/g lw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SFC</td>
<td>UHPLC</td>
<td>SFC</td>
</tr>
<tr>
<td>SCCPs</td>
<td>92% (RSD 11%)</td>
<td>83% (RSD 16%)</td>
<td>95% (RSD 19%)</td>
</tr>
<tr>
<td>MCCPs</td>
<td>95% (RSD 6%)</td>
<td>85% (RSD 14%)</td>
<td>92% (RSD 16%)</td>
</tr>
<tr>
<td>LCCPs</td>
<td>84% (RSD 10%)</td>
<td>80% (RSD 16%)</td>
<td>85% (RSD 17%)</td>
</tr>
</tbody>
</table>
significant in SFC (with a response decrease of up to 69% for the MCCPs). The signal suppression was presumably caused by methyl esters of fatty acids, as these esters are sometimes a dominant form of fatty acids in concentrated fish oil-based dietary supplements (19). The free fatty acids released from ester bonds by sulfuric acid were then separated from CPs on the chromatographic column, decreasing effectively the matrix effects, which was verified by successful validation.

**SFC and UHPLC Method Comparison:** For the developed and validated methods final comparison, the methods were used for determination of CPs in four fish oil-based dietary supplements (Figure 2). Chromatograms illustrating the separation differences of several chromatographic systems are shown in Figure 3. The concentrations of SCCPs and MCCPs obtained employing SFC and UHPLC systems were comparable (considering 30% uncertainty). The concentrations were in the range of 1.01–37.22 µg/g lw (SCCPs) and 0.83–37.76 µg/g lw (MCCPs), respectively. These results were lower than concentrations obtained by GC–HRMS in our previously published study (16). The differences may have been caused by still remaining matrix effects, even after sulfuric acid treatment of the samples. Similarly, concentrations of LCCPs obtained by UHPLC–HRMS (0.10–0.36 µg/g lw) were in the samples 3 and 4 lower than those obtained by SFC–HRMS (0.20–1.59 µg/g lw). The differences might have been caused by slightly higher matrix effects (Figure 1).

**Conclusions**

This article describes a validation of two analytical approaches
(employing SFC- and UHPLC-based methods) with a single sample preparation procedure for the analysis of SCCPs, MCCPs, and LCCPs in fish oil-based dietary supplements. The recoveries ranged from 80 to 96% (with RSDs <19%). The UHPLC instrumentation showed lower LODs than SFC, but SFC demonstrated a good robustness because acetonitrile or isooctane extracts could be analyzed under the same conditions.

The methods were then used to determine CPs contamination in four samples of dietary supplements. The SCCP and MCCP concentrations obtained by both systems were comparable, while the LCCP concentrations differed (with UHPLC yielding lower results). The SCCP and MCCP levels were also compared with results obtained employing GC–HRMS (which were previously published). The results obtained by SFC and UHPLC were slightly lower than those obtained by the GC-based method. This might have been caused by matrix effects in some samples, and in the following studies further research is needed (that is, selection of a 13C-labelled CP internal standard and/or use of a more complex clean-up procedure). Finally, the methods are planned to be verified in interlaboratory studies.

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5) H. Li, S. Gao, M. Yang, et al., Chemosphere 244, 125393 (2020).

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Quantitative Analysis of PFAS in Milk, Infant Formula, and Related Ingredients Using Liquid Chromatography–Tandem Mass Spectrometry

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Eurofins Food Chemistry Testing, Madison, Wisconsin, USA, Agilent Technologies, Inc., Little Falls, Delaware, USA

Per- and polyfluoroalkyl substances (PFAS) are a large group of anthropogenic chemicals that have been applied in a wide range of industrial, commercial, and domestic products since the 1950s. Because of their toxicity, persistence, and bioaccumulation potential, PFAS have become global environmental pollutants. Besides the environment, the food chain represents another source of exposure, and the risk to consumers related to the presence of PFAS in foods has recently become of increased interest. In this respect, whole milk, infant formula, and ingredients used in infant formula production represent important foodstuffs that require sensitive methods with reporting limits at low parts per billion (ppb) levels or lower for multiple PFAS. This article summarizes optimization experiments and the validation of a complete workflow, including sample preparation and a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, for the determination of 16 priority PFAS analytes listed by the U.S. Food and Drug Administration (FDA).

Rapid, sensitive, and reliable analytical techniques are required for the monitoring of PFAS in the above matrices. Liquid chromatography coupled to tandem quadrupole mass spectrometry (LC–MS/MS) with electrospray ionization (ESI) operated in negative ionization mode has been the instrumental technique most frequently used for the analysis of PFAS in foods as well as in other biotic matrices (3).

In this study, an LC–MS/MS assay based on the U.S. FDA method C-010.01 was optimized and validated for the analysis of 16 PFAS compounds in whole milk, ready-to-feed (RTF) and powdered infant formula, milk powder, soybean oil, and maltodextrin matrices (4).
Standards, Reagents and Samples:
The target PFAS and the stable isotope-labelled internal standards employed in this study are listed in Table 1. The PFAS standards, including isotopically-labelled analogues, were purchased from Wellington Laboratories. The formic acid (99%, for analysis) was from Thermo Scientific. LC–MS-grade ammonium formate, acetonitrile, and methanol were from MilliporeSigma. LC–MS-grade isopropanol and HPLC-grade hexane were from Thermo Fisher Scientific. Ultrapure water (UPW) was obtained from Elga Purelab Ultra purification system. QuEChERS salts (6 g anhydrous MgSO₄ and 1.5 g NaCl) and dispersive-solid phase extraction (SPE) sorbents in 15-mL polypropylene (PP) centrifuge tubes (900 mg MgSO₄, 300 mg PSA, 150 mg graphitized carbon black (GCB)) were supplied by UCT. PP centrifuge tubes were from Sarstedt. Disposable PP syringes were obtained from Becton Dickinson. Nylon syringe filters (15 mm, 0.2 µm) and PP vials (1 mL) with caps were from Agilent Technologies.

Method validation was performed with the use of whole milk, RTF and powdered infant formula, milk powder, soybean oil, and maltodextrin samples obtained from the local retail market and previously demonstrated to be free of target PFAS. Sample Preparation: Sample preparation followed a modified QuEChERS method based on the U.S. FDA method C-010.01. Homogenous samples (2 g for solids and oils and 5 g for liquids) were weighed into a 50-mL polypropylene (PP) tube followed by the addition of UPW (15 mL for solids and oils and 5 mL for liquids). Hexane (10 mL) was added to oil samples prior to aliquoting UPW. Each sample was spiked with 10 ng of isotope-labelled PFAS, added.

**Table 1:** PFAS compounds analyzed, with software-optimized MRM parameters and stable isotope-labelled internal standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acronym</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ion (s) (m/z)</th>
<th>RT (min)</th>
<th>CE (V)</th>
<th>Internal Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfluoro-n-butanoic acid</td>
<td>PFBA</td>
<td>213</td>
<td>169</td>
<td>1.83</td>
<td>6</td>
<td>M3PFBA</td>
</tr>
<tr>
<td>Perfluoro-n-pentanoic acid</td>
<td>PFPeA</td>
<td>263</td>
<td>219</td>
<td>3.04</td>
<td>4</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoro-n-hexanoic acid</td>
<td>PFHxA</td>
<td>313</td>
<td>269 (119)</td>
<td>3.78</td>
<td>6,18</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoro-n-heptanoic acid</td>
<td>PFHpA</td>
<td>363</td>
<td>319 (169,119)</td>
<td>4.54</td>
<td>6,16,20</td>
<td>MPFHxA</td>
</tr>
<tr>
<td>Perfluoro-n-octanoic acid</td>
<td>PFOA</td>
<td>413</td>
<td>369 (219,169)</td>
<td>5.42</td>
<td>8,12,16</td>
<td>M8PFOA</td>
</tr>
<tr>
<td>Perfluoro-n-nonanoic acid</td>
<td>PFNA</td>
<td>463</td>
<td>419 (269,219,169)</td>
<td>6.32</td>
<td>8,12,16,17</td>
<td>M8PFOA</td>
</tr>
<tr>
<td>Perfluoro-n-decanoic acid</td>
<td>PFDA</td>
<td>513</td>
<td>469 (269,219,169)</td>
<td>7.21</td>
<td>8,16,16,20</td>
<td>M8PFOA</td>
</tr>
<tr>
<td>Perfluorobutane-1-sulfonic acid</td>
<td>PFBS</td>
<td>299</td>
<td>99 (80)</td>
<td>3.53</td>
<td>33,40</td>
<td>M3PFBS</td>
</tr>
<tr>
<td>Perfluoropentane-1-sulfonic acid</td>
<td>PFPeS</td>
<td>349</td>
<td>80 (119,99)</td>
<td>4.25</td>
<td>48,40,36</td>
<td>MPFHxS</td>
</tr>
<tr>
<td>Perfluorohexane-1-sulfonic acid</td>
<td>PFHxS</td>
<td>399</td>
<td>80 (169,119,99)</td>
<td>5.10</td>
<td>45,35,40,40</td>
<td>MPFHxS</td>
</tr>
<tr>
<td>Perfluoroheptane-1-sulfonic acid</td>
<td>PFHpS</td>
<td>449</td>
<td>80 (169,99)</td>
<td>6.01</td>
<td>60,40,44</td>
<td>MPFHxS</td>
</tr>
<tr>
<td>Perfluoroctane-1-sulfonic acid</td>
<td>PFOS</td>
<td>499</td>
<td>80 (99)</td>
<td>6.90</td>
<td>50,50</td>
<td>M8PFOA</td>
</tr>
<tr>
<td>4-Bis-Dioxa-3H-perfluorononanoic acid</td>
<td>ADONA</td>
<td>377</td>
<td>251 (85)</td>
<td>4.77</td>
<td>8,32</td>
<td>M8PFOA</td>
</tr>
<tr>
<td>2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptfluoropropoxy)-propanoic acid</td>
<td>HFPO-DA (Gen X)</td>
<td>285</td>
<td>169 (185,119)</td>
<td>4.04</td>
<td>4,16,30</td>
<td>M3HFPO-DA</td>
</tr>
<tr>
<td>9-Chlorohexadecafluoro-3-oxanone-1-sulfonic acid</td>
<td>9CI-PF3ONS</td>
<td>531</td>
<td>351 (99,83)</td>
<td>7.52</td>
<td>28,40,32</td>
<td>MPFHxS</td>
</tr>
<tr>
<td>11-Chloro(polyfluorooctyl)oxo-1-sulfonic acid</td>
<td>11CI-PF3OUdS</td>
<td>631</td>
<td>451 (199,83)</td>
<td>9.06</td>
<td>32,30,32</td>
<td>MPFHxS</td>
</tr>
<tr>
<td>Perfluoro-n-[2,3,4,13C3] butanoic acid</td>
<td>M3PFBA</td>
<td>216</td>
<td>172</td>
<td>1.82</td>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>Perfluoro-n-[1,2,13C2] hexanoic acid</td>
<td>MPFHxA</td>
<td>315</td>
<td>270</td>
<td>3.78</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>Perfluoro-n-[13C8] octanoic acid</td>
<td>M8PFOA</td>
<td>421</td>
<td>376 (172)</td>
<td>5.39</td>
<td>8,18</td>
<td>NA</td>
</tr>
<tr>
<td>Perfluoro-1-[2,3,4,13C3] butanesulfonic acid</td>
<td>M3PFBS</td>
<td>302</td>
<td>80 (99)</td>
<td>3.53</td>
<td>41,35</td>
<td>NA</td>
</tr>
<tr>
<td>Perfluoro-1-hexane[18O2] sulfonic acid</td>
<td>MPFHxS</td>
<td>403</td>
<td>84 (169,103)</td>
<td>5.10</td>
<td>50,35,45</td>
<td>NA</td>
</tr>
<tr>
<td>Perfluoro-1-[13C8] octane sulfonic acid</td>
<td>M8PFOS</td>
<td>507</td>
<td>80 (99)</td>
<td>6.87</td>
<td>58,50</td>
<td>NA</td>
</tr>
<tr>
<td>2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptfluoropropoxy)-13C3-propanoic acid</td>
<td>M3HFPO-DA</td>
<td>287</td>
<td>185 (169)</td>
<td>4.04</td>
<td>18,4</td>
<td>NA</td>
</tr>
</tbody>
</table>

Experimental

Standards, Reagents and Samples:
The target PFAS and the stable isotope-labelled internal standards employed in this study are listed in Table 1. The PFAS standards, including isotopically-labelled analogues, were purchased from Wellington Laboratories. The formic acid (99%, for analysis) was from Thermo Scientific. LC–MS-grade ammonium formate, acetonitrile, and methanol were from MilliporeSigma. LC–MS-grade isopropanol and HPLC-grade hexane were from Thermo Fisher Scientific. Ultrapure water (UPW) was obtained from Elga Purelab Ultra purification system. QuEChERS salts (6 g anhydrous MgSO₄ and 1.5 g NaCl) and dispersive-solid phase extraction (SPE) sorbents in 15-mL polypropylene (PP) centrifuge tubes (900 mg MgSO₄, 300 mg PSA, 150 mg graphitized carbon black (GCB)) were supplied by UCT. PP centrifuge tubes were from Sarstedt. Disposable PP syringes were obtained from Becton Dickinson. Nylon syringe filters (15 mm, 0.2 µm) and PP vials (1 mL) with caps were from Agilent Technologies.

Method validation was performed with the use of whole milk, RTF and powdered infant formula, milk powder, soybean oil, and maltodextrin samples obtained from the local retail market and previously demonstrated to be free of target PFAS. Sample Preparation: Sample preparation followed a modified QuEChERS method based on the U.S. FDA method C-010.01. Homogenous samples (2 g for solids and oils and 5 g for liquids) were weighed into a 50-mL polypropylene (PP) tube followed by the addition of UPW (15 mL for solids and oils and 5 mL for liquids). Hexane (10 mL) was added to oil samples prior to aliquoting UPW. Each sample was spiked with 10 ng of isotope-labelled PFAS, added.
with 10 mL acetonitrile and 150 µL formic acid. The tube was vortexed for 1 min. Pre-weighed QuEChERS salt mixture was added to the tube, and the mixture was immediately shaken to ensure crystalline agglomerates were broken up sufficiently. Samples were further vortexed on a multi-tube vortexer set to 1500 rpm for 5 min. Following centrifugation at >2000 × g, the upper acetonitrile layer was transferred into a 15-mL tube while avoiding lipid layer or hexane. In the next step, a pre-weighed dispersive-SPE sorbent mixture (900 mg MgSO₄, 300 mg PSA and 150 mg GCB) was added to the sample extract, and the tube was vortexed for 2 min and centrifuged (>2000 × g, 5 min). A 5-mL aliquot of the supernatant was transferred into a fresh 15-mL centrifuge tube, and evaporated at 60 °C to near dryness using a gentle stream of nitrogen. The residues were reconstituted in 500 µL of methanol and briefly vortexed to mix. The sample was filtered through a 0.2-µm nylon filter into a PP autosampler vial, and capped.

