APPNOTE

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GERSTEL AppNote 239 - updated

# Fully Automated Determination of 3-MCPD, 2-MCPD and Glycidol in Edible Oils and Fats based on ISO 18363-4 - Zwagerman/Overman Method

Jasmin Zboron and Oliver Lerch

*GERSTEL GmbH & Co.KG, Eberhard-Gerstel-Platz 1, 45473 Mülheim an der Ruhr, Germany*

## Keywords

3-MCPD, 2-MCPD, Glycidol, edible oil and fat, ISO 18363-4, Zwagerman/Overman method, laboratory automation

## Abstract

Fatty acid esters of 3- and 2-monochloropropanediol (3-MCPD-e, 2-MCPD-e) and glycidol (Gly-e) are process contaminants that are formed, for example, during the refining of edible oils and fats. After ester cleavage during digestion in the human body, they pose a relevant health risk and must therefore be determined in edible oils and fats and in fatty foods.

For the indirect analysis of 3-MCPDe and Gly-e, several standardized analytical methods are currently available, which are defined in various standard method documents. GERSTEL previously developed and presented a comprehensively automated analysis system following ISO 18363-1, AOCS Cd 29c-13, DGF C-VI 18 (10) (GERSTEL AppNote 191). In this document, we present a system for comprehensive automation of the ISO 18363-4 (Zwagerman/Overman) method, the most recent standard method available for 3-MCPD-e, 2-MCPD-e and Gly-e determination.

When performing sample preparation and sample introduction with the GERSTEL MultiPurpose Sampler (MPS), fitted with the required modules and options, the only manual steps the user needs to perform are weighing an aliquot of oil into a sample vial, capping it, and placing it in the MPS sample tray. All other sample preparation steps listed in the standard method and the subsequent GC-MS/MS determination are performed automatically. Limits of quantification (LOQs) of 19 µg/kg for 3-MCPD, 17 µg/kg for glycidol and 16 µg/kg for 2-MCPD are achieved, which is well below the LOQs of 100 µg/kg required in the ISO standard for all three compounds. The precision (repeatability) is below 6% relative standard deviation for all three compounds. The accuracy was verified by analyzing a sample from a proficiency test and a sample for which external analysis results were available. The system and method proved to be robust and reliable when processing real samples. The complete setup and method parameters are bundled and made available for end-users to enable rapid method setup and analysis of 3-MCPD, 2-MCPD and glycidol according to ISO 18363-4.

## Introduction

Fatty acid esters of 3- and 2-monochloropropanediol (3-MCPD-e, 2-MCPD-e) and glycidol (Gly-e) are process contaminants found in a variety of fatty foods. These compounds are formed in the presence of sodium chloride in fatty foods when high temperatures are applied during processing, such as the refining of edible oils and fats. During digestion in the human body, ester cleavage occurs, releasing 3- and 2-monochloropropanediol (3-/2-MCPD) as well as glycidol (Gly).

The International Agency for Research on Cancer (IARC) has classified glycidol in category 2A as probably carcinogenic to humans and 3-MCPD in category 2B as possibly carcinogenic to humans [1]. These relevant health risks have prompted the European Union (EU) and many countries worldwide to set maximum acceptable concentrations for edible oils and fats as well as for fatty foods

[2]. The maximum level for glycidol in oils and fats is 1000 µg/kg. For foods intended for infants and young children, the maximum levels range between 6 and 500 µg/kg. For 3-MCPD the maximum admissible levels for various oils and fats are between 1250 and 2500 µg/kg. In the case of foods intended for infants and young children, the maximum allowable concentration is between 15 and 750 µg/kg. As the toxicological assessment for 2-MCPD has not yet been completed, there are no maximum levels set for this compound so far.

There are already a couple of standardized analysis methods for indirect analysis of 3-MCPD-e and Gly-e, which are laid down in various documents of the International Organization for Standardization (ISO), the American Oil Chemists' Society (AOCS) and the German Society for Fat Science (DGF). All these methods are based on similar chemical processes and workflows, but differ in the reaction conditions, the internal standards used. Furthermore, the calculation methods differ. Fats are saponified under alkaline or acidic conditions, the target analytes are released from their esters and the resulting fatty acids are converted to their corresponding methyl esters (FAMEs). The reaction is quenched in the presence of an aqueous sodium chloride or sodium bromide solution, which convert glycidol into 3-MCPD or 3-monobromopropanediol (3-MBPD), respectively. In method ISO 18363-3 (equivalent to AOCS Cd 29a-13), these steps are carried out in reverse order, first the transformation of glycidol esters to 3-MBPD esters and then the ester cleavage. The FAMEs are subsequently removed by extraction with a non-polar solvent, while the analytes remain in the aqueous phase. In the next step the analytes are derivatized with phenylboronic acid (PBA). Finally, the derivatives are extracted with a non-polar solvent and determined by GC-MS(/MS).

IISO 18363-2 (equivalent to AOCS Cd 29b-13) [3] and ISO 18363-3 (equivalent to AOCS Cd 29a-13) [4] are known to yield extremely accurate analytical results with high precision. However, these methods require a considerable amount of time, including a transesterification step at -22 to -25°C or at 40°C for 16 h. These methods are often used by contract laboratories as a kind of gold standard. Both methods can be automated by the GERSTEL Multi-Purpose Sample (MPS). ISO 18363-1 (equivalent to AOCS Cd 29c-13 and DGF C-VI 18 (10)) [5] is the most commonly used method, also known as the C-method. Although it requires two assays per sample, it does not require overnight incubation and is therefore much faster. The glycidol content is determined via a differential calculation based on the results of assay A and B. This calculation is the main disadvantage of the method, as statistical errors of both assays accumulate in the glycidol determination. In addition, the method tends to overestimate the glycidol content at high 3-MCPD concentrations. During transesterification, 3-MCPD is partially converted into glycidol, which is not taken into account by the method. Despite these weaknesses, the method is widely used, especially in production laboratories where rapid results are required for the release of products or supplied raw materials. It has been fully automated using the GERSTEL MPS, see AppNote 191 [6].

ISO 18363-4 [7] is the latest standard in the ISO series, published in August 2021. It is based on the work of Ralph Zwagerman and Pierre Overman from Bunge Loders Croklaan company in the Netherlands [8,9]. They were searching for a fast alternative to the AOCS C-method which, as explained above, has disadvantages in the glycidol determination. Zwagerman and Overman found a way to determine glycidol directly from the sample and to compensate for the deviations caused by glycidol formation from 3-MCPD during the transesterification step. All three analytes are determined in one assay based on calibration curves generated prior the analysis. To accurately quantify the amount of 3-MCPD that is converted to glycidol, which would otherwise lead to glycidol overestimation, the method applies a correction via $^{13}$C-labelling. In short, a known amount of 3-MCPD-$^{13}C_3$ ester is used to quantify the amount of glycidol-$^{13}C_3$ formed during transesterification. The amount of glycidol-$^{13}C_3$ formed is then used to calculate the amount of glycidol formed from 3-MCPD ensuring that any overestimation can be corrected.

