

# Certification of the mass fraction of 3- and 2-MCPD fatty acid esters in infant formula
ERM®-BD087

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JRC REFERENCE MATERIALS REPORT

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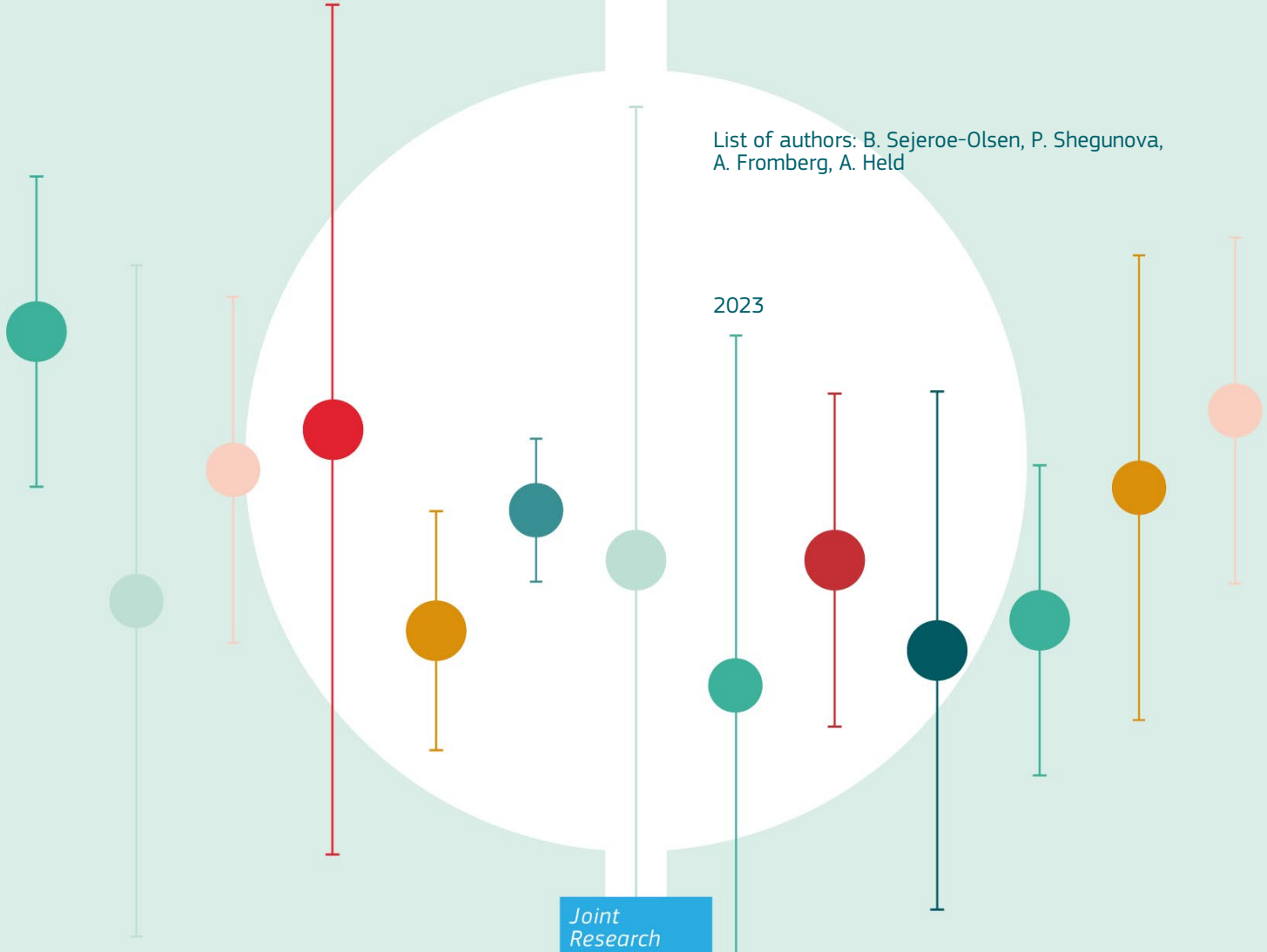
2023



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# Contents

## Abstract

ERM-BD087 is an infant formula reference material certified for the mass fraction of the fatty acid esters of 3-chloro-1,2-propanediol (3-MCPD fatty acid ester also called bound 3-MCPD) and the sum of 3-chloro-1,2-propanediol (free 3-MCPD) and bound 3-MCPD, the fatty acid esters of 2-chloro-1,3-propanediol (2-MCPD fatty acid ester also called bound 2-MCPD) and the sum of 2-chloro-1,3-propanediol (free 2-MCPD) and bound 2-MCPD. Glycidyl fatty acid esters (GEs), are also present in the material. However, no mass fraction value was assigned to this compound due to indications of instability. ERM-BD087 is produced within the scope of ISO 17034:2016 [1] and in accordance with ISO Guide 35:2017 [2].

Infant formula containing 3-MCPD fatty acid esters, 2-MCPD fatty acid esters and glycidyl fatty acid esters was produced, using a contaminated coconut oil in the recipe. ERM-BD087 is available in glass vials containing at least 30 g of spray-dried infant formula powder, which were sealed under an atmosphere of nitrogen.

Between-unit homogeneity was quantified and stability during transport and storage was assessed in accordance with ISO Guide 35:2017 [2]. The minimum sample size for one sample preparation is 0.4 g.

The material was characterised by an inter-laboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017 [3]. Technically invalid results were removed but no outlier was eliminated since no technical reason for deviations was found.

Uncertainties of the certified values were calculated in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is intended for the quality control and assessment of method performance.

Before release of the CRM, the certification project was subjected to peer-review involving both internal and external experts.