**LC–MS/MS Instrumentation:**

LC–MS/MS analysis of the PFAS was carried out using an Agilent 1290 Infinity II liquid chromatograph (LC), coupled with an Agilent 6495 triple quadrupole LC–MS system. The LC system was equipped with an Agilent Zorbax Eclipse Plus rapid resolution high definition (RRHD) C18 column (2.1 × 50 mm, 1.8-µm) with an Agilent Zorbax Eclipse Plus guard column. Gradient elution was performed with 1 mM ammonium formate in UPW (A) and a mixture of 1:1 (v/v) acetonitrile and methanol (B) at a flow of 600 µL/min. The eluent was diverted to waste during the first 1.0 min and after 10 min of the run. The total run time from injection to injection was approximately 12.5 min. Specific parameters are listed in Table 2.

To eliminate PFAS background, an Agilent perfluorinated compound (PFC)-free high-performance liquid chromatography (HPLC) conversion kit was installed on the Agilent 1290 Infinity II LC system (S). Additionally, an Agilent Poroshell 120, 3.0 × 30 mm, 1.9-µm delay column was connected between the solvent mixer and injector module to further control potential background contamination from the mobile phases. To reduce contamination because of sorption after injection, the needle wash procedure consisted of 10-s washes with 1:1 (v/v) acetonitrile–isopropanol, followed by methanol and 9:1 (v/v) UPW–methanol.

The LC–MS parameters are listed in Table 3. The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in negative ion and multiple reaction monitoring (MRM) modes. The MRM parameters (listed in Table 1) were optimized for best response using the Agilent MassHunter Optimizer software. Quantification of target analytes was performed with the use of calibration standards prepared in solvent.

Table: Additional LC parameters

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>A: 1 mM ammonium formate in UPW</th>
<th>B: 1:1 (v/v) acetonitrile–methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (µL/min)</td>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

Table: Mass spectrometer parameters

<table>
<thead>
<tr>
<th>Ionization mode</th>
<th>Negative electrospray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying gas temperature (°C)</td>
<td>150</td>
</tr>
<tr>
<td>Gas flow rate (L/min)</td>
<td>11</td>
</tr>
<tr>
<td>Nebulizer (psi)</td>
<td>20</td>
</tr>
<tr>
<td>Sheath gas temperature (°C)</td>
<td>400</td>
</tr>
<tr>
<td>Sheath gas flow (L/min)</td>
<td>12</td>
</tr>
<tr>
<td>Capillary voltage (V)</td>
<td>2500</td>
</tr>
<tr>
<td>High-pressure ifunnel RF voltage (V)</td>
<td>130</td>
</tr>
<tr>
<td>Low-pressure ifunnel RF voltage (V)</td>
<td>40</td>
</tr>
</tbody>
</table>

Isotope-labelled internal standards were employed to normalize analyte response. Analyte identification in samples was accomplished via retention time and qualifier-to-quantifier MRM ratio comparison with calibration standards.

**Results and Discussion**

**Method Optimization:** The main focus of the optimization efforts was on high throughput of the analysis. To meet this requirement, a 50 mm ultrahigh-pressure liquid chromatography (UHPLC) C18 column with sub-2-µm particle size and a corresponding 5 mm guard column maintained at 50 °C was used. Under the optimized conditions, baseline separation of all analytes was achieved in approximately 9 min, a significant improvement over the U.S. FDA method C-010.0 (4). The analyte peak widths at the baseline were below 0.2 min. A representative chromatogram of RTF infant formula extract spiked with target...
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analytes at a concentration of 1 µg/kg is presented in Figure 1.

Target precursor ions represented deprotonated molecules for all analytes except for hexafluoropropylene oxide-dimer acid (HFPO-DA, or Gen X) that fragmented during the ionization to form a decarboxylation product. Considerable time was spent evaluating the type and concentration of modifier in the aqueous mobile phase component A. Various concentrations of ammonium acetate, ammonium fluoride, and ammonium formate were tested. There was only a minimal impact on separation, but the type and concentration of the modifier impacted the response of certain analytes, especially HFPO-DA. When 1 mM ammonium formate was used as mobile phase A, it allowed for the best response for HFPO-DA, while not sacrificing response for the other PFAS.

**Method Validation:** The method validation evaluated selectivity, linearity, accuracy and precision, limit of quantification (LOQ), and method detection limit (MDL).

Selectivity of the method was demonstrated based on analyses of unspiked samples and a method (procedural) blank in each validation batch. The responses of target PFAS in matrix and method blank injections were <30% of the response in samples spiked at the LOQ. Analytes present in validation samples at or above the LOQ were successfully identified. The analyte retention times in samples were well within 0.1 min of the retention time in standards. Ion ratio in samples were within ±30% of the average ratio established in calibration standards for at least one quantifier/qualifier transition pair. Ion ratio-based identification could not be performed for perfluorobutyrate (PFBA) and perfluoro-n-pentanoic acid (PFPeA) because only a single MRM transition was available for each of these analytes.

**TABLE 4:** Method LOQs and MDLs determined in validation matrices

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MDL (ng/kg)</th>
<th>LOQ (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Milk</td>
<td>RTF Infant Formula</td>
</tr>
<tr>
<td>PFBA</td>
<td>82.5</td>
<td>18.7</td>
</tr>
<tr>
<td>PFPeA</td>
<td>4.80</td>
<td>5.50</td>
</tr>
<tr>
<td>PFHxA</td>
<td>7.50</td>
<td>5.80</td>
</tr>
<tr>
<td>PFHpA</td>
<td>7.00</td>
<td>4.50</td>
</tr>
<tr>
<td>PFOA</td>
<td>6.10</td>
<td>9.50</td>
</tr>
<tr>
<td>PFNA</td>
<td>4.50</td>
<td>10.1</td>
</tr>
<tr>
<td>PFDA</td>
<td>11.9</td>
<td>4.80</td>
</tr>
<tr>
<td>PFBS</td>
<td>4.40</td>
<td>6.20</td>
</tr>
<tr>
<td>PFPeS</td>
<td>10.3</td>
<td>11.9</td>
</tr>
<tr>
<td>PFHxS</td>
<td>10.2</td>
<td>6.20</td>
</tr>
<tr>
<td>PFHpS</td>
<td>4.40</td>
<td>6.10</td>
</tr>
<tr>
<td>PFOS</td>
<td>11.6</td>
<td>10.4</td>
</tr>
<tr>
<td>ADONA</td>
<td>4.90</td>
<td>6.30</td>
</tr>
<tr>
<td>HFPO-DA</td>
<td>7.90</td>
<td>13.7</td>
</tr>
<tr>
<td>9Cl-PF3ONS</td>
<td>4.10</td>
<td>3.00</td>
</tr>
<tr>
<td>11Cl-PF3OUS</td>
<td>4.50</td>
<td>3.20</td>
</tr>
</tbody>
</table>
Linearity was evaluated with each validation batch at eight concentration levels ranging from 0.1 to 50.0 ng/mL injected at minimum at the beginning and at the end of the measurement sequence. This concentration range corresponded to 0.050–25 µg/kg and 0.020–10 µg/kg in 2-g and 5-g samples, respectively. Linear regression calibration curves with 1/x weighting were constructed by plotting the analyte-to-internal standard peak area ratio against the analyte concentrations. The coefficients of determination ($R^2$) were consistently above 0.995 for all analytes with residuals within ± 20%.

Accuracy (marginal recovery) and precision (repeatability as relative standard deviation [RSD] and intermediate precision [RSD$_{INT}$]) were determined based on replicate analyses of unspiked samples and samples spiked with target PFAS at multiple concentrations ranging from 0.05 to 5.0 µg/kg. The spiking experiments were performed at minimum in six replicates over two different days. The mean recoveries calculated at individual spiking levels above method LOQ were within 70–120% with RSD, and RSD$_{INT}$ values ≤20%, indicating robust recoveries in a variety of matrices with high reproducibility. The mean recoveries obtained at LOQ for each analyte are shown in Figure 2.

MDL was determined for each analyte/matrix combination based on accuracy and precision data after samples were taken through the entire workflow including sample extraction and LC–MS/MS analysis. Standard deviation was calculated based on precision data at low level spiking concentration and the obtained value was multiplied by t-value corresponding to the appropriate degrees of freedom (6). LOQ was determined for each PFAS as the lowest spiking level for which the recoveries within 70–120% and RSDs ≤20% were achieved in all matrices while successfully identifying the analyte. MDLs and LOQs are summarized in Table 4. MDLs were typically lower than 25 ng/kg for most PFAS analytes in all matrices except for PFBA, which was significantly higher in matrices other than infant powder.

### Conclusions

This paper has described optimization and validation of a sensitive, robust, and high-throughput LC–MS method based on a modified QuEChERS procedure for the determination of 16 priority U.S. FDA PFAS analytes including perfluoroalkyl carboxylic acids, perfluoroalkyl sulfonic acids, and major replacement chemicals in whole milk, infant formula, and related ingredients. The validation experiments demonstrated adequate performance of the method in the studied matrices that proves to be robust, reliable, and reproducible with scope to expand the list of PFAS in the future.

### References

Poly- and perfluoroalkyl substances (PFAS)—a.k.a. “forever chemicals”—are widely used in everyday applications, and there are approximately 5000 known PFAS structures. This article presents a targeted approach for detecting and quantifying 24 PFAS commonly found in paper and board matrices. Using accelerated solvent extraction to extract PFAS, identification and quantification was done using high-performance liquid chromatography coupled with triple quadrupole mass spectrometry. Apparent recovery values were in the 84–94% range, with method detection limit values in the 0.1–0.5 ng/g range.

Poly- and perfluoroalkyl substances—PFAS—are man-made chemicals that are widely used in a range of applications, from water-resistant clothing to food packaging materials. Often called “forever chemicals”, these compounds have a special chemical structure: multiple fluorine atoms attached to an alkyl chain. The bond between carbon (C) and fluorine (F) is one of the strongest chemical bonds known. Additionally, the size of the fluorine atom is just right to pack closely around a carbon chain and shield it from interaction with other atoms (1). This is both good news and bad news. Because of their properties, PFAS repel water, fat, and dirt. This makes them very useful for various applications. But because PFAS are highly resistant towards chemical and physical strains, the bad news is that they are long-lasting, bio-accumulative, and unfortunately toxic.

In recent years, PFAS have been increasingly detected in water, soil, air, as well as in wildlife and human beings. For instance, in Belgium in 2021, the Flemish government issued a warning to residents to stop eating produce from their own gardens because of the high levels of PFAS in their water, soil, and food. PFAS have also been found in packaging products from many fast food chains (2). As a result, some PFAS have recently begun to be regulated or even phased out. But so far only a few of the substances have been assessed for risk by the European Food Safety Authority (EFSA) or the Environmental Protection Agency (EPA) (3,4).

Assessing the Threat of PFAS in Food Packaging

PFAS can enter the human body through various sources, but one of the major pathways is food consumption (5). As a result, in recent years much focus has been placed on PFAS found in packaging materials that are in direct contact with food. There are several different analytical methods used for detecting and quantifying PFAS in food packaging materials, depending on the desired type of analysis. However, the most selective and sensitive method is liquid chromatography coupled with triple quadrupole mass spectrometry (LC–MS/MS). Recently, researchers at the Institute of Analytical Chemistry and Food Chemistry at the Graz University of Technology in Austria, tested a simple method for extracting PFAS from paper and board matrices using accelerated solvent extraction (ASE), followed by quantification using LC–MS/MS.

Experimental

Twenty-four PFAS substances most commonly found in paper food contact material were analyzed. A mixture of these, with a concentration of 2000 ng/mL, was purchased from Wellington Laboratories (Guelph, Ontario). For each substance, the precursor ion and product ion, as well as the retention time, were identified. Two series of calibration solutions were prepared by dilution with 50:50 (v/v) methanol–water. For high concentration calibration, a series of nine calibration solutions at concentrations of 100, 50, 25, 15, 10, 5, 2.5, and 1.25 pg/µL was prepared. For low-concentration calibration, 10 calibration solutions at concentrations of 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, and 0.01 pg/µL were prepared. As a sample, an unprinted recycled paper (70 g/m²), which had previously been analyzed for the presence of PFAS, was used. The paper was cut into small pieces, and 2 g were used for experiments. The paper was spiked with a PFAS mixture with different concentrations of the standard solution—100, 50, 20, 10, 5, 2.5, 1, 0.5, and 0.1 ng/g. After drying, the samples were extracted with methanol using ASE. The collected extracts were placed in a nitrogen evaporator and evaporated to dryness under a gentle stream of nitrogen, and then reconstituted in 50:50 (v/v) methanol–water. Finally, the solutions were filtered using 0.22-µm regenerated cellulose filters and transferred into polypropylene vials for LC–MS/MS measurement.

Analytical Conditions

An extraction method in combination with a chromatographic separation process and mass-selective detection was used. Specifically, the PFAS analysis was performed by injecting 5 µL of the prepared solutions into a Shimadzu LCMS-8050 system (Figure 1) with the parameters detailed in Table 1 and Table 2. The analytes were chromatographically separated using a Restek Raptor C18 column. To separate PFAS that could potentially leach out from the instrument upstream of the injector, a Restek Delay column installed between the mixer and autoinjector was used.

Calibration Curve Linearity

The calibration solutions were analyzed at five injections for each concentration. Both at high-range and low-range concentration solutions, the regression coefficient (R²) was above 0.99 for most analytes. However,
the standard deviation between the five measurements at low-range concentrations was for some analytes—especially higher PFAS—somewhat higher.

All the samples were measured five times, and linearity was determined from five measurements for each analyte. Recovery of the analyte was calculated using the calibration curves from previous experiments (Table 3). As can be seen in the table, even after extraction the regression coefficient was above 0.99 for the majority of analytes, except for higher PFAS. It is evident that PFAS with 10 carbon-atom chains have lower linearity, which decreases with the length of the carbon chain. The average recovery between all the analytes is 88.8%, which is within the required criteria (80–120% of the true value) set by the EU Reference Laboratory for Halogenated POPs (6).

**Conclusion**

Despite their many benefits, PFAS currently pose a risk worth measuring. That is why a method was developed to specifically identify and precisely quantify PFAS in food packaging. It can be concluded that the Shimadzu LCMS-8050 reliably measures PFAS concentration ranges up to 0.01 pg/µL, and that its combination of high sensitivity with outstanding speed parameters makes it well-suited for high-throughput multicomponent analysis. This means that the LCMS-8050 can be beneficially used as an integral part of a simple and efficient method for the monitoring and quantification of PFAS in paper-based food packaging materials.

**References**


(4) “Order on food contact materials and on provisions for penalties for breaches of related EU legislation,” European Commission.


(6) European Union Reference Laboratory for halogenated POPs in Feed and Food, “Guidance Document on Analytical Parameters for the Determination of Per- and Polyfluoroalkyl Substances (PFAS) in Food and Feed,” Union Reference Laboratory for halogenated POPs in Feed and Food.
This article describes the method development involved in the authentication of nutraceuticals, particularly those containing Panax ginseng, which is popular because of possible positive effects on human health. For this purpose, an analytical strategy based on a metabolomic approach was chosen. The aqueous methanolic extracts were analyzed using ultrahigh-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC–HRMS). Generated data were processed by advanced statistical methods that enabled the specific markers for Panax ginseng and other plants that can be used for its adulteration (Gynostemma pentaphyllum, Withania somnifera, Eleutherococcus senticosus) to be found. The developed method is primarily intended for verifying the presence of Panax ginseng and its adulterants in Panax ginseng-based herbal teas.