The following is the complete workflow of ISO 18363-4: The oil or fat sample is dissolved in toluene and methyl-tert-butyl-ether (MTBE). The internal standards 3-MCPD-$^{13}C_3$ diester, as internal standard for 3-MCPD and 2-MCPD, and pentadeuterated glycidyl ester as internal standard for glycidol, are added. The sample is then cooled to 10 °C before the alkaline transesterification is initiated by adding a sodium methoxide solution in methanol. After 12 minutes of incubation at 10 °C, the sample mixture is acidified with an acidic solution of sodium bromide to convert the released glycidol to 3-MBPD. The fatty acid methyl esters generated during transesterification are removed by extracting twice with iso-octane. The polar analytes remain in the aqueous phase and are derivatized with phenylboronic acid prior to GC-MS/MS determination. Figure 1 shows a representation of the described workflow.

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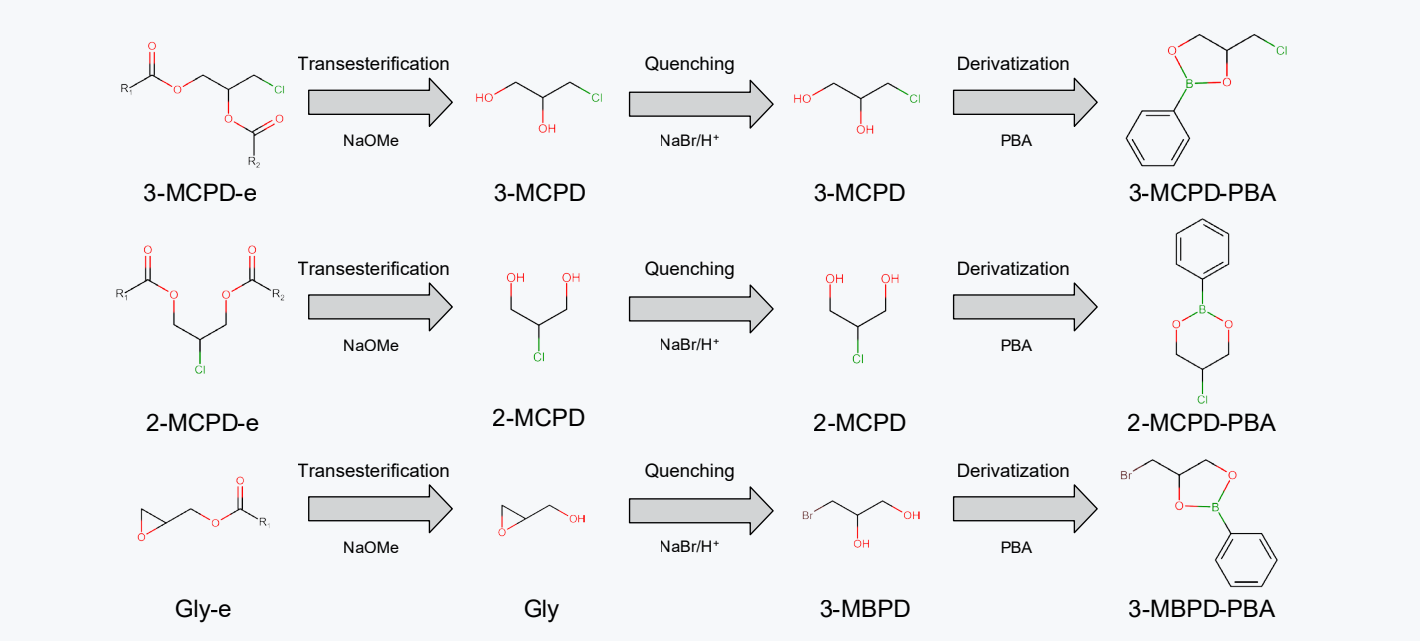


**Figure 1:** Overview of the chemical reactions performed in the sample preparation workflow of ISO 18363-4.

The quantification of ester-bound 2-MCPD and 3-MCPD is based on the signal ratios of 2-MCPD/3-MCPD-$^{13}C_3$ and 3-MCPD/3-MCPD-$^{13}C_3$, respectively. The quantification of ester-bound glycidol is based on the 3-MBPD/3-MBPD-$d_5$ signal ratio. The amount of 3-MBPD-$^{13}C_3$ formed after the transesterification reaction represents the amount of released 3-MCPD-$^{13}C_3$ that was degraded to glycidol due to the alkaline transesterification conditions. Since no difference in the rate of degradation between 3-MCPD and 3-MCPD-$^{13}C_3$ was observed, the observed change in the 3-MCPD-$^{13}C_3$ concentration can be used to correct for an overestimation of glycidol due to the degradation of 3-MCPD. Under the conditions used, 2-MCPD is considered stable and thus does not contribute significantly to a possible overestimation of glycidol. In contrast to the other parts of the ISO 18363 series, ISO 18363-4 requires GC-MS/MS instrumentation for unambiguous determination of the isotopically labelled 3-MBPD, which is necessary to ensure correct quantification of the glycidyl ester-related glycidol.

After weighing in a quantity of fat or oil, all remaining sample preparation steps, and the injection into the GC-MS/MS system are performed by the GERSTEL MPS equipped with the required modules. The chromatography system used for the analysis is equipped with a pre-column backflush system to prevent phenylboronic acid and major matrix constituents from entering and contaminating the analytical column and the mass spectrometer, and to shorten the overall analysis time. Such a backflush system is highly recommended.

## Experimental

### *Materials and Solvents*

1,2-dipalmitoyl-3-chloropropanediol (PP-3-MCPD), 1,3-distearoyl-2-chloropropanediol (SS-2-MCPD), $^{13}C$ labelled 1,2-dipalmitoyl-3-chloropropanediol (PP-3-MCPD-$^{13}C_3$), glycidyl stearate (Gly-S) and pentadeuterated glycidyl stearate (Gly-S-$d_5$) each 1000 µg/mL in toluene purchased from Campro Scientific, Berlin, Germany. Methanol, iso-octane, acetone, toluene, tert-butyl-methyl-ether (MTBE), water and concentrated sulfuric acid, all analytical grade, obtained from Merck, Darmstadt, Germany. Phenylboronic acid (PBA), sodium bromide, and sodium methoxide solution in methanol (NaOMe, 25% m/m) provided from Merck.

A 25% (v/v) aqueous sulfuric acid solution was prepared by transferring 25 mL of concentrated sulfuric acid to a 100 mL volumetric flask containing 50 mL of water and filling up to 100 mL with water. The sodium bromide solution was prepared as follows: 600 g sodium bromide was dissolved in 700 mL water, 36 mL of the 25% sulfuric acid was added and the solution was finally made up with water to 1000 mL in the volumetric flask. The 0.35 M NaOMe solution was prepared by adding 20 mL of the supplied 25% NaOMe solution to a 250 mL volumetric flask and topping up with methanol. For the saturated PBA solution 12 g of PBA was dissolved in a mixture of acetone and water (95/5, v/v) under vigorous shaking (the PBA is not completely dissolved).

