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Original scientific paper

Performance evaluation of the ISO 18363-4:2021 method for quantitative determination of chloropropanediols and glycidol in fats and oils by GC-MS/MS

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ABSTRACT

A new GC-MS/MS method from the ISO 18363 series of standards for determination of fatty acid bound chloropropanediols and glycidol was assessed for its performance characteristics. This method enables indirect simultaneous determination of free 2-monochloropropane-1,3-diol (2-MCPD), 3-monochloropropane-1,2-diol (3-MCPD) and glycidol in a single sample preparation and analysis protocol. Results of the validation study show satisfactory performance characteristics, well within required limits. Recently held proficiency testing also showed the adequate performance of the laboratories employing this method. Taking into consideration the simpler sample preparation and stringent quality control inherent to this protocol, wider use of ISO 18363:4:2021 for routine applications can be expected in the foreseeable future.

1. Introduction

2-monochloropropane-1,3-diol (2-MCPD), 3-monochloropropane-1,2-diol (3-MCPD) and glycidol (GLY) belong to the chemically diverse group of processing contaminants, i.e. compounds that can be found in foods either through direct introduction into the food processing at some technological stage, or due to the chemical changes in natural food ingredients during, e.g., thermal treatment of food. The latter represents the formation mechanism of 2-MCPD, 3-MCPD and GLY in plant and animal-originating fats and oils, after they are exposed to prolonged high temperatures for refining purposes. These compounds are present in foods predominantly chemically bound to fatty acids (MCPD and glycidyl esters). However, upon entering the gastrointestinal tract, they readily hydrolyse into free compounds due to gut lipase activity (*Beekmann et al*, 2022). MCPD and GLY content in processed food is dependent on temperature, time and type of oil/fat; plant-originating fats have higher concentrations. Ubiquitous palm oil and fats are especially susceptible to MCPD/GLY synthesis, and having in mind their widespread use in numerous processed food commodities, they are among the most significant contributors to human exposure (*EFSA*, 2018).

From the toxicological perspective, MCPD/GLY gained significant attention in the last decade; the Joint FAO/WHO Expert Committee on Food Additives in 2016 (*JECFA*, 2017) established a provisional group maximum TDI of 4 µg/kg b.w.

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for 3-MCPD. The European Food Safety Authority established a tolerable daily intake (TDI) of 2.0 µg/kg b.w. per day for 3-MCPD and its esters, attributing potential renal toxicity to 3-MCPD. Furthermore, 3-MCPD is listed as a threshold genotoxic carcinogen (*EFSA*, 2016, 2018). GLY was classified as probably carcinogenic to humans, so in Group 2A according to the International Agency for Research on Cancer, suggesting ALARA (as low as reasonably achievable) as a risk management strategy (IARC, 2000). Both 3-MCPD and GLY are considered especially harmful for infants having in mind high exposures due to their low body weight. Full assessment of 2-MCPD is not currently possible due to insufficient data availability.

As a result, maximum levels (MLs) for 3-MCPD and GLY were set by the European Union *(EU*, 2023) ranging from as low as 6 µg/kg of GLY for liquid baby food to 2500 µg/kg of 3-MCPD for animal and vegetable fats and oils. These MLs are set for the sum of 3-MCPD and its esters, i.e. GLY and glycidyl esters expressed as 3-MCPD and GLY respectively.

Available analytical methods are numerous and can be divided into direct and indirect categories. Direct determination is based on detection and quantification of MCPD and glycidyl esters in the sample. However, according to *Zheng et al.* (2021), such protocols can lead to significant underestimation due to the limited availability of esters sold as commercial analytical standards. Indirect determination after transesterification and analysis of the main compounds (2-MCPD, 3-MCPD, GLY) is considered more suitable, since the entire quantity in the sample, regardless of where the ester originated from, is accounted for. Another difficulty is related to the pronounced instability of GLY after transesterification; this is alleviated by immediate derivatization step with sodium bromide, transforming GLY into 3-monobromopropanediol (3-MBPD).

The American Oil Chemists' Society (AOCS) has developed several methods for indirect determination of MCPD and GLY (*AOCS*, 2017a; *AOCS*, 2017b; *AOCS*, 2017c), adopted eventually by International Organization for Standardization (ISO) and integrated into the ISO 18363 series of standards (*ISO*, 2021). These methods, all based on gas chromatography with mass selective detection, differ by ester cleavage mechanisms (fast or slow transesterification), and quantitative determination of GLY. However, they all require division of each sample into two portions (for separate determination of MCPD and GLY), which doubles the time and resources necessary for measurement.