Ginseng is a common name for medicinal plant species representing mainly Araliaceae genera Panax L. and Eleutherococcus Maxim. They are found in the Northern Hemisphere and East Asian countries, with China and Korea being the key producers (1). Currently, 13 ginseng species have been recognized, however, new taxa of species, subspecies, and variety levels are continuously reported (2,3). P. ginseng together with P. quinquefolium and P. notoginseng are the dominating species used in herbal medicine (4–6). The major therapeutic effects of ginseng are associated with unique secondary metabolites, ginsenosides. More than 30 compounds of this group have been identified, with the highest quantities occurring in the root (1,7). Regarding ginseng quality and its curative properties, cultivar, locality, growth conditions (cultivated vs. harvested from the wild), and plant age (roots from older plants are more valuable) are the most important factors for product value (6). Huge price differences exist among the main Panax processed varieties, and so it is not surprising that ginseng-based products are prone to economically driven fraud. As has been confirmed by the recent review on literature reports of 507 ginseng-containing commercial herbal products from different countries worldwide (6), the adulteration rate is as high as 24% (both botanical and chemical identity tested).

It is worth noting that authentication of ginseng-labelled food supplements, extracts, and other products is a fairly challenging task. This is mainly due to the diversity of Panax varieties and the means of their processing (1,3). Most of the authentication strategies are based on the analysis of ginsenosides patterns in the respective sample (4,5,8,9). Target analysis/screening employing liquid chromatography coupled to mass spectrometry (LC–MS) is commonly employed for this purpose (4,8–10); alternatively, the use of nuclear magnetic resonance (NMR) has been reported (11,12), along with molecular biology method options (2,13–16).

As well as the use of low-grade material from the above-ground parts or processing waste, substitution by alternative plant species is a common fraudulent practice. This article documents the potential of metabolomic fingerprinting employing ultrahigh-performance liquid chromatography coupled with tandem high-resolution mass spectrometry.
spectrometry (UHPLC–HRMS/MS) to detect plant adulterants other than the Panax genus, but with similar biologically active compounds. In this particular case, the authentication, contrary to other studies, was not based on ginsenosides profiling only, but on the assessment of the entire fingerprint metabolome.

Materials and Methods

Chemicals: Methanol was of HPLC-grade and obtained from Millipore. The other chemicals were of analytical-grade. Formic acid was supplied by VWR Chemicals, leucine-encephalin and ammonium formate from Honeywell. Deionized water was obtained from a Milli Q purification system. All the analyzed samples were prepared in duplicates. The instrumental setup system supplied by Millipore.

Samples: Samples of dried root of P. ginseng (n = 2), the extract of the root of P. ginseng (n = 1), leaves and stem of G. pentaphyllum (n = 3), samples of dried E. senticosus (n = 3), and samples of dried W. somnifera (n = 3) were analyzed. Panax notoginseng was also analyzed but only one sample was available, so this sample was used for verification of the specificity of markers. For this purpose, the frequently used ingredients of herbal teas like green tea, ginger, peppermint, lemongrass, rooibos, orange peel, lemon balm, cinnamon, clove, cardamom, black pepper, and dead nettle were also analyzed. Two samples of each ingredient were available. All the analyzed samples were purchased at the Czech market from certified suppliers, except for the P. ginseng root extract. This sample came directly from Korea and is an authentic sample.

Sample Preparation: The samples were homogenized in the grinder Grindomix GM 200 (Retsch GmbH), and then were weighed (0.5 g) in 15-mL polypropylene tubes. The extraction solvent, 5 mL of 4:1 (v/v) methanol–water mixture, was added to the tube, shaken for 10 min on an automatic shaker (240 oscillations per min), and then was centrifuged (5 min, 5 °C, 10,000 rpm). The supernatant was collected using a 5-mL plastic syringe and filtered through a 0.22-µm filter. An aliquot of the filtrate was transferred to a 2-mL vial and prepared for UHPLC–HRMS/MS analysis. All samples were prepared in duplicates. The concentration control (QC) sample was a combined aliquot of samples and was analyzed at the beginning of the sequence, and always after a set of 10 tested samples to control the stability of instrument conditions. The extraction mixture was also analyzed within the sample sequence to check the absence of carryover effects.

Table 1: Potential characteristic markers of Panax ginseng, Withania somnifera, Eleutherococcus senticosus, and Gynostemma pentaphyllum. Ionization mode (ESI+ or ESI−), m/z, and retention time (min).

<table>
<thead>
<tr>
<th>Plant genus</th>
<th>ESI+</th>
<th>ESI−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panax ginseng</td>
<td>974.5323 (4.59), 1109.6108 (4.65) = ginsenoside Rb₁</td>
<td>793.4350 (5.11), 955.4903 (4.60), 1077.5845 (4.69) = ginsenoside Rb₂, Rb₃, or Rc</td>
</tr>
<tr>
<td>Withania somnifera</td>
<td>502.2913 (2.81), 505.2622 (4.28), 569.2420 (3.59), 586.2693 (3.63), 638.3899 (4.27), 650.3540 (3.80), 654.3844 (4.20), 721.3374 (3.56), 783.4167 (4.15), 784.4483 (4.61), 800.4439 (4.15), 816.4391 (4.05), 941.5424 (4.07), 958.5690 (4.27), 1077.5845 (4.69) = ginsenoside Rb₂, Rb₃, or Rc</td>
<td></td>
</tr>
<tr>
<td>Eleutherococcus senticosus</td>
<td>553.1528 (2.22), 760.3024 (2.66), 765.2576 (2.66), 769.2888 (2.39), 779.2367 (2.18)</td>
<td>529.1559 (1.76)</td>
</tr>
<tr>
<td>Gynostemma pentaphyllum</td>
<td>513.3552 (5.15), 825.4999 (4.87), 869.4904 (4.68), 953.7805 (5.76), 1079.6002 (4.51) = ginsenoside Rb₂, Rb₃, or Rc; 1165.6006 (4.46) = ginsenoside mRb₂ or mRc</td>
<td></td>
</tr>
</tbody>
</table>

Instrumental Conditions: The chromatographic analysis of sample extracts was performed using an Acquity UPLC system (Waters) on a 100 × 2.1 mm, 1.7-µm Acquity UPLC BEH C18 column (Waters) held at 40 °C. The mobile phase consisted of water with 5 mM ammonium formate and 0.1% formic acid (A) and methanol with 5 mM ammonium formate and 0.1% formic acid (B) with a gradient elution: 0–2 min
FIGURE 1: UHPLC–HRMS fingerprints in ESI+ and ESI- of (a) Panax ginseng, (b) Eleutherococcus senticosus, (c) Withania somnifera, and (d) Gynostemma pentaphyllum; 4:1 (v/v) MeOH–H₂O extracts.

95–60% (A); 2–5 min 60–0% (A); 5–10 min 0% (A); 10–13 min 0–95% (A). The sample injection volume was 2 µL for the positive mode and 5 µL for the negative mode of ionization. Mass spectrometric detection was performed using a Synapt G2 MS system (Waters) equipped with an electrospray ionization (ESI) source. In addition to MS³, MS² was performed to investigate precursor ions and product ions. The resolution was around 20,000 FWHM (full width at half maximum). Positive and negative ion spectra were recorded in the range of m/z 50–1200. The ion source conditions were as follows: capillary voltage, 1.0 kV in positive mode and -0.7 kV in negative mode; cone voltage, ±25 V; ESI source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 800 L/h. The instrument was tuned using leucine-encephalin (2 ng/µL, 50:50; [v/v] water–methanol with 0.1% formic acid), sodium formate was used for calibration. The LC–MS method was taken from the previous research in this department (17).

Data Processing and Statistical Analysis: The data processing was performed by MarkerLynx XS of MassLynx 4.1 software. This software was employed to pre-process raw data generated by the UHPLC–HRMS instrument. For the peak detection, the following parameters were applied: m/z range 50–1200; XIC window 0.05 Da; intensity threshold 1000 counts; mass window 0.05; retention time window 0.10; data deisotoping. The resulting data files were exported to the SIMCA software (version 13.0.3, Umetrics). Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used in this software. Before the statistical analysis, Pareto scaling was applied to all datasets. For sorting the marker ions according to their importance, the VIP (variable importance in the projection) plots were used. The ten most important variables with VIP score > 1 in a given model were chosen. Ions that were the most significant for the classification of the sample were selected with an S-plot generated by the SIMCA software. For confirmation of ions as the markers, a trend plot was used. The tentative identification of compounds selected as the potential marker ions were based on the calculation of the elemental formula (accurate mass and mass error for respective m/z values in MS³ were considered). To confirm the suggested identification of marker ions, their product ions were investigated in MS/MS spectra, which were obtained by using a collision energy ramp ranging from 10 to 30 V in the positive mode and 40 V in the negative mode of ionization. For compound identification, online databases such as mzCloud (https://www.mzcloud.org/home), Chemspider (http://www.chemspider.com/), PubChem (https://pubchem.ncbi.nlm.nih.gov/), or METLIN (https://metlin.scripps.edu/index.php) were employed.

Results and Discussion
In the first phase, the extraction procedure was optimized to achieve the highest possible representation of metabolites in tested samples. The column used and operated under the conditions described in the Material and Methods section was found to be suitable for sample extracts separation, and the HRMS detector was set for nontarget screening in the range of m/z 50–1200. Figure 1
clearly documents the differences in UHPLC–HRMS fingerprints obtained from the analysis of Panax ginseng, Eleutherococcus senticosus, Withania somnifera, and Gynostemma pentaphyllum extracts, both in the negative and positive ionization mode. The generated data were processed by PCA, an unsupervised statistical tool, to give a first view of the data structure. The PCA score plot showed clustering according to the plant species. In the next step, PLS-DA, a supervised multivariate classification tool, was applied to achieve better visualization of the discrimination of samples. The PLS-DA score plot is presented in Figure 2. Excellent parameters were obtained (recognition ability = 98.5%, prediction ability = 97.2%).

Based on the statistical analysis, possible characteristic marker ions were chosen; specifically, 105 potential unique metabolites of P. ginseng, 120 of W. somnifera, 99 of E. senticosus, and 109 of G. pentaphyllum were found in these individual plant species. However, P. ginseng is a component of herbal teas containing a mixture of different herbs. For this reason, it was necessary to verify the specificity of these “unique” metabolites. All of these compounds were searched in extracts of frequently used ingredients of herbal teas (green tea, ginger, peppermint, lemongrass, rooibos, orange peel, lemon balm, cinnamon, clove, cardamom, black pepper, dead nettle), and also in the extract of Panax notoginseng. Five markers of P. ginseng, 29 markers of W. somnifera, six of E. senticosus, and 17 of G. pentaphyllum were confirmed to be characteristic of ginseng and ginseng-like plant

**FIGURE 2:** PLS-DA score plot showing the classification of samples of Panax ginseng, Gynostemma pentaphyllum, Withania somnifera, and Eleutherococcus senticosus in ESI-.
species. The list of potential markers is summarized in Table 1. The extracted ion chromatograms of potential characteristic markers of *P. ginseng* are shown in Figure 3. Interestingly, a number of other ginsenosides were detected in *P. ginseng*, nevertheless only three were unique as they were not found in *P. notoginseng* or other ginseng-like plant species. The tentative identification of the ginsenosides was based on the estimation/calculation of the elemental formula, accurate mass, isotopic pattern, and mass error for the respective *m/z* values in MS
1. The suggested identification of the ginsenoside was confirmed by MS/MS spectra in online databases. Two *P. ginseng* unique markers were not possible to identify. No compliance between the measured fragmentation spectra and those in databases was found.

**Conclusion**
This study has focused on the development of a fast and reliable method for the detection of *P. ginseng* substitution in products by other plant species. The results are summarized below.

UHPLC–HRMS-based nontarget screening of aqueous methanolic extracts of *P. ginseng* and its potential adulterants—"ginseng-like" compounds containing plants represented by *W. somnifera, E. senticosus*, and *G. pentaphyllum*—showed differences in metabolomic fingerprints. The number of metabolites (detected as ions thereof), which enable recognition of tested species, was found.

Screening of identified unique metabolites (markers) occurring in the above-mentioned plants but not present in common ingredients of herbal teas allowed the verification of product teas allowing the declaration of *P. ginseng* content) or documentation of common adulterants such as *W. somnifera, E. senticosus*, and/or *G. pentaphyllum*.

A follow-up study is necessary to identify the minimal amount of *W. somnifera, E. senticosus*, and *G. pentaphyllum* that could be documented through the analysis of their potential characteristic markers.

Finally, the developed method will be used to control the declared content of *P. ginseng* in herbal teas from the Czech market.

**Acknowledgement**
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**References**

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Per- and polyfluoralkyl substances (PFAS) are a very large family of man-made chemicals that can be found in food and drinking water (1). PFAS have received considerable attention over recent years (2,3). The European Food Safety Authority (EFSA) has published a risk-assessment (4), setting very low safety levels (tolerable weekly intake) for the sum of four of these chemicals: PFHxS, PFOS, PFOA, and PFNA. To shed light on the actual human exposure through food consumption, and to anticipate possible future legislative limits, sensitive and easy-to-operate analytical approaches are needed for the detection of PFAS in foods at low ppt levels. Fruits were chosen in this study because limited data are available on fruits and vegetables and because they are a significant part of the human diet. As such, data are urgently needed. In this work, we compared dispersive solid-phase extraction (dSPE) with an approach using SPE columns based on a weak anion exchange (WAX). The latter method was already established in-house for milk, eggs (5), and fishery products (6), but not yet tested on apples. In the current study, we wanted to compare the analysis of apples with both methods.

With the continuous emergence of new PFAS, the need for new cleanup and analysis methods also increases. Conventional SPE and dSPE can both serve this need, but both have their advantages and disadvantages. SPE-WAX cannot capture all relevant PFASs in one processing and/or measurement method; neutral and cationic PFASs are lost during the SPE cleanup, and due to the high range of polarity, multiple separation methods are required. As an alternative to conventional SPE, dSPE ([QuEChERS] quick, easy, cheap, effective, rugged, and safe) is used in pesticide analysis to remove interferants such as fatty acids, sugars, and pigments (7). It has also shown to be very effective as a cleanup for PFAS analysis (8). Several benefits of dSPE over SPE apply: (i) Due to a larger surface area, recoveries of dSPE are, in theory, higher compared with conventional SPE. (ii) The adsorption material is directly added to the extract/matrix so that no compounds are lost due to any washing steps. As the SPE material is added to adsorb interferences rather than the PFAS, neutral and cationic compounds can also be analyzed in a single cleanup. In addition, clogging of SPE columns is omitted. (iii) The cleanup can be much faster and cheaper when pre-mixed dSPE salts are used. The challenge with dSPE, however, lies in achieving similar detection limits as a conventional SPE method. As the cleanup using dSPE is in general less effective, the concentration of the raw extract to a small end volume is also restricted as a result of ionization effects. Moreover, residual salts in the extracts may desolvate in the MS.
source (and LC column) leading to salt deposits and increased dead volume and peak tailing. Furthermore, due to the strong adsorptive properties of the dSPE-salts, it is a challenge to obtain 100% PFAS-free solid phases. In conventional SPE procedures, an initial washing step is included, which removes most PFAS-residuals present in the SPE-column. This preconditioning step is not possible for dSPE, potentially resulting in higher background concentrations. Another challenging aspect of dSPE is that the required amount of added salts is largely dependent on the water content in the matrix. This makes the method less robust for varying matrices.