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*Preparation of Calibration Standards and Samples*

As specified in the standard method, three calibration working solutions, an internal standard solution and a spiking solution were prepared. For calibration working solution I (Cal I) 42 µL PP-3-MCPD (1000 µg/mL), 42 µL SS-2-MCPD (1000 µg/mL) and 36 µL Gly-S (1000 µg/mL) were dissolved in 880 µL toluene resulting in 7.9 µg/mL 3-MCPD, 7.2 µg/mL 2-MCPD and 7.8 µg/mL Gly. To prepare the calibration working solution II (Cal II), the purchased individual standard solutions were diluted 1:10 resulting in a concentration of 100 µg/mL of each analyte. Using these solutions 168 µL of PP-3-MCPD, 168 µL of SS-2-MCPD, and 144 µL of Gly-S were mixed with 520 µL of toluene resulting in 3.2 µg/mL 3-MCPD, 2.9 µg/mL 2-MCPD and 3.1 µg/mL Gly. Calibration standard III (Cal III) was prepared by mixing 20 µL Cal I with 980 µL of toluene resulting in 0.16 µg/mL 3-MCPD, 0.14 µg/mL 2-MCPD and 0.16 µg/mL Gly. An additional calibration standard (Cal IV), not mentioned in the norm, was prepared by mixing 25 µL Cal II with 975 µL of toluene resulting in 0.08 µg/mL 3-MCPD, 0.07 µg/mL 2-MCPD and 0.08 µg/mL Gly.

The spiking solution was prepared using 1:10 dilutions of the purchased single standards: 56 µL PP-3-MCPD, 48 µL Gly-S and 40 µL SS-2-MCPD dissolved in 856 µL toluene resulting in 1.05 µg/mL 3-MCPD, 0.69 µg/mL 2-MCPD and 1.04 µg/mL Gly. The internal standard working solution (ISTD) was prepared by mixing 40 µL of the purchased single solution (1000 µg/mL) of Gly-S-$d_5$, 80 µL of the purchased single solution (1000 µg/mL) of PP-3-MCPD-$^{13}C_3$ and adding 9880 µL of toluene resulting in 1.54 µg/mL 3-MCPD-$^{13}C_3$ and 0.92 µg/mL Gly-$d_5$, respectively.

The calibration standards were prepared in a blank oil which was a non-thermally treated, cold pressed olive oil provided by a customer. It was checked for possible traces of the analytes before using it. Nevertheless, glycidol was present in very low levels in all tested potential blank oils.

Calibration standards were prepared by weighing in 100 mg of the blank oil into a 4 mL round bottom vial. and spiking it with the required volumes of calibration working solutions Cal I to III, respectively (see table A.1 in the ISO standard). Additional calibrators in the low concentration range were prepared by spiking the blank oil with the Cal IV solution. The vials were capped with a magnetic screw cap and placed in the MPS sample tray for further processing.

Real oil and fat samples (all in the amount of 100 mg in a 4 mL round bottom vial) and calibration samples were subjected to the following workflow steps, all performed by the MPS:

- Add 100 µL toluene (or the volume specified in table A.1 for the calibration standards)
- Add 200 µL MTBE
- Add 100 µL ISTD working solution
- Agitate at 250 rpm and 80 °C for 120 s to melt or solve the oil or fat
- Homogenize for 10 s in the quickMIX module
- Cool the vial in a cooled tray at 10 °C for 240 s
- Add 200 µL NaOMe solution to start the transesterification
- Homogenize for 10 s in the quickMIX module
- Cool the vial for exactly 12 min at 10 °C
- Add of 700 µL acidic sodium bromide solution to stop the reaction
- Homogenize for 30 s in the quickMIX module
- Keep the sample for 9 min at room temperature for complete conversion of Gly to 3-MBPD
- Add 300 µL iso-octane to remove the FAMEs
- Homogenize for 10 s in the quickMIX module
- Agitate at 400 rpm and 80 °C for 270 s (4.5 min) in order to dissolve potentially jellified or solidified upper layer
- After 3 min at room temperature remove and discard the upper iso-octane/toluene/MTBE layer with the FAMEs, while being careful not to extract any of the water layer
- Add 600 µL fresh iso-octane
- Homogenize for 10 s in the quickMIX module
- Remove and discard the upper iso-octane layer with the FAMEs
- Add 100 µL PBA solution to derivatize the analytes
- Add 600 µL iso-octane
- Homogenize for 1 min in the quickMIX module to extract the derivatives into the iso-octane layer
- Inject 2 µL of the iso-octane layer into the GC-MS/MS system

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*Instrumentation*

The analysis system used is shown in figure 2. The dual head MPS mounted on top of the GC-MS/MS performs all sample preparation steps. One head is equipped with a 10 µL syringe for sample introduction (left), the other one is equipped with a 1 mL syringe for sample preparation (right). From left to right the following modules are included:

A 10 mL wash station, tray holder for sample/extract vials, agitator, a quickMIX module (high power agitator), a high flow fast wash station, several 180 mL solvent reservoirs, and a cool-tray.

For online analysis an GC 8890 (Agilent Technologies) with GERSTEL Cold Injection System (CIS) inlet, cooled by a GERSTEL Universal Peltier Cooling (UPC) device, was used in combination with an 7000E triple quadrupole mass spectrometer (Agilent Technologies).