## Acknowledgements

The authors would like to acknowledge the support received from colleagues of JRC for the processing, organising of stability studies, measuring, reviewing of the certification project and distribution of this CRM.

Furthermore, the authors would like to thank the experts of the Reference Material Review Panel Tuija Pihlström (Swedish Food Agency, SE) and Katrin Vorkamp (Aarhus University, DK) for their constructive comments and the external review of the certification report and certificate.

***Authors***

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[2)] European Union Reference Laboratory for Processing Contaminants (EURL-PC), DTU, Kgs. Lyngby, DK

# 1 Introduction

## 1.1. Background

Monochloropropanediol (MCPD), its fatty acid esters and glycidyl fatty acid esters are food processing contaminants formed via different mechanisms during the thermal refinement process of vegetable and fish oils [4, 5, 6, 7, 8, 9]. Depending on the position of chlorine on the propane backbone, i.e. 2-MCPD or 3-MCPD, corresponding 2- and 3-MCPD fatty acid esters are formed. MCPD fatty acid esters derive from mono-, di-, and triglycerides in the presence of a chlorine donor [9]. Glycidyl fatty acid esters are mainly formed from mono- and di-glycerides via intramolecular rearrangements [10]. These substances can be present in refined vegetable oils such as palm oil, coconut oil or pomace olive oil, formed at the high temperature utilised in oil deodorisation processes [11, 12]. The chemical structures can be found in Annex 1.

Infant formula is used as an alternative to breast milk. Producers aim to prepare recipes with fat composition similar to that of breast milk. To achieve this, producers normally use vegetable oils, and as a consequence, infants has a risk to be exposed to MCPD fatty acid esters and glycidyl fatty acid esters through the infant formula consumption [13]. MCPD fatty acid esters and glycidyl fatty acid esters are hydrolysed in the human gastrointestinal tract into their corresponding free form MCPD and glycidol. According to the International Agency for Research on Cancer (IARC) [14, 15], 3-MCPD and glycidol are possibly and probably carcinogenic to humans, respectively, and this potential exposure is therefore of high concern.

Due to this concern, the Commission Regulation (EU) 2023/915 [16] has set maximum levels for 3-MCPD, 3-MCPD fatty acid esters and glycidyl fatty acid esters in foodstuffs and food ingredients. The maximum levels of 3-MCPD and 3-MCPD fatty acid esters are regulated as the sum of the compounds expressed as 3-MCPD. The same definition is used in this report for the sum.

For processed food such as vegetable oils, fish oils and oil from marine organisms the maximum levels are 1250 µg/kg and 2500 µg/kg for the sum of 3-MCPD and its fatty acid esters, expressed as 3-MCPD, depending on the type of oil [16]. For glycidyl fatty acid esters, the corresponding maximum levels are 500 µg/kg and and 1000 µg/kg, depending on the type of oil. The regulation stipulates a lower maximum level for infant formula, follow-up formula and food for special medical purposes intended for infants and young children. For the sum of 3-MCPD and its fatty acid esters, these maximum levels are 125 µg/kg and 15 µg/kg for powders and liquids, respectively. For glycidyl fatty acid esters the equivalent maximum levels are 50 µg/kg and 6.0 µg/kg for powders and liquids, respectively.

Different analytical approaches have been developed for the analysis of MCPD fatty acid esters and glycidyl fatty acid esters including direct methods by LC-MS/MS and indirect methods converting MCPD fatty acid esters and glydicyl esters into their free forms (MCPD and glycidol) determined using GC-MS(/MS). Regulation (EU) 333/2007 [17] lays down the methods of sampling and the minimum performance requirements for the analysis of 3-MCPD, 3-MCPD fatty acid esters and glydicyl fatty acid esters in foodstuffs. The regulation does not specify the analytical method, but states minimum performance requirements including for example requirements for recovery and limit of quantification (LOQ). The recovery performance criteria for powdered infant formula is 70-125% for 3-MCPD fatty acid esters and glydicyl fatty acid esters and the LOQ minimum performance requirements for powdered infant formula for 3-MCPD fatty acid esters is 50 µg/kg and for glycidyl fatty acid esters 20 µg/kg for samples with a fat content below 65%

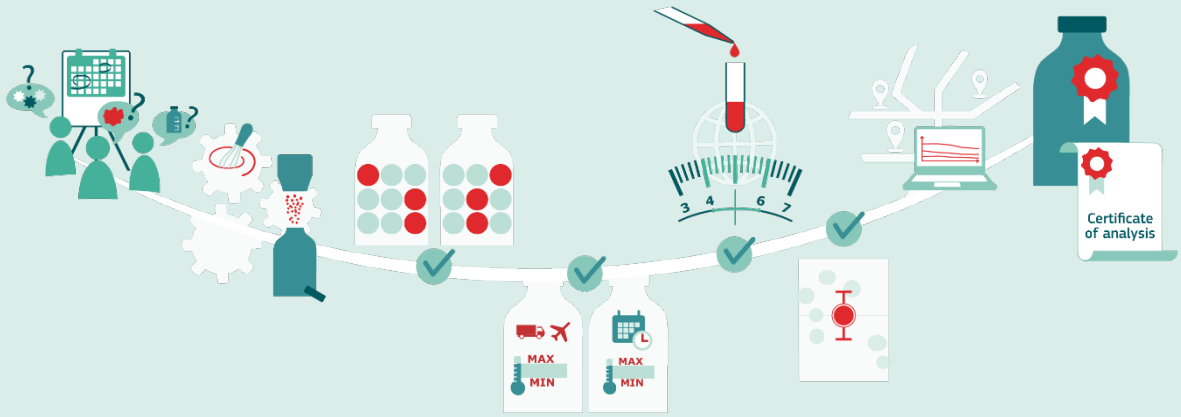
## 1.2. Choice of the material

The material was chosen for the purpose of being a quality assurance tool for the analytical performance in the routine laboratory. The material was produced using coconut oil containing MCPD fatty acid esters and glycidyl fatty acid esters following a recipe of the infant formula in compliance with Commission Directive 2006/141/EC [18] and Regulation (EU) 2016/127 [19].