**Methodology**

The PFAS compounds in the study are mentioned in Table 1 and included perfluoro carboxylic acids from C5 (PFPeA) to C14 (PFTeDA) and several sulfonates (C4, C6, C7, C8, and C10). Moreover, a non-ionic precursor (PFOSA) and GenX (successor of PFOA as a fluoropolymer polymerization aid) were added as target analytes to this study. Two sample cleanup procedures were tested: SPE-WAX and dSPE. The SPE-WAX method was based on an existing protocol (5), with adjusted sample intake (10 g) and final extract volume to increase the sample enrichment factor. The dSPE method was optimized as regards sample intake (2 and 5 g) and the composition of the adsorbents and salt. The final composition was magnesium sulfate, sodium chloride, and the sorbents C18, primary secondary amine (PSA), and granulated active carbon.

The instrumental analysis was achieved using ultrahigh-performance liquid chromatography (UHPLC) coupled to a Sciex 7500 tandem mass spectrometer, operated in multiple reaction monitoring (MRM) mode for (tandem mass spectrometry [MS/MS]). Separation was performed on a 100 × 2.1 mm, 1.6-µm Luna Omega Polar C18 100Å column (Phenomenex) with methanol and an ammonium acetate buffer (20 mM) as the mobile phase. The MS/MS conditions were optimized for maximum selectivity and sensitivity and an instrumental limit of detection (iLOD) of 10 femtograms or less on-column for most analytes was achieved, based on a 20-µL injection. All experiments for comparing both methods were performed in triplicate.

**Results and Discussion**

The SPE (WAX) method showed a low limit of quantification (LOQ) at the pg/g level for most components, as well as a low limit of confirmation (LOC)—the level where both diagnostic

<table>
<thead>
<tr>
<th>PFAS</th>
<th>dSPE</th>
<th>SPE (WAX)</th>
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<tbody>
<tr>
<td></td>
<td>LOQ</td>
<td>LOC</td>
</tr>
<tr>
<td></td>
<td>(pg/g)</td>
<td>(pg/g)</td>
</tr>
<tr>
<td>PFPA</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td>PFHxA</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>PFHpA</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>PFOA</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>PFNA</td>
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<tr>
<td>FDIA</td>
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<tr>
<td>PFUnDA</td>
<td>50</td>
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<tr>
<td>PFDa</td>
<td>40</td>
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<tr>
<td>PFTrDA</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>PFTeDA</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>PFBS</td>
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<td>2.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>PFHpS</td>
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<td>2.5</td>
</tr>
<tr>
<td>PFDS</td>
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</tr>
<tr>
<td>GenX</td>
<td>50</td>
<td>50</td>
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<tr>
<td>PFOSA</td>
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<td>5</td>
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</tbody>
</table>
ions met the ion-ratio criteria). The trueness, determined at the 50 pg/g spike level, was between 98–114% (except for GenX), with a relative standard deviation (RSD) lower than 10%, which is excellent. However, PFTrDA and PFTeDA did not meet the criteria, which was caused by the lack of an isotopically-labelled internal standard (PFTrDA) and low recoveries due to strong adsorption to surfaces. Moreover, the non-ionic PFOSA could not be retained by the SPE column.

dSPE, on the other hand, showed LOQs of 5 and 2.5 pg/g for about half the compounds. For the other half, high background signals were observed and yielded much higher LOQs. Further efforts are required to resolve the background signals for apples, and, if successful, lower LOQs can likely be achieved for the dSPE method as well. Moreover, excellent trueness values were obtained, as well as good RSDr values for most PFAS. Another advantage of the dSPE method over SPE-WAX was that the non-ionic PFOSA could be measured, as well as PFTrDA and PFTeDA.

Conclusion

Both methods showed advantages and disadvantages. dSPE was easy to operate and allowed the concurrent analysis of both ionic and nonionic PFAS (PFOSA). The LOCs were generally higher due to (i) a lower matrix-to-column ratio and (ii) background signals (by the presence of the actual PFAS in sorbents or unidentified interferences) on some of the PFAS. SPE based on WAX did not allow the analysis of PFOSA, PFTrDA, and PFTeDA at relevant levels, but a more powerful cleanup was achieved, allowing lower LOCs in most cases. Accuracies and RSDr values were excellent. Further optimization of the dSPE method will likely resolve the relatively high LOQs for some of the short-chain carboxylic acids. Both methods can be used for monitoring PFAS in fruits in the future, and, depending on the specific interests, one can choose either SPE or dSPE. These methods will contribute to a better understanding of human exposure to PFAS.

References

4) EFSA, Scientific Opinion on the risk to human health related to the presence of perfluoroalkyl substances in food, EFSA Journal 18(9), 6223 (2020).

Marise van der Vegt has obtained a B.Sc. in analytical chemistry. At Wageningen Food Safety Research, she investigated dSPE and SPE extraction and cleanup approaches for PFAS analysis in fruits, and mutually compared them. She has recently accepted a position as Junior Researcher Analytical Chemistry at Enza Zaden, Enkhuizen, The Netherlands. Ruben Kause is a scientist at Wageningen Food Safety Research (WFSR, Wageningen, The Netherlands). Ruben has a background in analytical chemistry and environmental sciences. His expertise is mainly in emission modelling and analysis of perfluorinated alkyl substances in food and environmental matrices. Bjorn Berendsen studied analytical chemistry at Amsterdam University and did his Ph.D. at Wageningen University whilst being affiliated to Wageningen Food Safety Research (WFSR). He is an expert in residue analysis of mainly veterinary drugs and perfluoro alkyl substances in food and environmental matrices. He has been working at WFSR for 23 years in several functions and is currently, besides researcher and project manager, the programme manager of the statutory tasks policy research programme and as such part of the institute’s management team. Stefan van Leeuwen is a senior scientist at Wageningen Food Safety Research (WFSR, Wageningen, The Netherlands). His current focus is on investigating the impact of environmental pollutants like PFAS, on food safety and our dietary exposure. He employs a range of complementary analytical techniques to unravel the complexity of the PFAS issue.

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Analysis of MCPD and Glycidyl Fatty Acid Esters in Refined Plant Oils by Supercritical Fluid Chromatography–High-Resolution Mass Spectrometry

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2- and 3-chloropropane-1,2-diol (2- and 3-MCPD) and their fatty acid esters (2- and 3-MCPDEs) together with glycidyl fatty acids esters (GEs) belong to a group of process-induced contaminants. They are formed in high temperature environments and most commonly occur in refined vegetable oils. Since 2021, maximum limits for GEs and 3-MCPDEs have been enforceable in the EU, and the requirement for adequate analytical methods is constantly increasing. The aim of this study was to develop an efficient method to enable a rapid determination of nine 3-MCPDEs and seven GEs in vegetable oils within a single run employing supercritical fluid chromatography coupled to high-resolution tandem mass spectrometry (SFC–HRMS/MS). Contrary to a routine gas chromatography mass spectrometry method aimed at determination of the total MCPD pool, the sample throughput when using SFC–HRMS/MS was greatly increased, as the “dilute-and shoot” approach did not require any hydrolysis and derivatization. Additionally, the pattern of natural MCPDEs was characterized. The performance characteristics of this new method met the criteria required by Commission Regulation 2019/2093 for all target analytes.

2- and 3-chloropropane-1,2-diol (2- and 3-MCPD) and glycidol belong to a group of process-induced chemical contaminants of edible oils. Chloropropanediols and glycidol are derived from glycerol, and, similar to glycerol, they occur mainly in their ester-bound form as 2- and 3-chloropropanediol mono- and diesters (2- and 3-MCPDEs) and glycidyl esters (GEs). The profile of bound fatty acids usually corresponds to that in the respective plant oil (1–2). Numerous studies have shown that they are not present in crude oils and occur only after high temperature treatment, such as during oil deodorization (2–6).

The International Agency for Research on Cancer classifies 3-MCPD as possibly carcinogenic to humans (Group 2A). Toxicity of 2-MCPD and its esters requires further investigation and is yet to be classified in terms of its carcinogenicity and dose-response assessment (7–12). Although most food-borne 3-MCPD occurs in its bound form (approximately 85% diesters and 15% monoesters), previous studies have shown that its free form is effectively released in vivo by enzymatic hydrolysis in the gastrointestinal tract (13). For the purpose of risk assessment, ingested bound 3-MCPD is usually considered as molar equivalent to its free form. In 2016, the Contaminants in the Food Chain (CONTAM) panel of the European Food Safety Authority (EFSA) assessed known risks associated with 2- and 3-MCPD and established a tolerable daily intake (TDI) of 2 μg/kg body weight per day for 3-MCPD and its fatty acid esters, which are expressed as MCPD equivalents. EFSA further concluded that adequate toxicological data for free and bound 2-MCPD are currently not available (9,11,13–16).

Following these findings, the Directorate-General for Health and Food Safety (DG SANTE) proposed maximum limits for GEs in several food commodities. These have been further expanded to include 3-MCPDEs, and since 2021, these limits are enforceable in the European Union as a part of Commision Regulation 2020/1322 (17). The requirements for adequate analytical methods are therefore constantly increasing.
There are two main approaches to MCPDEs and GEs analysis: either directly determine their content in intact ester forms or release them all into their free forms and determine them indirectly. The majority of currently used methods are based on the indirect approach, which involves analysis of 2- and 3-MCPD and glycidol after hydrolysis (either chemical or enzymatic) and conversion to volatile derivatives, followed by gas chromatography–mass spectrometry (GC–MS) analysis. Their main advantages lie in lower analytical standard requirements and applicability in a wider range of food categories (18–19). However, the accuracy and repeatability of these methods was disputed due to the harsh chemical conditions required during sample preparation, which can interfere with MCPD and glycidol content (20). In combination with often lengthy sample preparation of indirect methods, direct approaches are often promoted as a more suitable alternative (18).

Direct methods have the obvious advantage in providing profiles of individual MCPDEs and GEs. The fatty acid composition not only offers a better insight in MCPDEs and GEs formation and subsequent development of mitigation strategies but it is also suggested that individual esters might differ in their overall toxicity. On the other hand, a higher number of analytical standards and often more expensive instrumentation are required (18,21).

In most cases the direct method workflow involves liquid chromatography–mass spectrometry (LC–MS) analysis, either in a tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HRMS) arrangement. As the vast majority of these systems operate in reversed-phase mode, the major issue then arises in the determination of MCPDEs and GEs in oily matrices. The presence of large amounts of acylglycerols, particularly triacylglycerols (TAG), negatively affect the analysis. Therefore, most published methods include a sample purification step, usually by the use of solid-phase extraction (SPE), often in multiple stages (reversed-phase SPE, followed by normal-phase SPE), prior to LC–MS (4,18–19,22–23). These additional steps have a significant impact on sample throughput and the overall cost of these methods.

As a possible solution, methods based on supercritical fluid chromatography (SFC) for direct determination of MCPDEs have been published in recent years (24–25). The mobile phase of choice for modern SFC systems is supercritical CO₂, which displays characteristics similar to pentane or hexane, making it suitable for the separation of lipophilic compounds (26). Indeed, SFC coupled with MS/MS or HRMS was shown to provide rapid, high-throughput “dilute and shoot” methods for MCPDEs analysis. Obtained method performance characteristics indicate a potential of this approach to be used for quantification of these processing contaminants. Furthermore, the use of CO₂ instead of commonly used organic solvent as the mobile phase is considered green (24–25).

The aim of this study was to develop and optimize a method enabling a rapid, simultaneous determination of not only various intact esters of 3-MCPDEs but also GEs, in various vegetable oils and fats. The use of supercritical fluid chromatography coupled with high resolution mass spectrometry (SFC–HRMS) was addressed as a analytical challenge. Achieving criteria for performance characteristics set in European legislation (Commission Regulation 2019/2093 [27]) for methods targeting 3-MCPDEs and GE in plants was the ultimate objective.

Materials and Methods

Chemicals: Standards of racemic 3-MCPD fatty acids diesters were as follows: 1,2-dipalmitoyl-3-chloropropane-1,2-diol (PP-3-MCPD); 1-palmitoyl-2-linoleoyl-3-chloropropanediol (PL-3-MCPD); 1-palmitoyl-2-oleoyl-3-chloropropanediol (PO-3-MCPD); 1-palmitoyl-2-stearoyl-3-chloropropanediol (PSt-3-MCPD); 1,2-dilinoleoyl-3-chloropropanediol (LL-3-MCPD); 1,2-dioleoyl-3-chloropropanediol (OO-3-MCPD); 1-oleoyl-2-linoleoyl-3-chloropropanediol (OL-3-MCPD); 1-oleoyl-2-stearoyl-3-chloropropanediol (OSt-3-MCPD); 1,2-distearoyl-3-chloropropanediol (StSt-3-MCPD). Available glycidyl esters (GEs) with fatty acids were: glycidyl stearate (G-St); glycidyl palmitate (G-P); glycidyl laurate (G-La); glycidyl myristate (G-M); glycidyl linoleate (G-L); glycidyl linolenate (G-Ln); and glycidyl oleate (G-O). All of these analytical standards were purchased from Toronto Research Chemicals (the declared purity was ≥ 98%). HPLC-grade ethyl acetate, n-hexane, 2-propanol, and methanol were purchased from Honeywell. Deionized water was supplied by the Millipore (Milli - Q) purification system from Merck. Ammonium acetate for mass spectrometry was obtained from Merck. Nitrogen and carbon dioxide (purity 4.0) were purchased from SIAD. Two sets of stock standard solutions—one containing all MCPDEs standards, the other containing all GEs—were prepared in ethyl acetate at 1 μg/mL, labelled MCPDE_1, GE_1, and 10 μg/mL labelled MCPDE_10 and GE_10.

Samples: Refined vegetable oil samples (rapeseed and sunflower; n = 2 and n = 4, respectively) purchased at a local retail market were used for analyte screening and subsequent method validation.

Sample Preparation:

Samples for Target Analytes Screening: A 0.5-g measure of oil was accurately weighted into a 5-mL volumetric flask and the volume adjusted by n-hexane. Two 1-mL aliquots of obtained oil solution (100 mg of oil/mL) were then transferred...
into 2-mL glass vials for SFC–MS analysis. Samples for Method Optimization and Validation: A set of 2-mL vials was pre-loaded with either 15, 20, or 25 μL of both MCPDE_1 and GE_1, or 5 or 10 μL of both MCPDE_10 and GE_10. Vials were then dried in a block heater at 30 °C under a gentle stream of nitrogen. The residue was diluted in 1 mL of hexane to obtain “solvent standard”, or in 1 mL of previously prepared oil solution to get “matrix-matched standard” containing 100 mg of oil/mL (for oil solution preparation, see section above), capped, thoroughly shaken, and subsequently subjected to SFC–MS analysis.