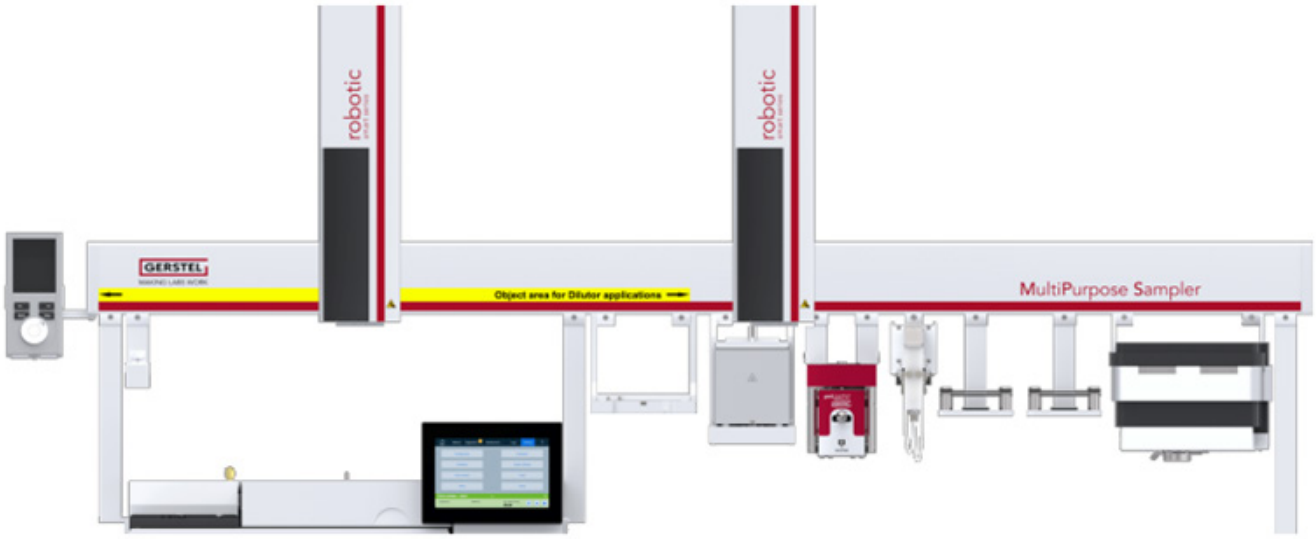


**Figure 2:** Analysis system used for automated determination of 3-/2-MCPD- and glycidol-esters from edible oils and fats according to ISO 18363-4. The MultiPurpose Sampler (MPS) mounted on top of the GC-MS/MS performs all sample preparation steps. The heads are equipped with a 10 µL syringe for sample injection (left) and a 1 mL syringe for sample preparation (right). From left to right the modules included are: 10 mL wash station, tray holder for sample/extract vials, agitator, a quickMIX module high power agitator, a high flow fast wash station, several 180 mL solvent reservoirs and cool-tray.

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*Analysis Conditions*

GC-MS/MS conditions were mainly set up as specified in the standard. Only a few parameters were further optimized or had to be adapted. The GC is equipped with a DB-5ms 10 m, 0.32 mm ID, 0.10 µm film pre-column connected to the analytical column, a DB-5ms Ultra Inert 20 m, 0.18 mm ID, 0.18 µm film using a purged ultimate union (all Agilent Technologies). Mid-column backflush is recommended to prevent excess PBA and FAMEs from entering and contaminating the analytical column and the mass spectrometer. In contrast to the standard method, in which a DB-5MS 10 m x 250 µm x 0.1 µm [Agilent 122-5511] pre column is mentioned, a 0.32 mm ID precolumn showed better performance in our setup.

*Inlet Conditions*

MPS

| | |
|---|---|
| Injection volume | 2 µL |

CIS 4

| | |
|---|---|
| Liner | baffled |
| Pneumatics mode | splitless |
| Splitflow | 100 mL/min @ 1.4 min |
| Temperature | 70 °C (1 min), 5 °C/sec to 165 °C (4.5 min), 5 °C/sec to 275 °C (5 min) |

*GC Agilent 8890 Parameters*

| | |
|---|---|
| Pre-column | 10 m DB-5ms (Agilent), $d_i$=0.25 mm, $d_f$=0.10 µm |
| Pneumatics | He; ramp flow 1.5 mL/min |
| Main column | 20 m DB-5ms ultra inert (Agilent), $d_i$=0.18 mm, $d_f$=0.18 µm |
| Pneumatics | He; constant flow 1.7 mL/min |
| Temperature | 70 °C (1 min), 15 °C/min to 120 °C (0.5 min), 40 °C/min to 275 °C (5 min) |
| Pneumatics | Midpoint Backflush start at 7.5 min with -6 mL/min |

*MS/MS Agilent 7010E Parameters*

| | |
|---|---|
| Source | High Efficiency Source |
| Ionization | Electron impact (EI) at 70 eV |
| Solvent delay | 6 min |
| Mode | Multiple Reaction Monitoring (MRM), for details see table 1 |
| Source temp. | 230 °C |
| Quad 1 temp. | 150 °C |
| Quad 2 temp. | 150 °C |
| Collision Gas Flow | $N_2$, 1.5 mL/min |
| Quench Gas Flow | He, 2.25 mL/min |
| Transfer line temp. | 275 °C |

**Table 1:** List of compounds with their mass spectral parameters.

| Analyte | ISTD | Precursor Ion [Da] | Product Ion [Da] | Purpose |
|---|---|---|---|---|
| 3-MCPD | | 196 | 147 | Quantification 3-MCPD |
| 3-MCPD | | 198 | 147 | Qualification 3-MCPD |
| 3-MCPD-$^{13}C_3$ | x | 199 | 149 | Quantification, ISTD 3-/2-MCPD |
| 3-MCPD-$^{13}C_3$ | x | 201 | 149 | Qualification, ISTD 3-/2-MCPD |
| 2-MCPD | | 196 | 104 | Quantification 2-MCPD |
| 2-MCPD | | 198 | 104 | Qualification 2-MCPD |
| 3-MBPD | | 240 | 147 | Quantification Gly |
| 3-MBPD | | 242 | 147 | Qualification Gly |
| 3-MBPD-$d_5$ | x | 245 | 150 | Quantification, ISTD Gly |
| 3-MBPD-$d_5$ | x | 247 | 150 | Qualification, ISTD Gly |
| 3-MBPD-$^{13}C_3$ | x | 243 | 149 | Quantification, correction Gly overestimation |

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## Results and Discussion

### *Sample Preparation Parameters*

During development of the automated sample preparation method, we adhered strictly to the volumes, times and other parameters listed in the ISO standard. The complete workflow was conveniently set up in the GERSTEL MAESTRO software. Each action was examined, and parameters, such as, for example, aspirating and dispensing speeds and penetration depths were optimized to achieve a fast, rugged, and repeatable workflow, as can be seen in figure 3.