The concentration level of the bound esters was chosen to be below, but close to the maximum allowed levels in infant formula. The content of free 3- and 2-MCPD is below LOQ of all analytical methods used, which are all indirect. The levels of the sum of 3-MCPD and its esters, as described in the EU Regulation 2023/915 [16], refer to lower bound concentrations, which are calculated on the assumption that all values below the LOQ are zero. Although not covered by the regulation, the sum of 2-MCPD and its esters is calculated in the same way.

## 1.3. Outline of the CRM project

**Box 1.** Reference material production




Reference material (RM) production is defined in ISO 17034 [1] as a project comprising planning and processing of the material, followed by homogeneity and stability testing, characterisation and assigning of one or more property values. Depending on the intended use of the RM a commutability study is carried out.

For certified reference materials (CRMs) a certificate is issued while for RMs a product information sheet is issued by the reference material producer (RMP).

CRMs and RMs are distributed globally and the stability of their assigned values is monitored throughout the life-time of the material.

Infant formula powder containing MCPD fatty acid esters and glydicyl fatty acid esters was produced by NIZO, NL. The recipe was developed in compliance with the EC regulations [18, 19] regarding the recommendations for the content of macronutrients (protein (and casein / whey ratio), carbohydrate, fat) and macro-mineral (calcium, magnesium, potassium, sodium, phosphorus). To reach the target levels of 3-MCPD fatty acid esters, 2-MCPD fatty acid esters and glycidyl fatty acid esters, contaminated coconut oil was chosen. The ingredients were dissolved in water and homogenised followed by pasteurisation and spray drying.

Homogeneity and stability testing was performed using validated and accredited analytical methods based on GC-MS/MS. As the content of glydicyl fatty acid esters showed signs of instability for a period of 24 months, the analytical results for these compounds are excluded from this report apart from the 24-month stability testing indicating the instability.

The characterisation was based on an inter-laboratory comparison between expert laboratories. The participants were requested to apply their own validated analytical method for the determination of MCPD, MCPD fatty acid esters and glycidyl fatty acid esters in infant formula, including both 2- and 3-MDCP and their esters.

The uncertainties of the certified values were estimated in compliance with ISO 17034:2016 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [20].

The CRM project, including the certification approach and the evaluation of the obtained measurement data, was subjected to peer-review involving both internal and external experts.

Certain commercial equipment, instruments, and materials are identified in this report to adequately describe the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

# 2 Participants

## 2.1 Project management and data evaluation

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM).

## 2.2 Processing

NIZO food research B.V., Ede, NL

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

## 2.3 Homogeneity measurements

SGS Germany GmbH, Hamburg, DE
(measurements under the scope of ISO/IEC 17025:2017 accreditation DAkkS No. D-PL-11020-04-01)

## 2.4 Stability measurements

AGROLAB Dr, Verwey B.V., Barendrecht, NL
(measurements under the scope of ISO/IEC 17025:2017 accreditation RvA No. L234)

SGS Germany GmbH, Hamburg, DE
(measurements under the scope of ISO/IEC 17025:2017 accreditation DAkkS No. D-PL-11020-04-01)

## 2.5 Characterisation measurements

Agència de Salut Pública de Barcelona, Barcelona, ES
(measurements under the scope of ISO/IEC 17025:2017 accreditation ENAC No. 227/LE459, 227/LE1338)

AGROLAB Dr, Verwey B.V., Barendrecht, NL
(measurements under the scope of ISO/IEC 17025:2017 accreditation RvA No. L234)

Danmarks Tekniske Universitet Fødevareinstituttet, Kgs. Lyngby, DK
(measurements under the scope of ISO/IEC 17025:2017 accreditation DANAK No. 350)

Dublin Public Analyst's Laboratory, Dublin, IE
(measurements under the scope of ISO/IEC 17025:2017 accreditation INAB No. 99T)

EUROFINS Lab Zeeuws Vlaanderen (LZV) B.V., Graauw, NL
(measurements under the scope of ISO/IEC 17025:2017 accreditation RvA No. L201)

EUROFINS WEJ Contaminants GmbH, Hamburg, DE
(measurements under the scope of ISO/IEC 17025:2017 accreditation DAkkS No. D-PL-14602-01-00)

I.A.P.R General Chemical State Laboratory, Athens, GR

Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Linz, AT
(measurements under the scope of ISO/IEC 17025:2017 accreditation AA No. 0452)

Prüfinstitut Chemische Analytik GmbH, Berlin, DE

Service Commun des Laboratoires, Massy, FR
(measurements under the scope of ISO/IEC 17025:2017 accreditation Cofrac No. 1-0162)

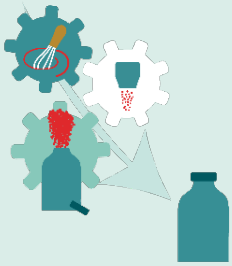
SGS Germany GmbH, Hamburg, DE
(measurements under the scope of ISO/IEC 17025:2017 accreditation DAkkS No. D-PL-11020-04-01)

TLR International Laboratories BV, Ridderkerk, NL

All datasets are identified by a code (e.g. D01). The numbering is not related to the order of the laboratories presented above.