**SFC–HRMS Analysis:** MCPDEs and GEs analysis was performed using an Aquity UltraPerformance Convergence Chromatography (UPC²) system coupled with Synapt G2-Si, which was equipped with ZSpray electrospray ion source with a hybrid quadrupole–travelling wave ion mobility–time-of-flight mass spectrometer (Waters). A make-up solvent was delivered by a HPLC 515 pump (Waters) and mixed with column effluent in a two T-piece coupling. The combined flow was then divided to an active atmospheric back pressure regulator (ABPR) module and the mass spectrometer. The best SFC–MS conditions found in our study were as follows.

**SFC Conditions:** Column: 100 × 3 mm, 1.8-μm Viridis BEH (Waters); mobile phase composed of supercritical CO₂ with 99:1 (v/v) methanol–water mixture as a co-solvent containing 30 mM of ammonium acetate as a modifier at a constant flow rate of 1.8 mL/min; the co-solvent followed a linear gradient curve: 0 min, 0.5% → 4 min, 5% → 8 min, 45% → 10 min, 45% → 11 min, 0.5% → 12 min, 0.5%; column temperature: 70 °C; ABPR pressure: 13.79 MPa (2000 psi); injection volume: 4 μL; injector needle was washed with a 7:3 (v/v) n-hexane–2-propanol mixture and pure 2-propanol after each injection.

**MS Ion Source Conditions:** As a make-up solvent, a mixture of 99:1 (v/v) methanol–water was used at a constant flow rate of 0.3 mL/min. A positive-mode electrospray ionization (ESI+) in resolution mode was used at the following settings of tuning parameters: capillary voltage: +4.5 kV; sampling cone: 40 V; source offset: 80 V; source temperature: 120 °C; desolvation temperature: 350 °C; cone gas flow: 50 L/h; desolvation gas flow: 800 L/h; nebulizer pressure: 4 bar.

**MS Acquisition Conditions:** MS data were acquired (i) in HRMS mode for SFC method development and ionization optimization, and (ii) in combined HRMS/MS mode for collision energy optimization and final method validation. The HRMS mode monitored ammonium adducts \([M + NH_4]^+\) of MCPDEs and protonated molecules \([M + H]^+\) of GEs. The HRMS/MS method used the \([M + NH_4]^+\) adducts of MCPDEs as precursor ions and monitored characteristic decarboxylated ions \([M - RCOO]^+\). The \(m/z\) values of characteristic ions are summarized in Table 1. The (i) HRMS data were acquired in \(m/z\) range 100–1000. For the (ii) combined HRMS/MS method, the HRMS function mass range was narrowed to \(m/z\) 250–400, and for the MS/MS functions for individual MCPDEs to \(m/z\) 300–400. MS/MS fragmentation was performed on a transfer cell.

**Method Optimization:** For method optimization, solutions of MCPDEs and GEs 100 ng/mL (for each analyte) in n-hexane and a spiked oil sample at the same concentration level (corresponds to 1000 μg of each ester/kg of oil sample) were prepared (see Samples for method optimization and validation), both in two replicates, and analyzed with SFC–MS under various conditions. The underlined values represent default method conditions. The outcome of modifications were evaluated based on average (mean of parallel measurements) percentage of response differences of \([M + NH_4]^+\) MCPD adducts and \([M + H]^+\) GEs adducts compared to the default settings. Each time, the best performing conditions were used for further steps.

**SFC Method Optimization:** SFC method parameters subjected to optimization included the following: co-solvent gradient (5% 30 mM of ammonium acetate in 99:1 (v/v) methanol–water co-solvent at 4/5/6/7 min), ABPR pressure (1600/1800/2000/2200 psi), and make-up solvent composition (pure methanol/99:1 [v/v] methanol–water/30 mM of ammonium acetate in 99:1 [v/v] methanol–water). MS Method Optimization: MS method parameters subjected to optimization included the following: ion source capillary voltage (1.0/1.5/2.0/2.5/3.0/3.5/4.0/4.5 kV), ion source temperature (100/120/150 °C), and collision energy for MS/MS analysis (0/10/20/30/40/50 eV).

**Method Validation:** To validate the previously optimized procedure, a standard addition method (SAM) approach utilizing spiked samples was chosen. Six parallel sets of selected oil solutions were spiked with MCPDEs and GEs stock standard solutions at concentration levels 30, 40, 50, 100, and 200 ng/mL (see Samples for method optimization and validation), which correspond to 300, 400, 500, 1000, and 2000 μg of each ester/kg of oil. Oil solutions without added MCPDEs or GEs (matrix blank samples) were also prepared in six parallels (see Samples for target analytes screening). All samples were then analyzed by SFC–MS. SAM calibration equations were expressed as \(y = ax + b\) by the least square method, where \(a\) is the slope of the curve and \(b\) is the intercept of the \(y\)-axis. Analyte concentrations were calculated as a negative horizontal axis intercept \((-b/a)\). Method performance characteristics represented by linearity, limits of quantification (LOQ), repeatabilities, recovery rates, and matrix effects were...
characterized and assessed against the criteria specified in Commission Regulation 2019/2093 regarding performance criteria for methods of analysis for 3-MCPDEs and GEs. Method linearity was expressed as coefficient of determination (R²) of SAM calibration equations. Recovery rates were evaluated by comparing the average theoretical and determined concentration in parallel sets. Repeatability was expressed as the relative standard deviation (RSD) between determined concentrations in parallel sets. The relative matrix effects were calculated as average ratios between theoretical and determined concentration in parallel sets. The relative matrix effects were selected from species naturally occurring in oil samples to have as low a retention time as possible, to mark the beginning of TAG elution zone. Since the analytes of interest in this study are eluted in the first 2 min, the initial mobile phase composition was lowered from the previously used 1% of co-solvent at 6 min. The largest increase of responses, measured in solvent standards (100 ng/mL), was observed with the steepest tested gradient (5% co-solvent at 4 min) and was on average 5.1%, with values ranging from +29.7% for G-M to -12.0% for StSt-3-MCPD. However, six out of nine MCPDEs showed a decrease in response. Considering that targeted MCPDEs ions are less intensive when analyzing solution with the same concentration than GEs, and the other two tested gradient settings

**Results and Discussion**

**SFC Method Optimization Results:**

As stated above, SFC–MS is a powerful tool for analysis of lipophilic compounds. For fast separation of target analytes from TAGs, the major matrix “interfering” components in refined oil, an ethylene bridged hybrid silica phase column with medium silanol activity was selected. Under optimized conditions, the “dilute and shoot” approach was enabled and both MCPDEs and GEs, which are more hydrophobic than TAGs, eluted at lower retention times and did not suffer from massive matrix effects. This is well outlined in Figure 1, where extracted ion chromatograms (EIC) of MCPD [M + H]+ and GEs [M + H]+ adduct ions (for m/z values see Table 1) in spiked rapeseed oil sample are shown, compared to EIC of selected m/z values calculated for [M + NH4]+ TAG adducts. EIC of displayed TAG peaks were selected from species naturally occurring in oil samples to have as low a retention time as possible, to mark the beginning of TAG elution zone. Since the analytes of interest in this study are eluted in the first 2 min, the initial mobile phase composition was lowered from 1.8 mL/min. This allowed for efficient separation of MCPDEs and GEs from the TAG matrix. The main contribution to matrix effect was therefore attributed to more lipophilic compounds, tentatively identified as [M + NH4]+ and [M - H2O + H]+ adducts of phytosterol and retinol esters (see Figure 1). The modified method was used as a basis for further optimization with regards to obtaining maximal responses and minimal LOQs.

The subject of gradient optimization was the initial slope from 0.5% to 5% of co-solvent at 6 min. The largest increase of responses, measured in solvent standards (100 ng/mL), was observed with the steepest tested gradient (5% co-solvent at 4 min) and was on average 5.1%, with values ranging from +29.7% for G-M to -12.0% for StSt-3-MCPD. However, six out of nine MCPDEs showed a decrease in response. Considering that targeted MCPDEs ions are less intensive when analyzing solution with the same concentration than GEs, and the other two tested gradient settings

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**TABLE 1: Accurate masses of ions employed for MCPDEs and GEs analysis**

<table>
<thead>
<tr>
<th>Elution Order</th>
<th>Name</th>
<th>Abbreviation</th>
<th>Elemental Composition</th>
<th>[M+H]⁺</th>
<th>[M+NH₄]⁺</th>
<th>[M-RCOO]⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>glycidyl laurate</td>
<td>G-La</td>
<td>C₃₀H₅₀O₆</td>
<td>257.212</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>glycidyl myristate</td>
<td>G-M</td>
<td>C₂₀H₄₀O₄</td>
<td>285.243</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>glycidyl palmitate</td>
<td>G-P</td>
<td>C₃₀H₅₀O₆</td>
<td>313.274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>glycidyl stearate</td>
<td>G-St</td>
<td>C₃₀H₅₀O₆</td>
<td>341.306</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>glycidyl oleate</td>
<td>G-O</td>
<td>C₂₀H₄₀O₄</td>
<td>339.290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>glycidyl linolenate</td>
<td>G-Ln</td>
<td>C₂₀H₄₀O₄</td>
<td>337.274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>glycidyl linoleate</td>
<td>G-L</td>
<td>C₂₀H₄₀O₄</td>
<td>335.259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1,2-dipalmitoyl-3-MCPD</td>
<td>PP-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>604.507</td>
<td>331.240</td>
</tr>
<tr>
<td>10</td>
<td>1-palmitoyl-2-stearoyl-3-MCPD</td>
<td>PST-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>632.539</td>
<td>359.272</td>
</tr>
<tr>
<td>11</td>
<td>1-palmitoyl-2-oleoyl-3-MCPD</td>
<td>PO-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>630.523</td>
<td>357.256</td>
</tr>
<tr>
<td>12</td>
<td>1,2-distearyloyl-3-MCPD</td>
<td>StSt-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>660.570</td>
<td>359.272</td>
</tr>
<tr>
<td>13</td>
<td>1-palmitoyl-2-linoleoyl-3-MCPD</td>
<td>PL-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>628.507</td>
<td>355.240</td>
</tr>
<tr>
<td>14</td>
<td>1-oleoyl-2-stearoyl-3-MCPD</td>
<td>OSSt-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>658.554</td>
<td>357.256</td>
</tr>
<tr>
<td>15</td>
<td>1,2-dioleoyl-3-MCPD</td>
<td>OOO-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>656.539</td>
<td>357.256</td>
</tr>
<tr>
<td>16</td>
<td>1-oleoyl-2-linoleoyl-3-MCPD</td>
<td>OLL-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>654.523</td>
<td>355.240</td>
</tr>
<tr>
<td>17</td>
<td>1,2-dilinoleoyl-3-MCPD</td>
<td>LL-3-MCPD</td>
<td>C₂₀H₄₀O₄ClO₂</td>
<td>-</td>
<td>652.507</td>
<td>355.240</td>
</tr>
</tbody>
</table>

*CE* corresponds to optimized collision energy values in eV
The first attempt was based on excluding composition. From the original make-up can also adversely impact the ABPR/ default gradient was kept unchanged. 

In the next phase, the ABPR pressure was optimized. Its higher values (and therefore higher CO₂ density) generally improve elution strength of supercritical CO₂, resulting in narrower and higher peaks. On the other hand, it can also adversely impact the ABPR/MS split ratio during solvent exchange. Nevertheless, in this case, the effect was only marginal, with the best result being an average 2.8% response increase (mean of all analytes) at 2000 psi.

Another factor influencing analyte responses is the make-up solvent composition. From the original make-up solvent (99:1 [v/v] methanol–water), two different approaches were tested. The first attempt was based on excluding water in addition to methanol. It was assumed that, thanks to the increased solubility of relatively nonpolar target analytes in make-up solvent, the ABPR/MS split ratio would be improved.

The second alternative involved modifying the existing make-up solvent with the addition of 30 mM ammonium acetate to support [M + NH₄]⁺ formation in the ion source, thus increasing MCPDEs responses. A rapeseed oil solution spiked at 100 ng/mL (1000 μg/kg) was used for this purpose to take into consideration potential matrix effects in this step. Unfortunately, the use of both modified make-up solvents resulted in reduced responses for all analytes, and therefore the original make-up solvent was kept unchanged.

A mention should be made that the SFC method presented here resulted in 2- and 3-MCPDEs coelution and, therefore, to some extent 3-MCPDE signals might be increased by 2-MCPDEs. Further method development steps were focused on analyte ionization. The source voltage was optimized on 100 ng/mL solvent standard. Compared to the default method with ionization voltage setting 3.5 kV, its increase to 4.5 kV resulted (with the exception of OL-3-MCPD) in some increase of analyte signal intensities, a maximum of 16.5% in the case of G-P.

**MS Method Optimization Results:**

**Quantification:** When targeting individual MCPDEs in chromatographically only partly resolved mixtures (which was the case for the developed high-throughput method), a quantification issue might occur. Like other organochlorine compounds, MCPDEs contain in their isotopic profile a relatively intensive M+2 ion, which may, supposing partial coelution, interfere with targeted ions of other analytes. For instance: the difference in the theoretical m/z value 632.5385 of PSI-3-MCPD ion [M + NH₄]⁺ and m/z 632.5220, calculated for M+2 isotope of PO-3-MCPD [M + NH₄]⁺ is only Δm/z = 0.0165. At a given instrument resolution (24,000–30,000 FWHM in “resolution” mode), for the m/z value 632.5385 ions in the range 632.5596–632.5174 cannot be resolved. To overcome this issue and to simultaneously improve on selectivity for MCPDEs, a MS/MS method was designed.

As described in Table 1, suitable product ions were identified. These ions correspond to decarboxylated [M - RCOO]⁺ fragments of [M + NH₄]⁺ precursors, in accordance with the available literature (24–25). Transfer cell collision energy was selected for each analyte based on responses obtained with each setting on 100 ng/mL hexane MCPDEs solution. With the exception of PP-3-MCPD (at 10 eV), the best results were achieved at 20 eV. An example MS/MS chromatographic record of rapeseed oil spiked with MCPDEs is shown.
in Figure 2. The resulting optimized method was subsequently validated.

**Validation Results:** Commission Regulation 2019/2093 specifies method performance criteria that have to be achieved when controlling the regulated levels of 3-MCPDEs and GEs in vegetable oils. These include a recovery rate of 70–125%, LOQ ≤ 100 μg of ester bound 3-MCPD or glycidol/kg of oil, and repeatability expressed as RSD derived from the modified Horwitz equation. LOQ of 100 μg of ester bound 3-MCPD or glycidol/kg of oil corresponds to 582–531 μg of MCPDEs/kg and 346–460 μg of GE/kg, depending on the particular ester form. At such concentration levels, the required repeatability equates to RSD ≤ 14.52%.

Samples of refined vegetable (rapeseed and sunflower, see Samples for target analytes screening) oils were screened for the presence of MCPDEs and GE. The rapeseed oil sample (labelled as “soft refined”) with the lowest contaminants content (G-O, G-L, and G-Ln were detected at estimated concentrations 5, 17, and 24 μg of bound glycidol/kg of oil, respectively) was selected as the blank matrix for SAM method validation (see Method validation). The responses of analytes occurring in such “blank” samples were subtracted from all concentrations determined in spiked samples. Achieved validation parameters are summarized in Table 2. LOQ for each analyte is expressed in μg of ester forms/kg of oil and equivalent μg of ester bound 3-MCPD or glycidol/kg of oil, and represent the lowest concentration of spiked oil sample at which acceptable recovery and RSD were achieved. Obtained LOQs were low enough, ranging between 65–87% of the proposed maximum limit—100 μg of ester bound 3-MCPD or glycidol/kg. Method linearity of SAM calibration equations R² < 0.99 was achieved for all studied compounds at all spiking levels. All the validation parameters met the required performance criteria.