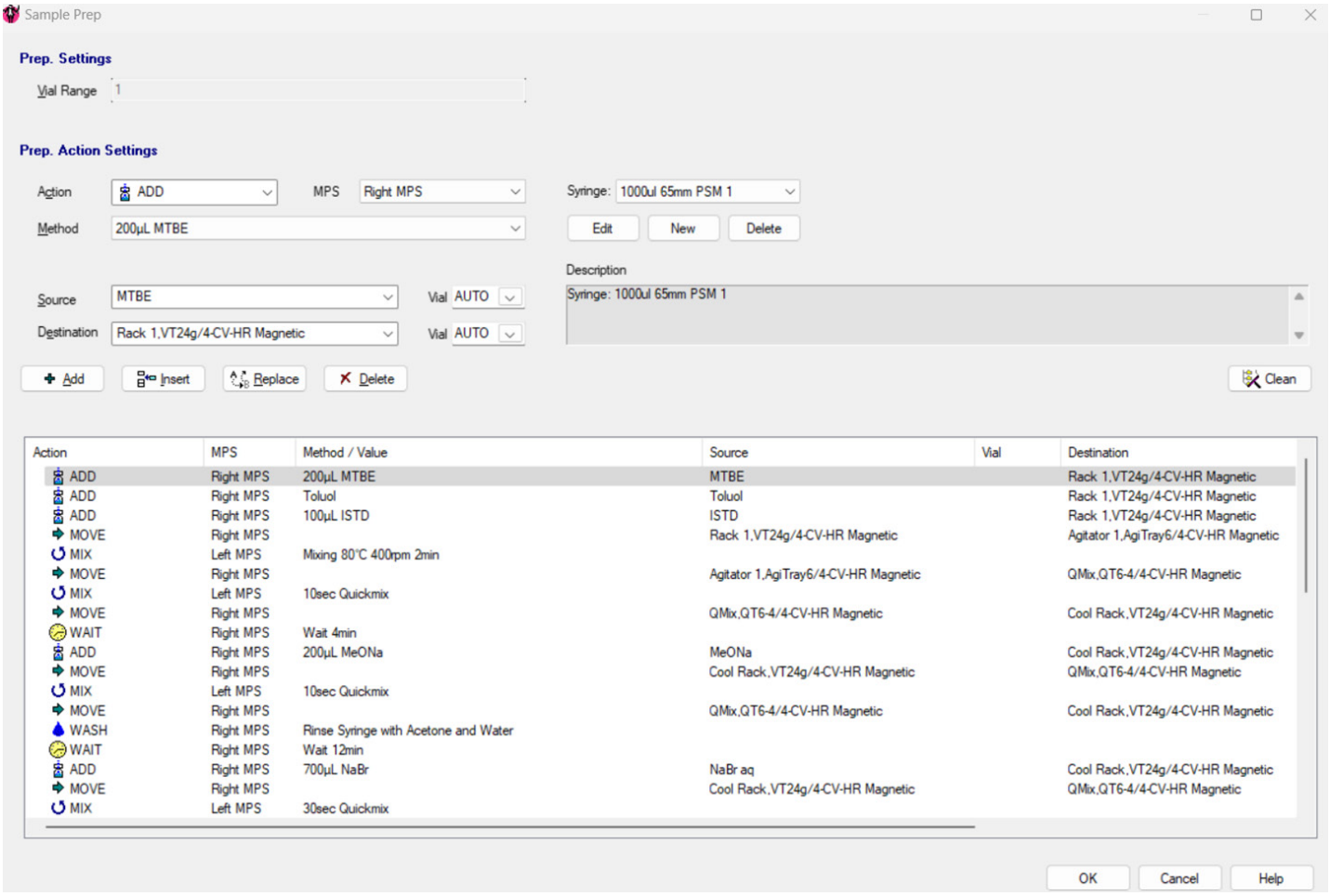


**Figure 3:** Excerpt of the MAESTRO sample preparation sequence.

Approximately 45 samples can be processed within 24 hours. A second tray holder is required for a sample throughput of 45 samples. With the MAESTRO Sequence Scheduler (figure 4), the wait times in sample preparation can be minimized, reducing the analysis time and maximizing throughput.

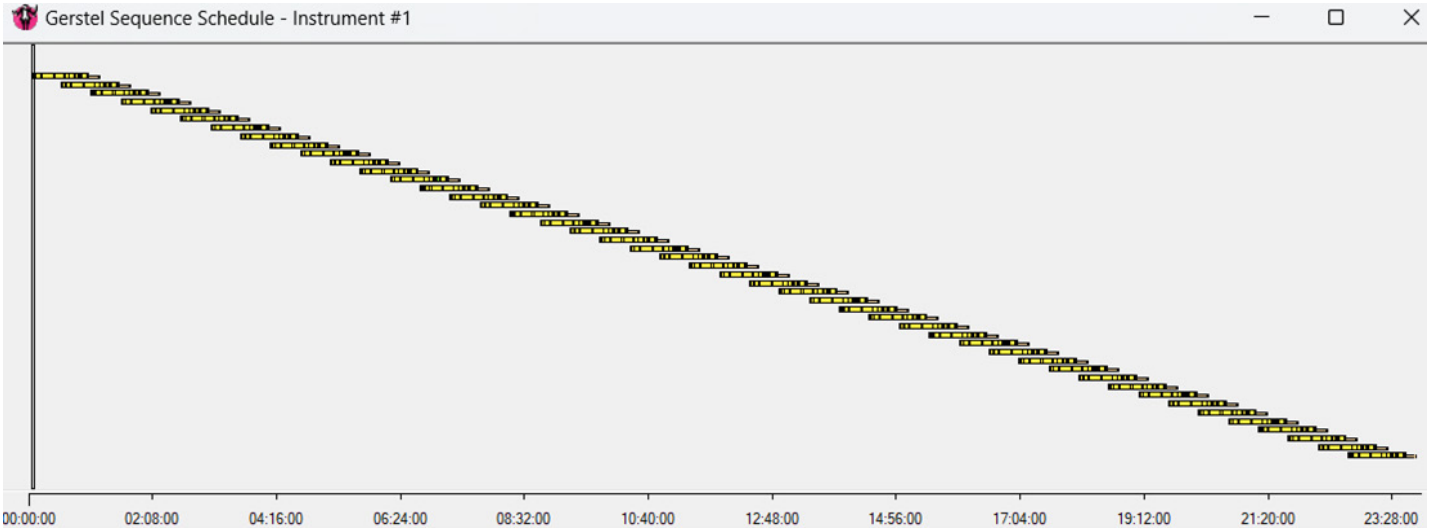


**Figure 4:** The MAESTRO Sequence Scheduler showing a total analysis time of 24 hours for 45 samples.

*Injection and Backflush Parameters*

IIn the lower calibration range of glycidol in combination with hot injection at 350 °C using a Split/Splitless inlet, deviations repeatedly occurred. To improve system performance, a GERSTEL CIS 4 inlet was installed. The CIS has several advantages. The injection takes place at 70 °C and the inlet is then heated to 165 °C in order to transfer the analytes to the separation column, while the PBA and matrix constituents are retained in the inlet and on the pre-column. These compounds are then backflushed while the CIS 4 is heated to 275 °C using a second ramp. The backflush system protects the separation column and the mass spectrometer from build-up of matrix compounds and derivatization reagent. The resulting improvement of the glycidol results is illustrated by four calibration curves, shown below, generated with a S/SL and a CIS configuration, respectively shown in figures 5 and 6. As can be seen, using a GERSTEL CIS 4 significantly improves reproducibility.

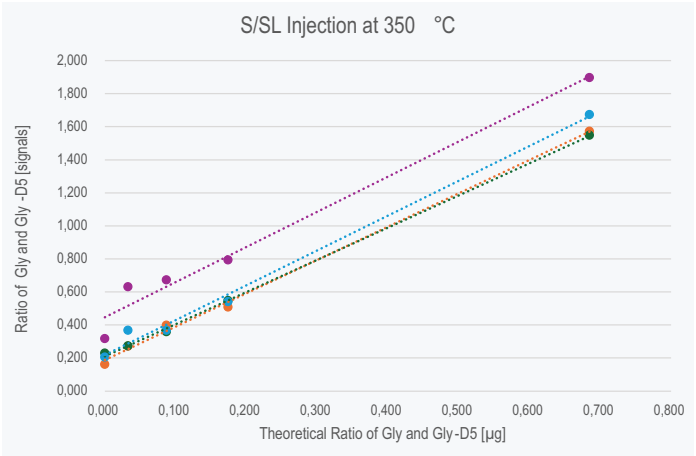


**Figure 5:** Four different calibration curves in the lower concentration range of 30 µg/kg to 630 µg/kg glycidol generated using a S/SL at 350 °C are shown. Deviations from linearity are clearly visible.