# 3 Material processing and processing control

**Box 2.** Reference material processing



RM processing covers the raw material conversion into a homogenous and stable material. It typically includes processing steps such as grinding or sieving and drying steps to enhance stability. When the processed material fulfils the specifications, the final material is filled into individual containers, referred to as RM units, such as bottles or ampoules and is labelled.

## 3.1 Origin of the starting material

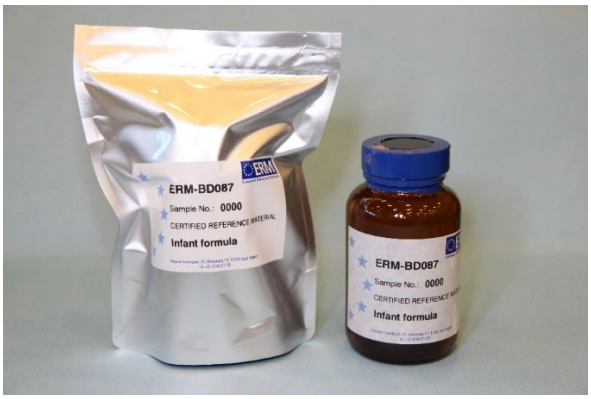
The infant formula was produced by NIZO, NL. The material was produced by dissolving the ingredients in water, adding minerals and adjusting the pH. After homogenisation, the infant formula was pasteurised and spray dried. The microbiological activity was measured as well as moisture content and content of 3- and 2-MCPD fatty acid esters and glycidyl fatty acid esters. The moisture content (1.5 %) and microbiological quality (*Enterobactericeae* and *B. cereus* <10 CFU/g, TPC 83 CFU/g) were found acceptable for infant formula powder. The material was packed in six paper bags lined with polyethylene (PE). In total approximately 60 kg of infant formula were produced for further processing at JRC-Geel.

## 3.2 Processing

After reception at JRC-Geel, the material was filled in a stainless steel drum and mixed using a Dynamix CM200 mixer (WAB, Basel, CH) for one hour. Next, the powder was filled in 100 mL bottles using a gravimetric doser (MCPI, Meythet, FR). The machine was placed in a glovebox flushed with nitrogen to avoid uptake of moisture. The amount filled per bottle was >30 g which was recorded using the balance of the gravimetric doser system. An antistatic blower was used to ensure correct weighing. After closing the bottles with a PE insert and screwcap they were removed from the glovebox. A crimp-film was placed over the cap to firmly keep it in place. The bottles were labelled and placed in a sachet which was also labelled and thermally sealed.

For the purpose of this report, the term 'unit' refers to one bottle of the ERM-BD087 material. One unit of ERM-BD087 is shown in Figure 1.

**Figure 1.** ERM-BD087. Final containment in sachet (left) containing a 100-ml glass bottle (right).

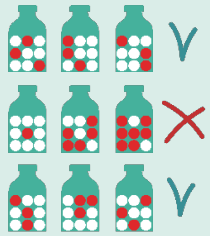



## 3.3 Processing control

The water content was measured by Karl-Fisher titration before filling in bottles. It was found to be 1.58 ± 0.09 %.

# 4 Homogeneity

**Box 3.** Homogeneity assessment



A key requirement for any RM produced as a batch of units is equivalence between those units. It is important to know how much the variation between units contributes to the uncertainty of the certified value. Consequently, ISO 17034:2016 [1] requires RMPs to quantify the between-unit variation in homogeneity studies.

The within-unit homogeneity is correlated to the minimum sample size, which is the minimum amount of sample that is, for a given measurand, representative of the whole unit and that should be used in an analysis. Using sample intakes equal to or above the minimum sample size guarantees the assigned value within its stated uncertainty.

The within-unit inhomogeneity does not influence the uncertainty of the certified value if the minimum sample size is respected, but determines the minimum size of sample that is representative for the whole unit. For this material the minimum sample size has been derived from the sample intakes used in the characterisation study.

## 4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRM are valid for all units of the material, within the stated uncertainties.

The number of units selected corresponds to approximately the cube root of the total number of units produced. Thirteen units were selected for the between-unit homogeneity test using a random stratified sampling scheme covering the whole batch. Random stratified sampling involves dividing the batch into 13 groups (with a similar number of units in each group) and selecting one unit randomly from each group. Three independent samples were taken from each selected unit and analysed by GC-MS/MS. The measurements were performed under repeatability conditions, and in a randomised manner to separate a potential drift in the measurement results from a potential trend in the filling sequence. The results are shown as graphs in Annex 2.

Regression analyses were performed to evaluate potential trends in the measurement sequence as well as trends in the filling sequence. Filling trends were detected for both measurands.

The dataset was assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. No outlying individual result was detected for the compounds.

The distribution of the individual results and the mean values per unit were visually tested for normality using histograms and normal probability plots. The results of all statistical evaluations are given in Table 1.

**Table 1.** Results of the statistical evaluation of the homogeneity study.

| | Trends [1] | | Outliers [2] | | Distribution | |
|---|---|---|---|---|---|---|
| | Measurement sequence | Filling sequence | Individual results | Unit means | Individual results | Unit means |
| 3-MCPD fatty acid esters | no | yes | none | none | normal | normal |
| 2-MCPD fatty acid esters | no | yes | none | none | normal | unimodal |

[1] 95 % confidence level.
[2] 99 % confidence level.