In 2021, the ISO 18363 family of standards was expanded with a new indirect analytical method for 2-MCPD, 3-MCPD and GLY determination in one unified preparation protocol and a single chromatographic run (*ISO*, 2021). This method employs fast alkaline cleavage of esters that is facilitated by sodium methoxide, immediate conversion of released GLY into 3-MBPD and subsequent derivatization of all three compounds with phenylboronic acid in order to make them suitable for GC analysis. Unlike previous methods that utilize GC/MS for measurement, ISO 18363-4 requires GC-MS/MS due to the specific quantification technique that has to be applied in order to accurately measure GLY concentration. Namely, during cleavage of esters in alkaline conditions, a certain amount of 3-MCPD is converted to GLY, leading to overestimation of GLY content (conversion of 2-MCPD is existent but negligible, hence not taken into account). In order to quantify the build-up of 3-MCPD-induced GLY, a specific internal standard ($^{13}C_{12}$ 3-MCPD) is used. The purpose of using isotopically labelled 3-MCPD (besides accurate quantification of ester-bound 3-MCPD) is to monitor formation of isotopically labelled GLY during transesterification (derivatized at a later stage to $^{13}C_{12}$ 3-MBPD). Using its concentration as a single calibration point, it is possible to quantify the amount of GLY originating from MCPD and subtracting this amount from glycidyl ester induced GLY compensates for the overestimation of GLY content. Quantification of 2-MCPD is performed using $^{13}C_{12}$ 3-MCPD as the internal standard as well, while GLY is quantified with its own labelled analogue (D5-GLY).

The aims of this study were to assess performance characteristics of the unmodified ISO 18363-4:2021 (*ISO*, 2021) method for quantitative determination of MCPD and glycidyl esters in fats and oils and to compare the results obtained with the data from the ISO collaborative study provided in the Standard, as well as with the performance criteria provided in the same document. For this purpose, authors utilised results from an in-house validation study. Additional confirmation of the method's fitness for purpose was judged by analysing the results of a proficiency test (PT) conducted in December of 2022. Having in mind that ISO 18363-4:2021 is a new method, the authors believe that assessment of the method's performance can encourage its wider

adoption, bearing in mind it's clear advantages over older and more established laboratory protocols.

2. Materials and methods

2.0.1. Reagents, standards and instrumentation

Methanol, isooctane, acetone, toluene, t-butyl methyl ether (TBME), sulphuric acid, sodium methoxide, sodium bromide were of p.a. quality. Analytical standards (1,3-distearoyl-2-chloropropanediol; 1,2-dipalmitoyl-3-chloropropanediol; glycidyl stearate; $^{13}C_{12}$ 1,2- dipalmitoyl-3-chloropropanediol and; glycidyl stearate-d5) were purchased from Toronto Research Chemicals (North York, ON, Canada). Blank matrix used for fortification was extra virgin olive oil purchased in a local supermarket and previously analysed for the absence of investigated analytes.

Analysis was performed on a Shimadzu GC-MS/MS system (Kyoto, Japan) consisting of GC 2030 oven, SPL 2030 split/splitless injector, AOC 20s auto sampler, AOC 20i auto injector and GCMS-TQ8050 NX triple quadrupole mass spectrometer using EI ionisation and operating in MRM mode. Separation was performed on Shimadzu SH-Rxi 5ms analytical column (30m × 0.25mm × 0.25 µm) equipped with a 1.5 m fused silica pre-column.

2.0.2. Analytical method

ISO (2021) provides a detailed analytical protocol for sample preparation and instrumental parameters. Briefly, 100 mg of fat or oil was weighted into a screw-capped glass vessel. After addition of internal standards, toluene and TBME, the mixtures were heated at 80°C. Vials were then cooled to 10°C prior to transesterification with sodium methoxide followed by vortex assisted homogenization and incubation at 10°C. The reaction was stopped by adding acidic sodium bromide. This also prevents decomposition of GLY, resulting in conversion to stable 3-MBPD. Removal of fatty acids and other co-extractives was accomplished by multiple liquid-liquid extractions with isooctane. After discarding the organic layer, phenylboronic acid was added for derivatization followed by another isooctane extraction. Non-polar derivatives are contained within organic layer, and so the isooctane fraction was transferred to GC auto sampler vial prior to chromatography.

The chromatographic program had the following parameters: splitless injection mode at 350°C, initial oven temperature 70°C, hold 1 min, ramp 15°C/min to 120°C, final ramp 40°C/min to 350°C, hold 2.5 min. The mass spectrometer operated in EI+ mode, and ion source and transfer line temperatures were 290°C and 315°C respectively. The following MRM transitions were monitored: 3-MCPD 196 > 147, 198 > 147; 2-MCPD 196 > 104, 198 > 104; 3-MBPD 240 > 147, 242 > 147; $^{13}C_3$ 3-MCPD: 199 > 149, 201 > 149; $^{13}C_3$ 3-MBPD: 243 > 149' D_5 3-MBPD 245 > 150, 247 > 150.