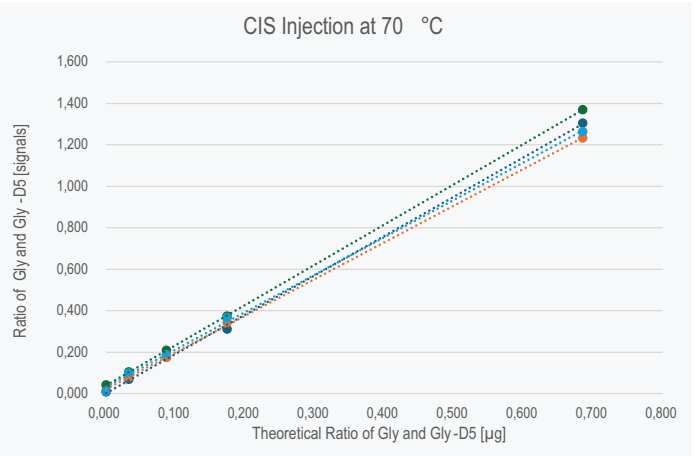


**Figure 6:** Four different calibration curves in the lower concentration range of 30 µg/kg to 630 µg/kg glycidol generated using a GERSTEL CIS 4. Excellent linearity and precision is achieved when using the CIS inlet at a starting temperature of 70 °C.

Apart from the inlet temperature, the splitless time was optimized to ensure a complete transfer of all analytes onto the analytical column, while preventing excessive transfer of PBA, FAMEs, or other matrix constituents to the analytical column. A splitless time of 1.4 min was found to be the optimum, in contrast with the 2 min mentioned in the ISO standard.

To implement pre-column backflush, a 2 m, 0.53 µm ID, 0.1 µm film pre-column was tested, as recommended in the standard. It was determined that this column diameter did not result in stable pressure conditions during the analytical GC run due to insufficient pre-column flow restriction. Very likely the backflush described in the ISO standard is an injector backflush only as described in the publications of Zwagerman and Overman [8,9]. This means that the pre-column is not backflushed and therefore its restriction is not relevant for the GC pneumatics. With a 10 m, 0.25 mm ID, 0.1 µm film pre-column reliable pressure conditions could be achieved, and pre-column backflush was successfully set up.

A midpoint backflush between the pre-column and the analytical column is more effective in protecting the analytical column and the mass spectrometer from unwanted matrix and PBA reagent. The time-point, at which all analytes had been transferred to the analytical column, was determined to be 7.5 min (see figure 7). A backflush activation time of 7.3 min was chosen to ensure reliable and complete analyte transfer to the analytical column, on the one hand, and minimal matrix transfer on the other.

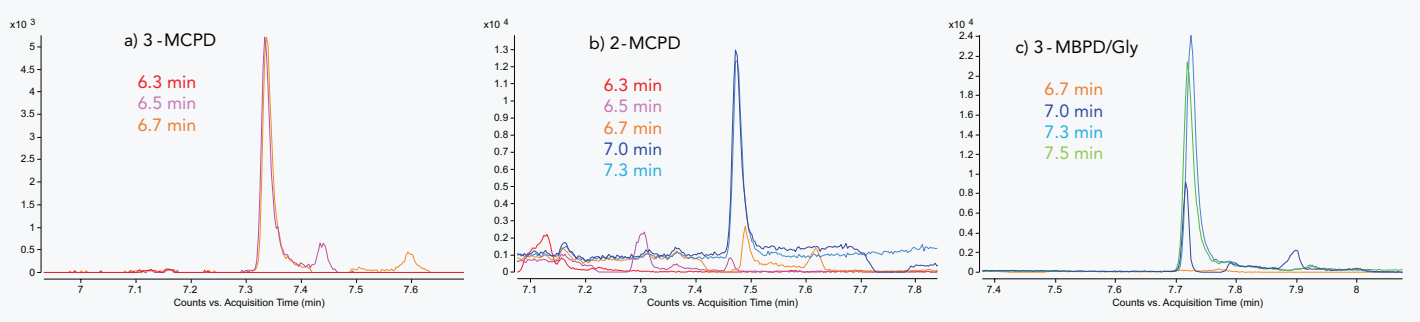


**Figure 7:** Optimization of backflush activation time. Starting the backflush at 7.3 min ensures that there is no loss of 3-MCPD (a) and 2-MCPD (b), and only minimal loss of 3-MBPD/Gly (c).

Figure 8 reveals the protective effect of pre-column backflush. Full scan chromatograms of an extracted chili oil sample with and without pre-column and inlet backflush were recorded. By using backflush, large amounts of matrix are prevented from entering the analytical column and the mass spectrometer, enabling extended maintenance intervals and ensuring overall method ruggedness, while significantly shortening the analysis time and improving throughput.

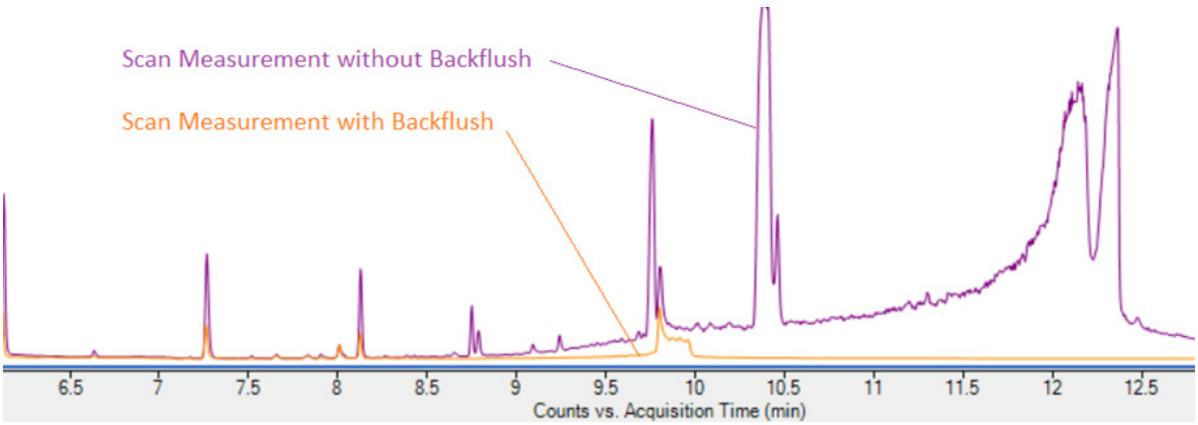


**Figure 8:** Full scan chromatograms of extracted chili oil sample with (orange) and without (purple) pre-column backflush illustrating the protective effect of pre-column and inlet backflush. Backflush prevents large amounts of matrix compounds and derivatization reagent from entering the analytical column and the mass spectrometer, enabling extended maintenance intervals, and ensuring overall method ruggedness. Backflush also significantly shortens the analysis time and improves throughput.