Since a trend in the filling sequence was significant at least at a 95 % confidence level for both compounds, the uncertainty was estimated as $u_{rec,\ rel}$ using a rectangular distribution between the highest and lowest unit mean [20]. In case of a significant trend in the filling sequence, the corrected uncertainty is given as:

$$u_{rec,\,rel} = \frac{|\bar{x}_{max} - \bar{x}_{min}|}{2 \cdot \sqrt{3} \cdot \bar{y}}$$ **Equation 1**

$\bar{y}$ mean of the mean values of the homogeneity study
$\bar{x}_{max}$ highest unit mean of the homogeneity study
$\bar{x}_{min}$ lowest unit mean of the homogeneity study

The results of the evaluation of the between-unit variation are summarised in Table 2.

**Table 2.** Results of the homogeneity study.

| | $u_{rec,\,rel}$ [%] |
|---|---|
| 3-MCPD fatty acid esters | 1.0 |
| 2-MCPD fatty acid esters | 1.1 |

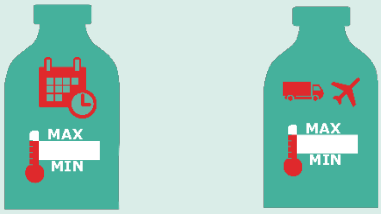
Although trends in the filling sequence were found, the inhomogeneity, quantified as $u_{rec}$, is still sufficiently small to make the material suitable for its intended use. Therefore, $u_{rec}$ was used as estimate of the beween-unit uncertainty $u_{bb}$ associated with inhomogeneity.

## 4.2 Within-unit homogeneity and minimum sample size

The minimum sample size was established based on the technically valid results of the characterisation study, using the method information supplied by the participants. The smallest sample intake that still yielded results with acceptable precision to be included in the respective studies was taken as minimum sample size. Using the data from Annex 5 the minimum sample size is 0.4 g.

# 5 Stability

**Box 4.** Stability assessment




Stability testing is necessary to establish the conditions for storage as well as the transport conditions of the RMs to the customers. During transport, especially in summer, temperatures up to 60 °C can be reached, and stability under these conditions must be demonstrated if the RMs are to be transported without any additional cooling.

Time, temperature, light (including ultraviolet radiation) and water content were regarded as the most relevant influences on the stability of the material. The influence of ultraviolet and visible light was minimised by storing the material in containers and sachets, which eliminate light exposure. In addition, materials are stored in the dark, thus removing any possibility of degradation by light during storage. The water content was reduced to a minimum (by spray-drying) during production and the content was confirmed before bottling. Additionally, the material was pasteurised to decrease microbial growth. Therefore, only the influences of time and temperature needed to be investigated.

The stability studies were carried out using an isochronous design [21]. In this approach, units are stored for a particular length of time under different temperature conditions. Afterwards, the units are moved to conditions where further degradation can be assumed negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Analysis of the material (exposed to different temperatures over different time periods) under repeatability conditions greatly improves the sensitivity of the stability tests.

## 5.1 Transport stability

The conditions for the transport of the material to the customers were established in a short-term stability study. To this end, units were stored at 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by GC-MS/MS. The measurements were performed under repeatability conditions, and a randomised sequence was used to differentiate any potential drift in the measurement results from a potential trend over storage time. The data were evaluated individually for each temperature.

The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %.

In addition, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases or decreases of the measurands due to shipping conditions. The slopes of the regression lines were tested for statistical significance.

The results of the measurements are shown in Annex 3. The results of the statistical evaluation of the short-term stability are summarised in Table 3.

**Table 3.** Results of the short-term stability tests.

| | Number of individual outlying results [1)] | | Significance of the trend [2)] | |
|---|---|---|---|---|
| | 18 °C | 60 °C | 18 °C | 60 °C |
| 3-MCPD fatty acid esters | none | none | no | no |
| 2-MCPD fatty acid esters | none | none | no | no |

1) 99 % confidence level.
2) 95 % confidence level.

No technically unexplained outliers were detected for any of the compounds. None of the trends were statistically significant at a 95 % confidence level for either of the temperatures.

Based on the statistical evaluation, it is concluded that the material can be dispatched without further precautions under ambient conditions.

## 5.2 Storage stability

Storage conditions and shelf life guaranteeing the stability of the material and the certified values were established in a long-term stability study.

To this end, units were stored at -20 °C for 0, 8, 16 and 24 months. The reference temperature was set to -70 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by GC-MS/MS. The measurements were performed under repeatability conditions and in a random sequence to be able to separate any potential drift in the measurement results from a potential trend over storage time.

The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %.

In addition, the data were plotted against storage time and linear regression lines of mass fraction versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage).

The results of the long-term stability measurements are shown in Annex 4. The results of the statistical evaluation of the long-term stability study are summarised in Table 4.

**Table 4.** Results of the long-term stability tests at -20 °C.

| | Number of individual outlying results [1)] | Significance of the trend [2)] |
|---|---|---|
| 3-MCPD fatty acid esters | none | no |
| 2-MCPD fatty acid esters | none | no |
| Glycidyl fatty acid esters | none | yes |

1) 99 % confidence level.
2) 95 % confidence level.

No technically unexplained outliers were detected for any of the compounds. A significant trend at a 95 % confidence level was found for glycidyl fatty acid esters, indicating instability. It is concluded that glycidyl fatty acid esters are not stable over a time period of 24 months and it is therefore not possible to assign any certified value for these compounds. For 3-MCPD fatty acid esters and 2-MCPD fatty acid esters, the trends were not statistically significant at a 95 % confidence level for the tested temperature. The material can therefore be stored at -20 °C.