3. Results and discussion

3.1.1 Method validation

This validation study was conducted according to the principles provided in the IUPAC (International Union for Pure and Applied Chemistry) harmonised protocol (*Thompson et al*., 2002). A 10-point calibration was performed (0.003–0.790 mg/kg of oil), during a three-day experiment for linearity assessment. Accuracy and precision were determined by analysing six replicates of three certified reference materials (CRM), vegetable oils, one of which (T2672) was also previously used in the proficiency test, and all of which were obtained from the Food Analysis Performance Assessment Scheme (FAPAS T2664, T2669 and T2672). Table 1 shows certified values of these reference materials with the ranges of acceptable values for z-score < 2.

Intralaboratory reproducibility was expressed as RSDr and was determined in the same experiment used for bias assessment, since certified values of

Table 1. Certified values of MCPD/GLY reference materials

	T2664 (µg/kg)	T2669 (µg/kg)	T2672-PT (µg/kg)
3-MCPD esters	1285 (889–1681)	431 (275–588)	174 (101–246)
GLY esters	448 (286–610)	61.4 (34.4–88.4)	99.9 (56.0–143.9)
2-MCPD esters	651 (429–873)	176 (103–249)	62.0 (34.7–89.3)

the three CRMs were within the range of required MLs and encompassed both lower and higher concentrations for each compound. Limit of quantification (LoQ) was determined by analysing six blanks and determining S/N ratio at the retention times of interest.

3.1.2 Validation parameters

Table 2 summarizes the results of the single-laboratory validation study. Requirements specified in ISO 18363-4:2021 are provided for comparison.

The results show satisfactory degrees of accuracy and precision according to the requirements of the ISO and established criteria for analytical method performances. Between-day reproducibility was noticeably higher for GLY compared to 2-MCPD and 3-MCPD, which is in accordance with the requirements and can be explained by the unpredictability of the conversion of released 3-MCPD into 3-MBPD. Although this process is accounted for, through monitoring of the isotope-labelled 3-MCPD conversion, much attention should be paid to the calculation of results and analysis of at least one CRM in each sample batch, which is advisable for confirmation. Bearing in mind the stability of the esters and low sample weight, a single CRM can be used for a considerable time, significantly contributing to the quality of the results. The duration of transesterification and overall timing in sample preparation steps should be as precise and consistent as possible, since they are the key factors determining method performance.

3.1.3. Proficiency test analysis

A proficiency test for determination of MCPD/GLY esters in vegetable oil was conducted in October-December 2022. According to the PT report (FAPAS, PT 2672), only 4 out of 87 participating laboratories applied the ISO 18363-4:2021 method (4.6%). Such a low figure is expected, since the several older methods are very well established. However, the performance of this new method was satisfactory; according to the PT report, all four laboratories employing ISO 18363-4 performed adequately, with z-scores ranging from –0.4 to +0.9 for 3-MCPD, from –0.9 to +1.2 for GLY and from –0.6 to +1.6 for 2-MCPD. Considering the relatively wide distribution of acceptable results (Table 1) and overall overestimation of all compounds seen in the distribution of reported results, especially in the case of GLY, this new method clearly outperforms the average reported results.

4. Conclusion

ISO 18363-4:2021 (*IOS,* 2021) is a new analytical method for determination of MCPD/glycidyl esters in fats and oils, and based on validation results, demonstrates satisfactory performance.

Table 2. Results of the single-laboratory validation study

	3-MCPD esters expressed as free 3-MCPD	Glycidyl esters expressed as free GLY	2-MCPD esters expressed as free 2-MCPD	ISO 18363-4 requirements (*ISO*, 2021)
Calibration curve slope	0.995	1.005	0.999	3-MCPD: 0.95–1.05 GLY: 1–1.25 2-MCPD: 0.8–1
Repeatability (RSD, %)	4.6	5.1	2.8	3-MCPD: 9 GLY: 11 2-MCPD: 12
Between-day reproducibility (RSDr, %)	9.3	19.1	12.5	3-MCPD: 15 GLY: 38 2-MCPD: 27
Recovery (%)	95.8	91.3	102.2	80–120
LoQ (µg/kg)	20	18	21	100
MU (%)	20.4	38.4	25.4	n/a

Moreover, this new method also offers significant advantages regarding sample preparation time and resources, with an elaborate quantification scheme and stringent quality control due to the use of two internal standards and the GC-MS/MS technique. Having in mind all this, much wider use of ISO 18363-4:2021 in routine applications can be expected in the foreseeable future.

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