*Validation*

Figure 9 shows a typical chromatogram. All analytes are well separated while the deuterated internal standard elutes slightly before the respective analyte and the $^{13}C$-marked internal standards elute exactly with the native compound. No interfering matrix peaks are visible in the vicinity of the analyte or internal standard peaks. Due to the optimization of the analysis method regarding the change of the injector from S/SL to CIS and the associated adjustment of the inlet heating rate to be used, 3-MCPD elutes at a retention time of 7.7 min, 2-MCPD at 7.85 min and glycidol at 8.1 min.

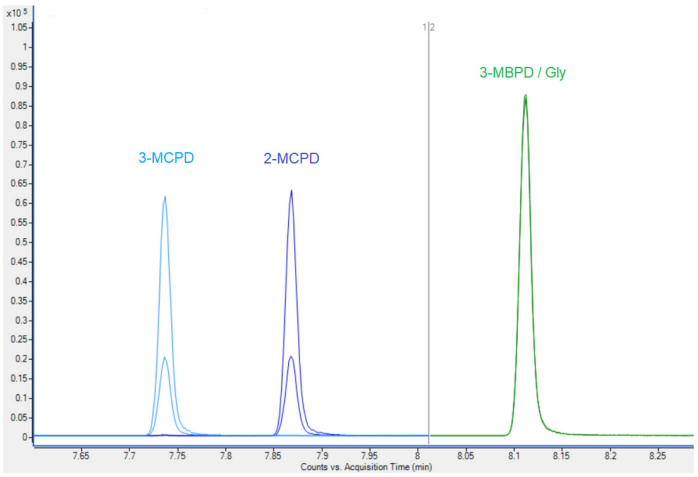


**Figure 9:** Representative chromatogram of a medium concentration calibration sample with 3-MCPD at 1050 µg/kg, 2-MCPD at 690 µg/kg and Gly at 1040 µg/kg.

Limits of quantification (LOQs) and limits of detection (LODs) were calculated according to DIN 32645 [10] employing a calibration based on from spiked blank oils with equially distantc ed points around the expected LOQ. Resulting LOQs and LODs are summarized in table 2. They were well below the 100 µg/kg level required by the ISO standard. Figure 10 shows representative chromatograms near the determined LOQs for all analytes. No interfering peaks are visible, and quantifier/qualifier ratios are adequate.

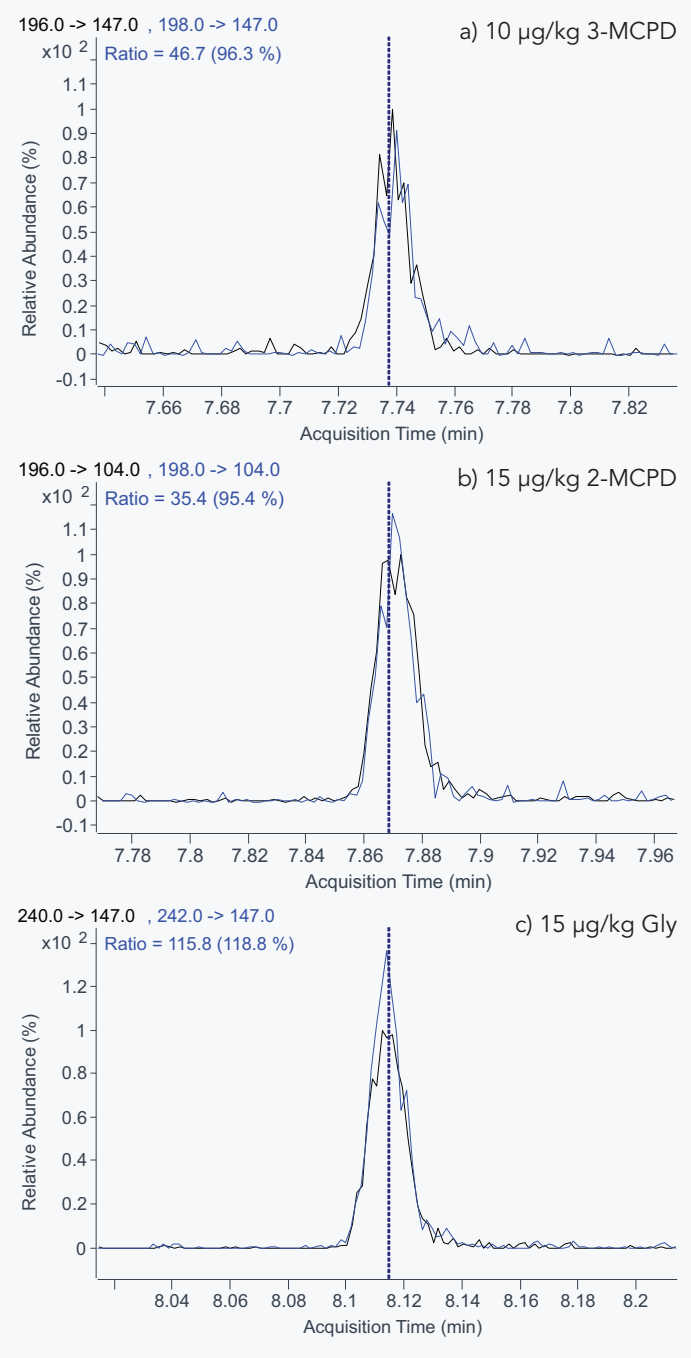


**Figure 10:** Typical chromatogram traces, quantifier and qualifier, for each compound near the respective limits of quantification. 3-MCPD a) at 10 µg/kg, 2-MCPD b) at 15 µg/kg and Gly c) at 15 µg/kg.

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Typical calibration curves are shown in figure 11. Coefficients of determination are larger than 0.999 for 3-MCPD and Gly and larger 0.99 for 2-MCPD.

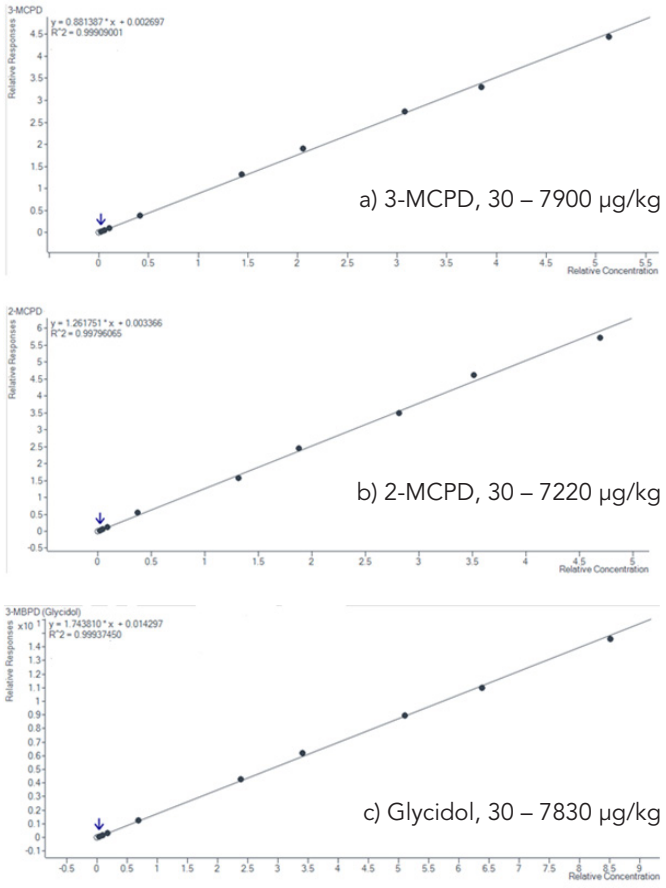


**Figure 11:** Typical calibration curves achieved for each compound: a) 3-MPCD, b) 2-MCPD, c) Gly.

In table 2 collected validation data for the automated analysis method based on using the GERSTEL MPS are listed. These were determined for low and high 3-MCPD values, especially concerning the resulting glycidol content. Additionally, table 3 contains precision and trueness values for spiked blank oil at high and low concentrations, as well as for vegetable oil.