## 5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability or intermediate precision, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during transport and storage were estimated, as described in [22] for each target compound. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions $u_{sts}$ and $u_{lts}$ were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,\,rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{tt}$$ **Equation 2**

$$u_{lts,\,rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{sl}$$ **Equation 3**

$s_{rel}$ relative standard deviation of all results of the stability study
$t_i$ time elapsed at time point *i*
$\bar{t}$ mean of all $t_i$
$t_{tt}$ chosen transport time (1 week at 60 °C)
$t_{sl}$ chosen shelf life (24 months at -20 °C)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of stability during transport. This was estimated from the 60 °C study. The uncertainty describes the possible change during a transport at 60 °C lasting for one week.
- $u_{lts,rel}$, the uncertainty of stability during storage. This uncertainty contribution was estimated from the -20 °C study. The uncertainty contribution describes the possible degradation during storage for 24 months at -20 °C.

The results of these evaluations are summarised in Table 5.

**Table 5.** Uncertainties of stability during transport and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 24 months.

| | $u_{sts,rel}$ [%] | $u_{lts,rel}$ [%] |
|---|---|---|
| 3-MCPD fatty acid esters | 0.8 | 0.6 |
| 2-MCPD fatty acid esters | 0.9 | 2.6 |

No significant degradation was observed for 3-MCPD fatty acid esters and 2-MCPD fatty acid esters even at 60 °C. Therefore, the material can be shipped at ambient conditions without special precautions.

The material is included in the JRC's regular stability monitoring programme, to control its further stability.

**Box 5.** Stability monitoring

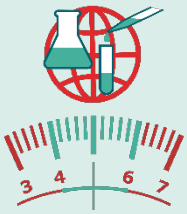
RMs are produced as batches that should last for ten years or longer. This long lifetime means that a storage stability study of limited duration cannot provide a definite “use by” date for the material. It therefore needs to be complemented by stability monitoring throughout the lifetime of the RM.

Therefore, the stability of RMs whose assigned values might change is regularly monitored. The monitoring frequency depends on the outcome of the storage stability assessment.

If the tests confirm the stability of the assigned values, the material remains on sale. If not, possible actions include the retraction of the value in question, retraction of the complete material or a change of the certified value. Customers are notified if the change is larger than the uncertainty of the assigned value.

# 6 Characterisation

**Box 6.** Reference material characterisation




Material characterisation is the process of determining the property value(s) of a RM. While ISO 17034 [1] allows to characterise a RM in various ways, quality management procedures of the JRC are more stringent and allow characterisation only by either interlaboratory comparison or the use of a primary method confirmed by independent analysis.

The material characterisation was based on an interlaboratory comparison, i.e. the properties of the material were determined by combining independent datasets obtained in laboratories applying different measurement procedures, to demonstrate the absence of a measurement bias. This approach converts the systematic bias of each dataset into a random variable, the combined effect of which is reduced by averaging over several datasets. Due to the nature of the compounds, the same analytical method (GC) was applied for all datasets though.

## 6.1 Selection of participants

Twelve laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participating laboratory was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of MCPD fatty acid esters and glycidyl fatty acid esters measurements in relevant matrices, based on interlaboratory comparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025:2017 [3] was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

## 6.2 Study setup

Laboratories received two units of ERM-BD087 per dataset and were requested to provide for each dataset six independent results, three per unit. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be done on at least two days to ensure intermediate precision conditions. The participants were instructed to keep the material at -20 °C until analysis. An independent calibration was performed for each day of analysis. The water content had to be determined in each unit, however the results are reported on as-is mass basis.

Laboratories were also requested to give estimations of the expanded uncertainties of the mean value of the six results. No approach for the estimation was prescribed, i.e. top-down and bottom-up [20] were regarded as equally valid procedures.

## 6.3 Measurement procedures used

A variety of extraction methods with different quantification steps (all using GC-MS or GC-MS/MS) were used to characterise the material. The combination of results from methods all targeting the same compound, based on different extraction principles aiming to overcome the challenges with extracting the potentially present protein-linked and microencapsulated fat mitigates undetected method bias.

All methods used during the characterisation study are summarised in Annex 5 The dataset code (e.g. D01-GC-MS/MS) is a random number and does not correspond to the order of laboratories in Section 2. The dataset code consists of a number assigned to each dataset (e.g. D01) and abbreviation of the measurement method used (e.g. GC-MS/MS).

## 6.4 Dry mass determination

All the results presented refer to the material as it is, without dry mass correction.

## 6.5 Evaluation of results

The characterisation study resulted in twelve datasets for 3-MCPD fatty acid esters and glycidyl fatty acid esters and nine datasets for 2-MCPD fatty acid esters. As glycidyl fatty acid esters was withdrawn from certification, the data were not evaluated further for these compounds. Not all laboratories participated with results for all parameters. The laboratories who also reported the content of free 2- or 3-MCPD all reported these compounds as <LOQ. All individual datasets of the participating laboratories, grouped per compound, are displayed in tabular and graphical form in Annex 6.

### 6.5.1 Technical evaluation

The obtained data were first checked for compliance with the instructions and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- Compliance with the instructions given: sample preparations and measurements performed on two days.
- Compliance with ISO/IEC 17025:2017, accredited or (minimum requirement) validated methods were used.
- Absence of values given as below limit of quantification (LOQ)
- Obvious technical issues

Based on these criteria, the following datasets were rejected as not technically valid (Table 6). Technical problems were encountered by the laboratory assigned to code D12. The participant changed the initially validated method from GC-MS to GC-MS/MS. To accept this dataset, a further proof of the trueness and validation would be needed to comply with ISO/IEC 17025:2017 requirements, which was not provided by the laboratory in the timeframe of this study. Consequently, the method validation was not complete. The dataset was therefore excluded for technical reasons.

For D11, the method is not sufficiently sensitive and has inferior precision close to the LOQ in contrast to the results of the other data sets, indicating that the measurements were out of control. The laboratory did not find any technical reasons for the lack of sensitivity and for this reason the dataset is excluded from the evaluation.

Both D11 and D12 did not submit results for 2-MCPD fatty acid esters.