**TABLE 2:** Performance characteristics of SFC–HRMS/MS method for MCPDs and GEs analysis obtained by validation on spiked refined rapeseed oil samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Ester Form (Spiked Sample) (μg/kg)</th>
<th>3-MCPD / Glycidol Bound in Respective Ester Form (μg/kg)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Relative Matrix Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP-3-MCPD</td>
<td>400</td>
<td>75.3</td>
<td>104.0</td>
<td>9.0</td>
<td>0.53</td>
</tr>
<tr>
<td>PL-3-MCPD</td>
<td>400</td>
<td>72.3</td>
<td>99.4</td>
<td>12.9</td>
<td>0.36</td>
</tr>
<tr>
<td>PO-3-MCPD</td>
<td>400</td>
<td>72.1</td>
<td>99.8</td>
<td>11.9</td>
<td>0.52</td>
</tr>
<tr>
<td>PST-3-MCPD</td>
<td>400</td>
<td>71.8</td>
<td>106.6</td>
<td>10.0</td>
<td>0.65</td>
</tr>
<tr>
<td>LL-3-MCPD</td>
<td>400</td>
<td>69.6</td>
<td>88.1</td>
<td>9.3</td>
<td>0.82</td>
</tr>
<tr>
<td>OL-3-MCPD</td>
<td>400</td>
<td>69.4</td>
<td>91.1</td>
<td>11.1</td>
<td>0.76</td>
</tr>
<tr>
<td>OO-3-MCPD</td>
<td>400</td>
<td>69.1</td>
<td>86.8</td>
<td>6.9</td>
<td>0.42</td>
</tr>
<tr>
<td>OS-3-MCPD</td>
<td>400</td>
<td>68.9</td>
<td>103.4</td>
<td>11.0</td>
<td>0.37</td>
</tr>
<tr>
<td>SiSt-3-MCPD</td>
<td>400</td>
<td>68.7</td>
<td>115.5</td>
<td>10.8</td>
<td>0.49</td>
</tr>
<tr>
<td>G-St</td>
<td>300</td>
<td>71.1</td>
<td>119.7</td>
<td>6.8</td>
<td>0.56</td>
</tr>
<tr>
<td>G-P</td>
<td>300</td>
<td>65.3</td>
<td>106.0</td>
<td>8.0</td>
<td>0.50</td>
</tr>
<tr>
<td>G-La</td>
<td>300</td>
<td>65.6</td>
<td>85.6</td>
<td>12.9</td>
<td>0.75</td>
</tr>
<tr>
<td>G-M</td>
<td>300</td>
<td>86.7</td>
<td>103.9</td>
<td>10.5</td>
<td>0.64</td>
</tr>
<tr>
<td>G-Ln</td>
<td>300</td>
<td>78.1</td>
<td>88.8</td>
<td>10.1</td>
<td>0.56</td>
</tr>
<tr>
<td>G-L</td>
<td>300</td>
<td>66.0</td>
<td>96.2</td>
<td>12.0</td>
<td>0.57</td>
</tr>
<tr>
<td>G-O</td>
<td>300</td>
<td>66.4</td>
<td>94.8</td>
<td>7.3</td>
<td>0.63</td>
</tr>
</tbody>
</table>
Conclusion
This study presents a newly developed and validated high-throughput “dilute and shoot” analytical approach for determination of nine esters of 3-MCPD and seven esters of glycerol in refined vegetable oil using SFC–HRMS/MS. The key advantages of the described method, in comparison with conventionally used procedures, consist in minimal sample preparation requirements and no need for chemical modification of analytes or sample clean-up prior to instrumental analysis. The green nature associated with a minimized solvent consumption is another benefit.

Selected SFC and HRMS conditions, namely co-solvent gradient, ABPR pressure, make-up solvent composition, ion source voltage, and temperature, were optimized to achieve the lowest LOQs and other method performance characteristics. As all of them meet the criteria of European legislation (Commission Regulation 2019/2093), the method is suitable for an official control of both groups of processing contaminants of plant oils.

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Analysis of UV-Treated Mushrooms: Dietary Source of Vitamin D$_2$?

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In recent years, dietary intake of vitamin D has become an issue of high concern because this bioactive molecule boosts the immune system and is presumed to provide some protection against Covid-19. Under these conditions, a search for nontraditional dietary sources has appeared as a new challenge. One of the possibilities is irradiation of champignons that contain a high amount of ergosterol, a vitamin D$_2$ precursor. In our study, a fast and sensitive liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) method for the determination of vitamin D$_2$ in fresh mushrooms and its metabolite 25(OH)D$_2$ in the blood of volunteers regularly consuming UV-treated mushrooms has been introduced. For extraction of desiccated mushrooms, solid-liquid extraction $n$-hexane–ethyl acetate was used, and $n$-hexane was employed for blood plasma samples. Separation of target analytes was performed on a polymeric bonding C18 phase column. Satisfactory limits of quantification (LOQs) were reached both for the control of vitamin D$_2$ content in mushrooms (LOQ = 10 ng/g) and for the monitoring of vitamin D$_2$ and D$_3$ metabolite in human blood (LOQ = 2.5 ng/mL). For accurate quantification, isotopic dilution was employed.

Vitamin D plays an important role in many aspects of human health, such as skeletal and cardiovascular diseases, neuromuscular problems, diabetes, and autoimmune and chronic diseases. Today, this vitamin is also very often mentioned in connection with the support of the immune system against Covid-19 (1,2). Although vitamin D can be partially synthesized by the human body from its precursor 7-dehydrocholesterol, vitamin D deficiency is currently a global health problem (3). The level of this vitamin in the body can be increased by a diet rich in fish, eggs, milk, and dairy products. In the case of a diet suitable for vegetarians and vegans, it is possible to use UV-treated mushrooms with an increased content of vitamin D (4). Mushrooms, like human skin, can produce vitamin D when exposed to UV radiation. During this process, the content of ergosterol on the surface of the fungus is converted to ergocalciferol (vitamin D$_2$) by a series of photochemical and thermal reactions. Thus, the amount of vitamin D$_2$ formed in mushrooms is considerably higher than the content of vitamin D in fortified food products (5). Also, many products containing fresh or powdered mushrooms with increased vitamin D$_2$ content are available on the retail market. Because vitamin D is a lipophilic vitamin and its excessive intake can cause problems associated with hypervitaminosis, bone loss, and kidney damage (if not treated), it is important to monitor levels of this vitamin in fortified foods (6). For this reason, it is necessary to have accurate and fast methods available for its quantification. Currently, methods used for vitamin D$_2$ determination in mushrooms are based on solid–liquid extraction (SLE) (7–10) or saponification (11,12) of a lyophilized sample, followed by a solid-phase extraction (SPE) clean-up and determination by liquid chromatography with UV detection (LC–UV) (8,9,11) or LC–tandem mass spectrometry (MS/MS) (7,10–12). For vitamin D determination in blood plasma, methods based on protein precipitation with a subsequent liquid–liquid extraction (LLE) and LC–MS/MS determination of target analytes are usually employed (13–15). The aim of this study was to develop a fast and easy LC–MS/MS method suitable for the determination of vitamin D$_2$ and ergosterol in fresh button mushrooms, and also a method for the analysis of the vitamin D metabolites 25(OH)D$_2$ and 25(OH)D$_3$ in blood plasma.

Materials and Methods

Chemicals: Acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), and ethyl acetate (EtOAc) were purchased from Honeywell. Isopropanol (iPro), $n$-hexane ($n$Hex), mobile phase modifiers (ammonium formate, formic acid), ergocalciferol (vitamin D$_2$), ergosterol, cholecalciferol (vitamin D$_3$), and internal standard (20S) were purchased from Sigma–Aldrich. The mobile phase consisted of acetonitrile and a solution of ammonium formate and formic acid in water at pH 4.0 (50:50, v/v). The column was a polymeric bonding C18 phase column (100 mm x 4.6 mm i.d., 3.5 µm, Agilent).
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**FIGURE 1**: Example chromatogram of a standard solution (c = 10 ng/mL, Zorbax Eclipse PAH column).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>25(OH)D3</th>
<th>25(OH)D2</th>
<th>ergosterol</th>
</tr>
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<tbody>
<tr>
<td>3.00</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>4.00</td>
<td>401.3 &gt; 383.3</td>
<td>3974 &gt; 379.4</td>
<td>379.4 &gt; 69.0</td>
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<td>5.00</td>
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<tr>
<td>10.00</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

D$_3$, 25-hydroxyergocalciferol (25(OH)D$_3$), 25-hydroxycholecalciferol (25(OH)D$_2$), d$_6$-25-hydroxyergocalciferol (25(OH)D$_3$-d$_6$), d$_6$-25-hydroxycholecalciferol (25(OH)D$_2$-d$_6$), butylhydroxytoluene (BHT), and magnesium sulfate were obtained from Merck. Hydromatrix was obtained from Varian and anhydrous sodium sulfate was purchased from Lach-Ner. Deionized water (dH$_2$O) was obtained from a Milli-Q Integral system (Millipore). The elution gradient was: 0 min (85% A, 0.2 mL/min), 1 min (85% A, 0.2 mL/min), 8 min (100% A, 0.2 mL/min), 12 min (100% A, 0.2 mL/min), 13 min (85% A, 0.2 mL/min), 16 min (85% A, 0.2 mL/min). The injection volume was set to 5 µL. The mass spectrometer was operated in the positive ionization mode (ESI+) using MRM acquisition mode with the following conditions: capillary voltage, 3.5 kV; extractor voltage, 4 V; source temperature, 120 °C; cone gas flow, 100 L h$^{-1}$; and desolvation gas flow, 1000 L h$^{-1}$ (both gases were nitrogen). Argon was used as a collision gas (3.3 × 10$^{-3}$ mbar). MRM transitions (collision energy) for the target analytes were: for vitamin D$_3$, 397.4>379.4 (13), 397.4>159.0 (35); for ergosterol, 379.4>69.0 (18), 397.4>379.4 (13); for vitamin D$_2$, 385.4>367.2 (15), 385.4>159.0 (29); for 25(OH)D$_2$ 413.5>395.5 (10), 413.5>159.1 (25); and for 25(OH)D$_3$, 401.3>383.3 (11), 401.3>365.5 (13).

**Validation Study**: The recovery and repeatability of the method were tested using a vitamin D$_3$ artificially contaminated sample (“spike”) of unirradiated fresh mushrooms at two concentration levels (50 and 200 ng/g) in six replicates. For the validation of the method for the determination of 25(OH)D$_2$ and 25(OH)D$_3$ in blood plasma, the standard reference material (SRM) 1950 Metabolites in Frozen Human Serum in 10 replicates and artificially contaminated sample (25(OH)D$_2$ 5 ng/mL) of bovine serum were used.

**Results and Discussion**: In this study, methods for the determination of vitamin D$_3$ in mushrooms, and 25(OH)D$_2$ and 25(OH)D$_3$ in blood plasma were developed. First, the LC–MS/MS method was optimized to fully separate ergocalciferol and ergosterol and to achieve a sufficient limit of quantification (LOQ) for the detection of 25(OH)D$_2$ and 25(OH)D$_3$. The tested analytical columns were: 3.6 mm × 2.1 mm, 1.8-µm Zorbax Eclipse PAH RRHD (Agilent Technologies); 4.6 mm × 2.1 mm, 2.7-µm Poroshell 120 EC-C18 (Agilent Technologies); and 3.6 mm × 2.1 mm, 1.7-µm Arion Plus C18 UHPLC (Chromservis s.r.o.). and 100 mm × 2.1 mm, 1.0-µm Zborax Eclipse PAH RRHD (Agilent Technologies).

**Sample Preparation**: Fresh Mushrooms: For the experiments and validation study, samples of fresh button mushrooms (*Agaricus bisporus*) and vitamin D$_2$-enriched button mushrooms were harvested the day before the experiments were performed. Previous to the extraction, the samples were homogenized using a laboratory blender. A homogenized sample (1 g) was thoroughly grounded with 5 g of anhydrous sodium sulfate and transferred to the 50 mL PP falcon tube. Vitamin D$_3$ standard (10 µL, 10 µg/mL) was added to the sample as an internal standard. Subsequently, 6 mL 4:6 (v/v) nHex–EtOAc extraction mixture with 0.2% BHT was added to the tube, and the tube was vigorously shaken (2 min, 1000, Geno/Grinder 2010 [Spex Sample Prep]). The upper layer was transferred into a 15-mL falcon tube and the sample was twice reextracted using 3 mL of the 4.6 (v/v) nHex–EtOAc extraction mixture. From the combined extracts, 6 mL was taken and evaporated. Subsequently, the residues were dissolved in methanol containing 0.2% BHT and transferred to the vial for LC–MS/MS analysis.

Blood Plasma: Blood plasma (0.2 mL) was transferred to the 15-mL falcon tube. A 0.02 mL (c = 500 ng/mL) measure of 25(OH)D$_3$-d$_6$ and 25(OH)D$_2$-d$_6$ was added as an internal standard. Subsequently, 0.6 mL of MeCN with 0.2% of BHT was added for the protein precipitation. The tube was mixed using a vortex and left in a fridge to precipitate proteins (0.5 h, 4 °C). The sample was then three times reextracted using 1 mL of nHex containing 0.2% of BHT. The combined extracts were evaporated and residues were dissolved with 0.4 mL of MeOH with 0.2% of BHT. The mass spectrometer was operated in the positive ionization mode (ESI+) using MRM acquisition mode with the following conditions: capillary voltage, 3.5 kV; extractor voltage, 4 V; source temperature, 120 °C; cone gas flow, 100 L h$^{-1}$; and desolvation gas flow, 1000 L h$^{-1}$ (both gases were nitrogen). Argon was used as a collision gas (3.3 × 10$^{-3}$ mbar). MRM transitions (collision energy) for the target analytes were: for vitamin D$_3$, 397.4>379.4 (13), 397.4>159.0 (35); for ergosterol, 379.4>69.0 (18), 397.4>379.4 (13); for vitamin D$_2$, 385.4>367.2 (15), 385.4>159.0 (29); for 25(OH)D$_2$ 413.5>395.5 (10), 413.5>159.1 (25); and for 25(OH)D$_3$, 401.3>383.3 (11), 401.3>365.5 (13).

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For 25(OH)D3, the blood plasma. To meet these requirements, the three columns described in the "Materials and Methods" section were tested and two mobile phases consisting of MeOH–iPro–dH2O and MeCN–iPro–dH2O were tested. To improve ionization, modifiers ammonium formate and formic acid were added to the mobile phase. Two of the tested columns were commonly used C18 columns. The last one was a polymeric bonding C18 phase column that separates on the basis of shape, and this selectivity made it suitable for separating vitamin D2 and ergosterol. The best separation of ergosterol and vitamin D3 was achieved on this column in combination with a mobile phase consisting of MeCN–iPro–dH2O (see Figure 1), and therefore this column and mobile phase were used for subsequent experiments. For 25(OH)D2 and 25(OH)D3, the limits of quantification reached using this setup were 2 ng/mL resp. 1 ng/mL of blood plasma, which is sufficient to monitor these analytes in this matrix.