**Table 2:** Collected validation data for the automated analysis method.

| Compound | LOQ [µg/kg] | Calibration range [µg/kg] | Coefficient of Determination $r^2$ |
|---|---|---|---|
| 3-MCPD | 8 | 5 - 7900 | 0.9993 |
| 2-MCPD | 14 | 5 - 7220 | 0.9984 |
| Gly | 13 * / 100** | 5 - 7830 | 0.9996 |

*low 3-MCPD values, ** high 3-MCPD values

**Table 3:** Repeat analyses for determination of precision and trueness were performed (n=6). Referred concentrations are: "Low" 55 µg/kg 3-MCPD, 51 µg/kg 2-MCPD, 55 µg/kg Gly; "High" 4740 µg/kg 3- MCPD, 4330 µg/kg 2-MCPD, 4700 µg/kg Gly; "vegetable oil" 1020 µg/kg 3-MCPD, 531 µg/kg 2-MCPD, 920 µg/kg Gly.

| Compound | Precision low RSD [%] | Trueness low [%] | Precision high RSD [%] | Trueness high [%] | Precision vegetable Oil RSD [%] | Trueness vegetable Oil [%] |
|---|---|---|---|---|---|---|
| 3-MCPD | 4.1 | 96 | 0.6 | 101 | 1.0 | 106 |
| 2-MCPD | 5.4 | 99 | 5.3 | 97 | 3.5 | 117 |
| Gly | 5.0 | 109 | 4.6 | 102 | 2.1 | 96 |

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*Application to Real Samples*

Using the developed method, no interfering matrix peaks were seen, and the sample preparation method worked reliably. Deviations in the lower calibration range for glycidol could be reproducibly corrected when using the GERSTEL CIS instead of a S/SL injector. The analyte concentrations were calculated according to chapters 9.2 and 9.3 of the standard method. The official analysis results of a round robin sample "Mix of refined and crude vegetable oils" were in agreement with our results, as shown in Table 4. "The "used frying oil" sample is a round robin test sample. The required analysis results for 2- and 3-MCPD were achieved without any problems. The stated analysis value of glycidol of 85 µg/kg was determined as 3-MCPD-induced glycidol with an analysis value of 83 µg/kg. In the ISO 18363-4 method, the glycidol induced from 3-MCPD is calculated and subtracted from the glycidol analysis value. If the 3-MCPD value is high and the glycidol value is low or not present in the sample, we recommend using a limit of quantification for glycidol of 100 µg/kg. For low 3-MCPD analysis values, a limit of quantification of 13 µg/kg can be used for glycidol (see Table 2). In addition, this table shows some other selected results for samples without a reference value. All samples from the supermarket were in compliance with EU regulations.

**Table 4:** Analysis results for real samples, analyzed in accordance with ISO 18363-4 using the developed automated workflow. Reference values are given wherever available.

| | 3-MCPD [µg/kg] | 3-MCPD reference value [µg/kg] | 2-MCPD [µg/kg] | 2-MCPD reference value [µg/kg] | Gly [µg/kg] | Gly reference value [µg/kg] | Comment |
|---|---|---|---|---|---|---|---|
| Mix of refined and crude vegetable oils | 67 | 75.8 +/- 34.3 | 12.3 | 28.8 +/- 16.6 | 114 | 115.6 +/- 35.0 | Proficiency test (Robin test sample) |
| Used frying oil | 633 | 601.2 +/- 68.0 | 257 | 266 +/- 55.2 | 23 | 85.1 +/- 58.1 | Proficiency test (Robin test sample) |
| Pasta oil | < LOQ | N/A | < LOQ | N/A | < LOQ | N/A | Purchased in supermarket |
| Clarified butter | < LOQ | N/A | < LOQ | N/A | < LOQ | N/A | Purchased in supermarket |
| Virgin olive oil | < LOQ | N/A | < LOQ | N/A | < LOQ | N/A | Purchased in supermarket |
| Lard | < LOQ | N/A | < LOQ | N/A | < LOQ | N/A | Purchased in supermarket |
| Linseed oil | < LOQ | N/A | < LOQ | N/A | < LOQ | N/A | Purchased in supermarket |
| Sunflower oil | < LOQ | N/A | < LOQ | N/A | 37 | N/A | Purchased in supermarket |
| Rapeseed oil | 50 | N/A | < LOQ | N/A | < LOQ | N/A | Purchased in supermarket |
| Frying oil | 23 | N/A | < LOQ | N/A | 137 | N/A | Purchased in supermarket |
| Fried, olive, sunflower oil | 20 | N/A | < LOQ | N/A | 78 | N/A | Purchased in supermarket |
| Walnut oil | 717 | N/A | 312 | N/A | 709 | N/A | Purchased in supermarket |
| Germ oil | 260 | N/A | 94 | N/A | 326 | N/A | Purchased in supermarket |
| Omega three fatty acids | 472 | N/A | 59 | N/A | 65 | N/A | Purchased in supermarket |

The comparison of the glycidol values achieved with the Zwagerman method to the glycidol values using the A or C method has to be treated with caution. It has to be kept in mind that during the sample preparation, glycidol is formed as a side product from 3-MCPD. Using the Zwagerman method, this 3-MCPD induced glycidol is then subtracted from the total glycidol value. The more 3-MCPD is present in the sample, the stronger the influence of the correction factor becomes.

## Conclusions

In this work, we have shown that the ISO 18363-4 method can be fully automated using a GERSTEL MultiPurpose Sampler MPS equipped with appropriate modules. The limits of quantification are well below 100 µg/kg as required by the method, the relative standard deviations achieved were mostly well below 5% and the accuracy mostly between 90 and 110%. The pre-column backflush device keeps the analytical column and the mass spectrometer clean, allowing longer maintenance intervals and ensuring the overall robustness of the method, while significantly shortening the analysis time and improving throughput.

## Acknowledgements

Thomas Brandsch and Dominik Lucas from GERSTEL GmbH & Co. KG are acknowledged for fruitful discussions during the project.

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