**Table 6.** Datasets that showed non-compliance with the instructions given and technical specifications or having technical issues, and action taken.

| | Dataset code | Description of problem | Action taken |
|---|---|---|---|
| 3-MCPD fatty acid esters | D11 | insufficient precision close to LOQ | Not used for evaluation |
| 3-MCPD fatty acid esters | D12 | Insufficient data given for the validation of method | Not used for evaluation |

### 6.5.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations (both at a 99 % confidence level). Standard

deviations within ($s_{within}$) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 7.

**Table 7.** Statistical evaluation of the technically accepted datasets for ERM-BD087. *p*: number of technically valid datasets.

| | *p* | Outliers | | Normally distributed | Statistical parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | | Means | Variances | | Mean [µg/kg] | *s* [µg/kg] | $s_{between}$ [µg/kg] | $s_{within}$ [µg/kg] |
| 3-MCPD fatty acid esters | 10 | none | 0 | normal | 70.34 | 14.10 | 14.04 | 3.09 |
| 2-MCPD fatty acid esters | 9 | none | 4 | normal | 29.67 | 5.88 | 5.76 | 2.92 |

The dataset means follow normal distributions. None of the data contains outlying means. The datasets are therefore consistent and the mean of means is a good estimate of the true value. Standard deviations between dataset means are considerably larger than the standard deviation within datasets, showing that confidence intervals of replicate measurement results are unsuitable as estimates of measurement uncertainty.

The statistical evaluation flagged dataset D1, D3, D7 and D10 as outlying variances for 2-MCPD fatty acid esters. This merely reflects the fact that different methods have different intrinsic variability. As all measurement methods were found technically sound, all results were retained.

It should be borne in mind that the methods used in the characterisation are methods routinely applied for measuring MCPD fatty acid esters in infant formula. The agreement of results from different methods demonstrates that the processing did not affect any properties relevant for these methods and that ERM-BD087 behaves like a real sample.

The uncertainty related to the characterisation is estimated as the standard error of the mean of means ($s/\sqrt{p}$) (Table 8).

**Table 8.** Uncertainty of characterisation for ERM-BD087.

| | *p* | Mean [µg/kg] | *s* [µg/kg] | $u_{char}$ [µg/kg] |
|---|---|---|---|---|
| 3-MCPD fatty acid esters | 10 | 70.3 | 14.1 | 4.5 |
| 2-MCPD fatty acid esters | 9 | 29.7 | 5.9 | 2.0 |

# 7 Value Assignment

**Box 7.** Assignment of values to a reference material


Based on the outcome of characterisation measurements three types of values can be assigned, namely certified, indicative or additional material information values.

Certified values are values that fulfil the highest standards of accuracy. Procedures at JRC Directorate F require a sufficient number of datasets to assign certified values. Full uncertainty budgets in accordance with ISO 17034:2016 [1] and ISO Guide 35 [2] are required. Certified values of a CRM can be used for calibration and trueness controls.

Indicative values are values where either the uncertainty is deemed too large or too few independent datasets are available to allow certification. Indicative values of an RM can be used for statistical quality control (homogeneity and stability has been assessed) but not for calibration, demonstration of method or laboratory proficiency or method trueness.

Additional material information values are values for which homogeneity and stability has usually not been assessed and insufficient data for characterisation is available. Consequently, an estimate of the reliability of the values is not possible and no uncertainty is given. Additional material information values cannot be used for calibration, demonstration of method or laboratory proficiency or method trueness. They can be used to e.g. anticipate possible interferences in measurement processes.

Certified and additional material information values were assigned.

## 7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 7 was assigned as certified value for each parameter.

The assigned uncertainty consists of uncertainties relating to characterisation ($u_{char}$), potential between-unit inhomogeneity ($u_{bb}$), and potential degradation during transport ($u_{sts}$), and long-term storage ($u_{lts}$). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor $k$ given as:

$$U_{CRM,\,rel} = k \cdot \sqrt{u^2_{bb,\,rel} + u^2_{sts,\,rel} + u^2_{lts,\,rel} + u^2_{char,\,rel}} \qquad \textbf{Equation 4}$$

- $u_{char}$ was estimated as described in Section 6.5
- $u_{bb}$ was estimated as described in Section 4.1
- $u_{sts}$ and $u_{lts}$ were estimated as described in Section 5.3

Following JRC's procedures for assigning uncertainties to certified values, a coverage ($k$) factor of 2 can be chosen if the main uncertainty component has at least five degrees of freedom. As can be seen in Table 9, the $u_{char,\,rel}$ is the dominant contribution to the combined uncertainty and it has 8-9 degrees of freedom. Therefore, a $k$-factor of 2 was applied to obtain the expanded uncertainties.

The certified values and their uncertainties are summarised in Table 9.

Free 3- and 2- MCPD are not found in any of the studies for homogeneity or stability, and all laboratories submitting results for 3- and 2-MCPD in the characterisation reported the content to be below LOQ.

EU regulation 2023/915 [16] stipulates the maximum level based on the sum of 3-MCPD and 3-MCPD fatty acid esters. The regulations also specify to use lower bound concentrations, which are calculated on the assumption that all the values below the LOQ are zero. Therefore the mass fraction of the sum of 3-MCPD and 3-MCPD fatty acid esters is equal to the mass fraction of 3-MCPD fatty acid esters. Although not covered by the regulation, the sum of 2-MCPD and 2-MCPD fatty acid esters is calculated in the same way. Furthermore it is assumed that the uncertainty components in Eq. 4 are identical for MCPD fatty acid esters and the sum of MCPD and MCPD fatty acid esters.