In the next step, extraction methods for vitamin D2 in mushrooms were optimized. Several parameters were tested: i) sample processing procedure before extraction, ii) the composition of the extraction solvent, and iii) the number of extraction repetitions. Mushroom sample drying before extraction is a crucial step that has a significant impact on the retention of ergosterol and vitamin D3. In most previously published studies, mushrooms were lyophilized prior to extraction, but lyophilization is a time-consuming step, and so attention has been focused on eliminating this procedure. In this experiment, three different methods of drying the sample before extraction and their effect on vitamin D3 recovery were tested: i) lyophilization, ii) drying using sorbent hydromatrix, and iii) drying using anhydrous sodium sulfate. Furthermore, fresh mushrooms were also extracted for recovery comparison. Based on the published studies, the extraction solvent n-Hex and solvent mixture 4:6 (v/v) n-Hex–EtOAc were tested. The obtained results are shown in Figure 2.

As expected, the lowest recoveries were obtained for the fresh mushroom extraction (50% and 42%) (see Figure 2). During the extraction, a gel formed in the n-Hex extract, which made it difficult to remove the n-Hex layer for the subsequent analysis, and therefore lowered the recoveries (50%). If the sample was extracted with the mixture of 4:6 (v/v) n-Hex–EtOAc, the gel was not formed, but the recovery of the method was also significantly reduced (42%). Sufficient recoveries for lyophilized mushroom samples (76% resp. 82%) were achieved, but as already mentioned, this method is very time-consuming and lyophilization can lead to significant losses of naturally occurring vitamin D3 (up to 16%). Therefore, this method was not selected. In case of the drying sorbents (hydromatrix and anhydrous sodium sulfate), sufficient drying of the sample as well as recoveries of the analyte of interest (130 and 110% resp. 109 and 91%) were achieved. Based on the results obtained, anhydrous sodium sulfate was chosen as the most suitable sorbent for drying of the sample before extraction due to its easy handling, availability, and price. As an extraction solvent, a mixture of 4:6 (v/v) n-Hex–EtOAc was selected. The last optimized parameter was the number of extraction repetitions. Usually, the vitamins are extracted from the matrix at least three times in a repeated solvent extraction, therefore three, four, and five repetitions were tested in our study. Because further extraction did not increase the recovery of the method, three repetitions of extraction were chosen as optimal.

In addition, this study also examined the method for the determination of 25(OH)D2 and 25(OH)D3 in blood plasma and optimized it. The tested parameters were the amount and type of protein precipitation solvent and the time of precipitation. In published studies dealing with the determination of vitamins in blood plasma, methanol and ethanol are most often used in a volume 2–4 times higher than the sample volume. In our study, 200 µL of blood plasma was chosen as a suitable volume of the sample and the volume of the precipitation solvent—400, 600, and 800 µL—was tested. For the precipitation solvent, methanol, ethanol, and acetonitrile were tested, and the precipitation length of 20, 30, and 40 min was assessed. The best protein precipitation occurred with the addition of 0.6 mL of acetonitrile and a precipitation time of 30 min.

Optimized methods were subsequently validated. The obtained validation parameters are listed in Table 1. As can be seen from Table 1, satisfactory recoveries and repeatabilities were achieved for all
TABLE 1: Validation parameters obtained during validation study for mushrooms and blood plasma

<table>
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<th>Analyte</th>
<th>Mushroom (n = 6)</th>
<th>Blood plasma (n = 10)</th>
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<tr>
<td></td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>25(OH)D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>103</td>
<td>9</td>
</tr>
<tr>
<td>25(OH)D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>113</td>
<td>7</td>
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Jana Pulkrabová is a professor and also Head of the Department of Food Analysis and Nutrition, University of Chemistry and Technology, Prague (UCT Prague). She is a head of the research group concerned with separation science in the fields of environmental analysis and biomonitoring.
Q. You recently developed a fast gas chromatography–mass spectrometry (GC–MS) method for efficacy assessment of natural antioxidants (1). Why is this type of analysis important?

A: As part of Unilever’s commitment to help people transition towards healthier diets, we are reducing the levels of unhealthy saturated fats in our products by replacing them with more healthy unsaturated oils. These unsaturated lipids are more prone to lipid oxidation, resulting in a poor smell and a rapid loss of product quality. To avoid this, antioxidants are needed. Up till now synthetic chemicals were used to this end, but we now try to use “nature’s ingenuity”. Nature has not only provided us with unsaturated fatty acids, but also with natural antioxidants! Many spices and herbs, for example, have antioxidant properties. It is just that you need to find which spices and herbs work and for that you need to test them. But because there are many potential natural antioxidant sources you need to test a lot of them. Hence the method needs to be fast.

Q. What is novel about the method you developed and what benefits does it offer the analyst?

A: In literature you will find many methods for testing antioxidant efficacy. Unfortunately, most of these methods are very far from reality. They test radical scavenging activity, metal chelation capacity, or oxygen scavenging. We wanted a test where we are much closer to reality. We wanted to monitor a lipid over its shelf life and monitor the products that are causing the rancid smell of oxidized lipids. Hexanal is one of these species. We have therefore developed an assay where we oxidize lipids under realistic, yet accelerated, conditions and can monitor the effects of antioxidants by following a key product of the oxidation reaction. The main novelty for me is the much more realistic nature of our assay. Moreover, the method is very fast. One measurement takes less than 2 min. The method is also safe for the analyst. There are no toxic chemicals involved. Just sunflower oil, salt, and air.

Q. Were there any particular difficulties you had to overcome when developing the method?

A: There were numerous difficulties. First, there are many potential food ingredients in nature that have an antioxidant capacity. So, we had to narrow the area in which we would search. Spices and herbs were a logical focus area because we...
GC being mature also means it is reliable, it has proven its value, and, just as for people, we should think of “lifelong learning and improvement”.

wanted to stabilize dry soups and sauces. But even with this focus there were still many possibilities to test and we also knew there could be rather strong synergy effects. We clearly needed a fast method. We also wanted to be realistic, meaning that we wanted to study the actual oxidation reaction, not some type of partial aspect of the complex process of oxidation. So, we needed to accelerate the ageing in a realistic way. We studied many routes for acceleration and asked our sensory experts to make sure that the off-smell in the accelerated ageing was the same as in real ageing. We had already fixed hexanal as the read-out for the assay, but we needed to find a fast method to measure it rapidly at very low levels. GC–MS with selected ion monitoring provided the solution here. A final problem was caused by the heterogeneity of many spices and herbs. We needed to take at least 50 to 200 mg to be representative. But this meant that a rather large volume of oil was needed. In the end our assay was done in 250-mL jars. There were many of them, some 200, so we needed a large climate cabinet for ageing.

Q. What were your main findings?
A: The main findings we have patented. We found mixtures of three to five common spices or herbs in specific ratios that exert very strong antioxidant effects. From an analytical perspective I would say the main finding was the high speed and reliability of headspace GC–MS measurements of hexanal. By now we are probably close to 10,000 injections without any major instrumental problems. The rapid hexanal method is now used not only to find new antioxidants, but also to study shelf life stability of our new range of plant-based meat alternatives. We are also thinking of using fast GC–MS on short columns in other applications, such as the dynamic release of flavours from ice cream or the formation of Maillard reaction flavours in cooking.

Q. Are there any specific method development strategies that can turn a “standard” GC method into a “fast GC–MS” method in food analysis?
A: We are very much used to GC runs that take half an hour and basically take that for granted. Admittedly, with fully automated systems this means you can still do some 50 analyses each day. Such a speed you can probably not meet with the sample preparation, data interpretation, and all the paperwork. I think very often the actual GC run is not the rate-determining step. But if you are interested in a shorter run time the use of a column with a slightly reduced inner diameter is a very simple option. We used a 150 micrometre column. Such a column is roughly twice as fast as the standard 320-µm column. Reducing the column length is also a simple way to reduce time, at least if you have over-resolution. Finally, GC–MS allows you to separate compounds that are not chromatographically resolved simply by exploiting mass spectral differences. This is also a good method for reducing the analysis time.

Q. GC is often regarded as a mature technology. Are there any technology developments in GC that offer the analyst “new horizons” in food analysis or any areas of GC that you find particularly innovative?
A: GC is absolutely a mature technology. But that should not be interpreted as the technique is dull, no longer important, and not relevant anymore. GC continues to be a very important workhorse in the analytical laboratory. GC being mature also means it is reliable, it has proven its value, and, just as for people, we should think of “lifelong learning and improvement” rather than allow people or techniques to dwindle into oblivion. Field-portable instruments that can be used by less-experienced users, stable retention times that can be repeated in other laboratories, and elimination of the need for calibration are just a few fields where there is still a lot of work to be done. Recent developments that I think are important include the better software we now have for comparisons of chromatograms, hyphenated systems for complex analyses such as mineral oil saturated hydrocarbons/mineral oil aromatic hydrocarbons (MOSH/MOAH) analysis, and high-resolution mass spectrometry. We should also not forget improvements in the sample preparation area, such as SPME Arrow and thin-film microextraction.

Q. Are there any recent drivers that you think will
Affect the direction of food analysis generally?
A: For sustainability reasons there is the desire to have people consume more plant-based foods. It is not that all people should become vegan, but a healthy balance of some days with meat and dairy and some without would be better. However, despite great progress made, plant-derived meat alternatives do not have the same taste and feel as animal meat. Why is that? The basis for this different taste must be based on the molecules present, so analysis is needed to shed light on this. Some of the off-taste compounds of plants are known, for example, the beany off-taste of plant products is caused by the aldehydes and ketones resulting from enzymatic lipid oxidation. The green taste of pea-derived products is caused by the presence of alkoxy pyrazines. The molecular reasons for other off-flavours and tastes are not known and require detailed chemical analysis and very low levels combined with sensory panel assessments. A complication there is that sensory panels are notoriously slow and in accurate. The alternatives GC-sniffing and LC-taste on the other hand are experimentally demanding. Additionally, the food industry and the governments want to reduce food waste “between field and fork”. A great step forward could be made if food manufacturers would be able to rapidly adjust their processing depending on, for example, the degree of ripeness, of the incoming crop. That would require very rapid analysis of the ingredients, while “the truck waits at the gate”. This would also call for more analyses of the finished goods as variations in the processing conditions are likely to change quality and safety of the products. Finally, this would all have to be done in largely automated analyses because food production runs 24/7.

Reference

This is an updated version of an interview that was published in the July 2021 issue of LCGC Europe.
Taking the Red Pill: Alleviating Matrix Effects for Small Molecule Quantitation in Food and Feed

Matrix effects continue to be an issue in small molecule quantitation in food analysis, with researchers exploring different approaches to circumvent the issue. LCGC Europe talked to Rudolf Krska and Michael Sulyok from the University of Natural Resources and Life Sciences (BOKU), Vienna, Austria, about their work to develop a multi-analyte approach using liquid chromatography tandem mass spectrometry (LC–MS/MS), and the issues surrounding agrocontaminants in animal feeds.

Interview by Lewis Botcherby, Associate Editor, LCGC Europe

Q. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become ubiquitous for the quantitative determination of small molecules in food and feed samples. However, according to your 2020 paper (1), matrix effects continue to be an issue for those using an LC–MS/MS method—in particular for multi-analyte approaches. What are the issues surrounding matrix effects in this area?

Rudolf Krska: Matrix effects are an issue in various analytical techniques. For LC–MS using both high-resolution (HR)MS and tandem MS, matrix components coeluting with analytes of interest influence the ionization efficiency of the latter. This decreases—and on rare occasions increases—their analytical response in the sample extract compared with the same analyte concentration in a neat solvent standard, and thus compromises the accuracy of the method. The classical approach of dedicated sample clean-up (such as by an immune-affinity clean-up) to remove the matrix components is limited to single target analytes or a group of analytes, although there are approaches involving combining different antibodies (2,3). An example of this would be for the mycotoxins addressed by regulatory limits. The same is true for stable isotope labelled internal standards that compensate losses during ionization and are therefore the preferable option for official control analysis, but they are available for only approximately 20 compounds (4). This leaves the option of matrix matching, which can be defined as preparing the calibration curve in sample extracts instead of solvent-based standards for methods that target a broader range of compounds. However, there are some difficulties in executing this approach, including the lack of samples that are true blanks for all the analytes, or accommodating samples from different matrices in one analytical sequence.

Q. The method you published in your 2020 paper was a “dilute-and-shoot” method and covered more than 500 secondary metabolites, including all mycotoxins addressed by regulatory limits as well as emerging and masked mycotoxins in seven different food matrices. What did the obtained data indicate about the
applicability and practicability of the current guidelines for method validation on such a broad method? Michael Sulyok: Following non-LC–MS-specific guidelines results in a considerable consumption of time for data analysis, particularly for levels close to the estimated limit of detection/limit of quantification (LOD/LOQ), which requires manual inspection of each peak. As it took close to two years to evaluate and compile all data for our 2020 publication, it was our aim to come up with a proposal to reduce this burden without compromising the overall assessment of the method performance. We believe that spiking experiments at low concentration levels are not essential as we found recoveries to be independent of the concentration level, and also it is feasible to come up with a conservative estimation for LOD/LOQ that is independent of the matrix. Instead, the focus should rather be on absolute and relative matrix effects, which can be studied at high concentrations that result in peaks that are reliably processed by automatic peak integration. In addition, our data did not support the concept of matrix groups mentioned in some guidelines. Differences in apparent recovery between the individual nut, grain, and dried fruits matrices indicated that matrix effects should be characterized for each new matrix, even if it is similar to another commodity that has already been evaluated.

Q. What conclusions were reached on the current guidelines and what were the recommendations for improvement? RK: There is a lack of recommendation to what extent matrix effects are acceptable, which is true both for the absolute extent, namely the difference in signal intensity to the identical concentration of a given analyte in neat solvent, as well as for the difference in matrix effects between different individual samples, such as different varieties, brands, and so forth of a given matrix, which is known as relative matrix effects. Even extreme absolute matrix effects may be compensated for in principle by matrix matching, but it may be questioned whether a method can be considered valid in such a case. In contrast, the issue of relative matrix effects cannot be overcome by matrix matching and is neglected in many

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Animal feed is particularly challenging because the fractions of the main ingredients vary to a large extent. Publications focusing on method validation. This may be partially explained by the fact that many guidelines foresee determination of repeatability on technical replicates, such as “identical test items” or “18 aliquots of a blank material”, which implies to some extent that any individual sample is representative for all samples of the same matrix. In our work, we emphasize that different individual samples should be included in the validation set.

Q. A further paper published in 2020 presents a comprehensive quantitative LC–MS/MS approach for the analysis of agrocontaminants in animal feeds (1). What challenges exist in the analysis of animal feeds and why are they important?

MS: Animal feed is particularly challenging because the fractions of the main ingredients vary to a large extent, thus emphasizing the issue of relative matrix effects. In addition, finding samples that are true blanks for all analytes of interest is close to impossible, which makes it difficult to find a suitable sample set for method validation. We have proposed to prepare model samples from the related single feed ingredients mirroring the common ranges of the exact composition.

Q. The paper mentions the construction of a prevalence database. What is the function of this database?

RK: The database intends to link occurrences of mycotoxins and fungal metabolites with complementary data, such as origin, weather, or agricultural practice. This serves to identify particular hot spots of mycotoxin contamination in the global supply chain of feed ingredients and finished feed, and also to monitor changes in the pattern of mycotoxins/fungal metabolites on a larger time scale due to global warming.

Q. Did you encounter any challenges in carrying out this research with regards to the instruments and methodologies available?

MS: In our multi-class paper, we explored the limits of data acquisition in tandem mass spectrometry, which is strictly consecutive, meaning that for a given time period (dwell time) on the scale of tens of milliseconds, only one MS/MS signal of one given analyte is scanned. This runs into the problem of not having sufficient acquisition time in the case of hundreds of analytes, even if the “scheduled multiple reaction monitoring mode” is applied, namely scanning a particular analyte only within a pre-defined period at the expected LC retention time. We have shown that reducing the number of data points per peak while keeping the dwell time as high as possible results in an acceptable data quality. However, we think our method is close to the limit of what is feasible in terms of number of analytes—if an even faster scanning technology is not feasible in tandem mass spectrometry, methods comprising even more analytes need to be transferred to HRMS in full-scan mode.

Q. Does the method have applications in other areas of research?

RK: Our multi-class paper has proven that our “dilute-and-shoot” approach is not limited to mycotoxins, although other contaminants might pose problems, such as the solubility and stability issues of certain sub-classes of veterinary drugs. We believe that our considerations on method validation for such broad methods can be extrapolated to LC–MS-based methods dealing with other contaminant classes and matrices.

Q. What are you currently working on?

MS: Our current work in the area of LC–MS/MS method development focuses on various aspects. We are investing a lot of time and effort in the validation of a further extended multi-class method, including some toxic metabolites such as byssychlamic acid, in processed grain-based products. Considering the plethora of secondary metabolites of plants and fungi present in food crops, even the 1250+ method does not suffice. We are also studying the metabolization of mycotoxins by insects and plants by utilizing a stable isotope-assisted LC–HRMS metabolomics-based approach in a functional genomics context.

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This interview was first published in the May 2022 issue of LCGC Europe.
Q. How common is olive oil adulteration and how large a problem is it in monetary terms?

A: Olive oils are basically grouped into three quality grades, namely “extra-virgin olive oil” (EVOO), “virgin olive oil” (VOO), and “lampante olive oil” (LOO), on the basis of specific chemical and organoleptic criteria (1) from the Commission Delegated Regulation (EU) 2019/1604 of 27 September 2019 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis (2).

Referring to the European Regulation No. 2568/91 and subsequent amendments, VOO must be obtained using solely mechanical or other physical means, under conditions that do not alter the product’s integrity. In contrast, the LOO category includes those oils that do not fulfil the minimum VOO quality criteria and, therefore, are not intended for direct human consumption. Finally, EVOO represents the top-quality grade because of its superior sensory attributes and the claimed health-promoting effects. EVOO is one of the most important and expensive edible oils, and is therefore also one of the most adulterated food commodities in the global market: limited production, higher price, and growing consumer demand represent an explicit fraud driver for EVOO.

The International Olive Council has clearly defined the limits of the specific chemical parameters able to protect EVOO against potential adulterations with other edible oils (3). Spain is the world’s largest producer of olive oil, with more than 40% of the world’s production originating from there, particularly from Andalusia. Another 20% comes from Italy, 18% from Tunisia, and approximately 12% from Greece. These four countries, together with Portugal, are also the largest exporters of olive oil.

Producing one litre of olive oil requires four to five kilos of olives on average; furthermore, processing methods also significantly affect olive oil yield.
In 2019, European import volume surpassed 11 million tonnes, at a value of €1.6 billion, a decrease in comparison with previous years, caused by an overproduction in Europe that led to a significant drop in prices. In the long term, the European market for olive oil is expected to show stable growth of 3–5% over the next five years (4).

Q. What exactly are soft refined oils? And why do they represent a challenge to scientists to detect?  
A: The term soft-deodorization refers to a deodorization process performed at a lower temperature with respect to the conventional process, for example, 100 °C instead of 180–200 °C. It basically consists of a vacuum steam distillation using nitrogen to strip out all the volatile compounds responsible for the undesirable odours. Deacidification is instead designed to remove the free fatty acids (FFA), which speed up oxidation processes and are involved in the development of rancid flavour. This is normally achieved through the addition of alkali, such as sodium hydroxide, to the oil; this results in the precipitation of the FFA as an insoluble soap dreg, subsequently removed by centrifugation and/or filtration. Since the use of lower temperatures does not seem to produce substantial compositional and structural modification in the bulk, the resulting soft-refined olive oils (SROO) are best used to blend EVOO and create illicit mixtures no longer detectable by current methods (5). Typical fraud, such as adding other types of oils like seed oils or pomace oils, the presence of re-esterified oils, or the preparation of blends with refined oils, can be detected with standard methods. For this reason, fraudsters are now focused on developing more sophisticated adulterations, such as the use of soft refined oils, that would allow for the creation of blends that cannot be discovered with regular methods. Furthermore, another relevant issue is the misrepresentation of the geographical origin of the oils. While there are currently no recognized official methods for detecting these frauds, the literature suggests different approaches with promising results: gas chromatography–mass spectrometry (GC–MS), nuclear magnetic resonance (NMR), isotopic fingerprint studies, or liquid chromatography–mass spectrometry (LC–MS) coupled with chemometrics (6).

Q. You first approached the issue with a 2020 publication where a nontargeted high-resolution mass spectrometry (HRMS) method was used. Could you discuss your approach to selecting a method, and the positives and negatives of the selected approach?  
A: We developed a nontargeted LC–HRMS study for the detection of new chemical markers able to identify the addition of soft deodorized and soft deacidified low-quality virgin or lampante olive oils to EVOO (7). This approach has definite advantages in terms of effectiveness in detecting anomalies, even those not foreseen among those under examination, but it should be kept in mind that it entails high instrumental costs, technical staff of high competence, and is difficult to be directly implemented by an industrial control laboratory. The industry in this case is rather oriented to subcontract these analytical investigations to specialized external laboratories. In this specific case, “in-house” soft refined oils were created and analyzed together with a group of pure EVOOs. In addition, different mixtures of pure EVOO and adulterated oils were included in the sample set. The markers selected through the study via robust data elaboration—12 molecules, seven of them were selected as discriminative in both the laboratories—were compared between two laboratories equipped with two different types of mass spectrometers, a quadrupole (Q)-orbital trap and a quadrupole time-of-flight (QTOF), with the successful aim to assess the reproducibility of the proposed analytical approach.

Q. Your most recent publication took a slightly different approach to the issue and evaluated LC–MS, GC–ion mobility
spectrum detection (SROO), and flash gas chromatography electronic nose (FGC-Enose) for their suitability to detect olive oil fraud. What were your findings regarding their suitability?

A: In parallel to LC–MS studies, in the last couple of years we have explored the potentialities of two emerging headspace (HS)-based techniques, namely GC–IMS and FGC-Enose for the detection of EVOO blending with SROO (9). These two techniques offer rapid, minimal sample preparation, high-throughput, and non-destructive solutions for quality and authenticity testing, which are highly sought by modern food industries. They provide global information about the volatile organic compound (VOC) profile in a short time. The methods exhibited both robustness and stability over time, giving promising results for screening purposes.

Additionally, the strategy of targeting the volatile profile is of interest because the main changes due to soft refining (especially for deodorization) are not expected to occur in the bulk fraction.

Very recently we also aimed our efforts into deepening the potential of the latest generation of multivariate statistical analysis: low-level data fusion of GC–IMS and the FGC-Enose datasets were performed (9). The merged unique fingerprints were submitted to partial least-squares discriminant analysis (PLS-DA) and the extrapolated most informative variables were used to build support vector machine (SVM) classifiers. The results of this PLS-DA-SVM strategy on the combination of datasets demonstrated that the discriminatory capability of the two merged GC-based techniques was remarkably improved compared to the individual ones, improving, for example, the sensitivity, which means that 20% or lower adulteration percentages detection could be achieved in the future.

Q. What were the main challenges developing this approach and how did you overcome them?
A: It was, of course, rather expensive to set up in the laboratory samples appropriately adulterated to “teach machines the frauds to recognize”, as well as collect data with several instrumental systems and multiple approaches, but perhaps the most complex part was the final stage of data processing.

To face the increasingly complex analytical challenges placed by fraudsters in olive oil adulteration and other areas of food analysis, laboratories need to recruit specialized staff with cross-skills in analytical chemistry and statistics, and learn to “merge” effectively complementary data from techniques capable of providing orthogonal information and therefore capable of enhancing both selective and discriminatory potentialities.

Q. What other projects are you working on?
A: We are currently working on both rapid screening and confirmatory methods to address the geographical origin of cereals, with a particular focus on wheat (10,11). For example, in Italy it is mandatory to declare the origin of wheat on the pasta label, according to a specific ministerial decree (12). Durum wheat (Triticum turgidum subsp. durum) is in fact the preferred raw material for the production of pasta due to its technological and nutritional properties, and the declaration of its geographical origin, sometimes claimed on pasta labels, represents an added value to the commodity.

References
Analyzing Mineral Oil Hydrocarbons in Food

LCGC spoke to Andrea Hochegger, a postdoctoral researcher at the Institute of Analytical Chemistry and Food Chemistry at the Graz University of Technology in Austria, about her work analyzing mineral oil hydrocarbons (MOHs), and the importance of packaging to reduce the migration of MOH into foods.

Interview by Kate Jones, Managing Editor, LCGC Europe

Q. When did you first encounter chromatography and what attracted you to the subject?
A: I first encountered chromatography very early in my chemistry study, during a summer internship in a food safety laboratory. I was asked to integrate some new analytes into an existing high performance liquid chromatography (HPLC) method and was fascinated by the task. I really enjoyed “playing” with the different parameters and seeing the influence on the chromatography, and then finally applying my developed method onto real samples. That was when I decided to focus on analytical chemistry and food chemistry for my future career.

Q. What chromatographic techniques have you worked with?
A: I did my master’s degree and my PhD at the Institute of Analytical Chemistry and Food Chemistry under the supervision of Professor Leitner at the University of Technology Graz. The group mainly focuses on gas chromatography (GC)-based techniques with different detection systems, and I tried to get my hands on as many instruments as possible. For example, I worked with conventional one-dimensional GC systems with flame ionization detection (FID), flame photometric detection (FPD), or nitrogen–phosphorus detector (NPD), and quadrupole mass spectrometer (qMS and MS/MS). I also worked with two-dimensional comprehensive GC systems: a GC×GC-qMS and a GC×GC–time-of-flight (TOF). During my PhD, I also started working on the topic of mineral oil hydrocarbons (MOHs), where the state-of-the-art analysis is done using an online-coupled HPLC–GC–FID system.

Q. Can you tell us more about your PhD thesis and the work you published related to this?
A: During my PhD, I worked with cellulose-based packaging materials—mainly recycled paper and board. The problem with the recycling of materials for food contact is that during the recycling process contaminations are not removed but instead are enriched in the final product, from where they then can be transferred into the packed food. The only fast and practical way to prevent this migration seems to be the application of functional barriers in the packaging. Recently applied barriers include polyethylene (PE), polypropylene (PP), aluminium, or multilayer materials. The problem is that recycling of most of the resulting packaging is—
The work I published related to that describes the developed method and the application on to functional barriers made of the biopolymer chitosan acetate and conventionally applied polymer films such as PE or PP, and compares the barrier properties, showing that the chitosan acetate can work perfectly as a barrier material.

Q. You are currently focused on the analysis of MOHs, and your latest paper on mineral oil hydrocarbon risk assessment talks about knowledge gaps in analytical methods (2). Why is MOH analysis important and what knowledge gaps do you think exist?

A: Discussion about mineral oil residues in food began in the early 1990s, but it was only in 2008 that the public became aware of this, mainly because high concentrations were found in sunflower oil. Mineral oil hydrocarbons are divided into two groups: saturated (MOSH) and aromatic (MOAH) mineral oil hydrocarbons. The MOSH fraction is known to accumulate in the human organism, resulting in negative impacts on the respective tissue. The MOAH fraction contains potentially mutagenic and carcinogenic substances. So, the presence in food of both is a concern for human health and should be avoided.

Since then, a lot has been done in terms of reducing contamination levels and analytical detection. Back then we talked of several grams of mineral oil per kilogram of food, today we deal with a few milligrams. This—of course—increases the analytical challenge. Highly specific
methods, adapted to the complex matrix “food”, are necessary to meet the requirements. We are lacking a common sense of the best practice and therefore standardized and validated methods for different food types.

**Q. Can you comment further on the potential of mutagenic and carcinogenic substances being present in MOAHs.**

**A:** Probably the main statement to say about MOAHs is that they may contain potential mutagenic and carcinogenic substances. According to the scientific opinion of the European Food Safety Authority (EFSA, 2012), the three- to seven-ring MOAH may form carcinogenic DNA adducts. Therefore, those may be the actual substances of interest. Analytical methods should be applied to identify the presence of those substances routinely.

**Q. What cutting-edge analytical techniques are being used in MOAH analysis?**

**A:** The problem with state-of-the-art analysis using online-coupled HPLC–GC–FID is that MOSH and MOAH are only determined as a sum parameter, without any further information about what is actually present (2,3). Two-dimensional comprehensive GC×GC is therefore a key method to provide the information needed. It allows for a detailed characterization of the substances and substance classes present. Attempts have been taken to develop an LC–GC×GC system with MS and FID detector, allowing on the one hand identification of the present substance classes, and on the other hand quantification (4). The missing information is then which substances are the ones responsible for the carcinogenic character and in what amount are they present in the food? In my current work, I try to answer this question.

**Q. How do you see MOH analysis developing in the future?**

**A:** Very important in terms of sample preparation is the possibility of automation, for example, automated aluminium oxide clean-up for the MOSH fraction, or automated epoxidation for MOAH, which would save a lot of time and resources in routine analysis. On the other hand, the GC×GC method should be routinely applied when MOAH is detected to provide the needed information on present substance classes for risk assessment and hazard characterization.

**Q. Can the problems associated with MOH analysis be solved?**

**A:** We saw and still see a lot of advances in the field. Different groups are working on, for example, an update of the existing EN16995:2017 to adapt it to the state-of-art and to lower the limit of quantification, or on a method for MOAH in infant formula. Lately, the “Standing Committee on Plants, Animals, Food, and Feed” published a new statement regarding the presence of MOAH in food (5), and the EFSA is working on a new scientific opinion on the topic.

We also saw that a good collaboration of all involved stakeholders, from the food producer, the analyzing laboratories, the toxicologist, right up to the different authorities, is needed and favoured to find a common purpose and solve the “challenge” of mineral oil residues in food. The use of GC×GC-based techniques is and will continue to be a driving analytical technique to solve the challenge. Furthermore, we have seen a huge attempt to bring GC×GC to routine analysis in recent years, including lots of developments for easier data evaluation (6). Therefore, I strongly believe that it is possible to use it in routine analysis, and once it is implemented there, we will be able to use it to its full potential. There are already a lot of different methods and applications published (7), including petrochemical, environmental, or food and fragrance samples, and there will be many more.

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