**Table 9.** Certified values and their uncertainties for ERM-BD087

| | Certified value[1)] [µg/kg] | $u_{bb, rel}$ [%] | $u_{sts, rel}$ [%] | $u_{lts, rel}$ [%] | $u_{char, rel}$ [%] | $U_{CRM, rel}$ [%] | $U_{CRM}$ [2)] [µg/kg] |
|---|---|---|---|---|---|---|---|
| 3-MCPD fatty acid esters[3)] | 70 | 1.0 | 0.8 | 0.6 | 6.4 | 13.0 | 10 |
| 2-MCPD fatty acid esters[3)] | 30 | 1.1 | 0.9 | 2.6 | 6.6 | 14.5 | 5 |
| Sum of 3-MCPD and 3-MCPD fatty acid esters[3,4)] | 70 | 1.0 | 0.8 | 0.6 | 6.4 | 13.0 | 10 |
| Sum of 2-MCPD and 2-MCPD fatty acid esters[3,4)] | 30 | 1.1 | 0.9 | 2.6 | 6.6 | 14.5 | 5 |

1) Reported on material as is (Section 6.5.2).
2) Expanded ($k$ = 2) and rounded uncertainty; uncertainties are always rounded up [23] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty.
3) expressed as MCPD
4) lower bound concentrations, on the assumption that all the values below the limit of quantification are zero.

## 7.2 Additional material information

The data provided in this section should be regarded as informative only on the general composition of the material and cannot, in any case, be used as certified or indicative value.

The measurement analysis of MCPD fatty acid esters in infant formula is based on extraction of fat, which is analysed for the respective compounds. It is therefore relevant to know the content of fat for the analysis. The content of fat was measured in 14 units (single measurement) and the mean value was found to be 25.7 %. The water content was measured during characterisation on 16 units (single measurement) and the mean value was found to be 1.4 %.

The analysis of free MCPD resulted in all cases during homogeneity, stability and characterisation studies in values below LOQ. As sufficient participants have reported LOQ values below 5 µg/kg, it is concluded that the content of 3-MCPD and 2-MCPD is below this value.

# 8 Metrological traceability and commutability

## 8.1 Metrological traceability

**Box 8.** Metrological traceability

Metrological traceability of measurement results is a key requirement for ensuring the comparability of data. As CRMs are used to make measurement results traceable, metrological traceability of its certified values to a stated reference is essential.

The certified value of a CRM is metrologically traceable if the measurements used for establishing it can be related to a reference through an unbroken chain of calibrations.

This requires that these measurements

- refer to the same property (e.g. Pb) and the same (kind of) quantity (e.g. Pb content),
- result in a number and its uncertainty (e.g. 6 ± 2) expressed in the same measurement unit (e.g. µg/kg).

The concept of traceability rests on several anchor points, namely identity, quantity value and measurement unit. The identity of a measurand can be defined by its structure alone or can be operationally defined, the quantity value of the measurand can refer to the SI, or to other appropriate references.

### 8.1.1 Identity

3-MCPD fatty acid esters and 2-MCPD fatty acid esters are chemically defined as a group of substances. Different methods were used for the sample preparation as well as for the final determination, demonstrating absence of measurement bias. Nevertheless, since methods based on GC-MS and GC-MS/MS were used for all datasets, the quantity values for these measurands are operationally defined as obtained by GC-MS and GC-MS/MS after hydrolysis.

### 8.1.2 Quantity value

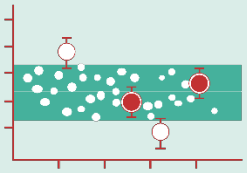
Only validated methods were used during the characterisation study. Different calibrants of known purity and specified traceability of their assigned values were used. Investigation of the method and measurement details of the individual results show that all relevant input parameters of each technically accepted dataset were properly calibrated. All technically accepted datasets are therefore traceable to the same reference, namely the International System of Units (SI). This traceability to the same reference is also confirmed by the agreement of results within their respective uncertainties. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

## 8.2 Commutability

**Box 9.** Commutability

Commutability is a prerequisite for RMs intended to be used for calibration or quality control of different measurement procedures targeting the same measurand. The concept of commutability of a RM is defined by the VIM [12] as:

"*property of a reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material, obtained according to two given measurement procedures, and the relation obtained among the measurement results for other specified materials*"



Commutability is a property of an RM indicating how well an RM mimics the characteristics of a typical routine sample in various measurement procedures for a stated measurand.

The same RM may be commutable for some measurement procedures but non-commutable for others. A commutability statement is therefore only valid for the mentioned measurement procedure(s).

ERM-BD087 was produced from an infant formula recipe, containing fat which was contaminated with MCPD fatty acid esters and glycidyl fatty acid esters. The CRM mimics as much as possible routine samples of infant formula powder which is dissolvable in water.

For sample matrices other than infant formula, it is the responsibility of the CRM user to assess the suitability of the reference material.

# 9 Instructions for use

## 9.1 Safety information

The usual laboratory safety measures apply.

## 9.2 Storage conditions

The materials should be stored at (-20 ± 5) °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open. The user should close any unit immediately after taking a sample. An opened unit can be reanalysed at least up to 7 days after opening. Further than this period of time has not been tested.

For more information regarding the shelf life of reference materials please consult ERM Application Note 7 [24].

Note that the European Commission cannot be held responsible for changes that may happen to samples after opening or when the material is stored differently from the stated storage conditions at the customer's premises.

## 9.3 Use of the material

The material must be re-homogenised by repeatedly turning over the unit and shaking for 2 min.

The determination of MCPD fatty acid esters and glycidyl fatty acid esters is performed in the extractable fat fraction of infant formula, as these compounds are lipophilic. However, milk-based infant formula ingredients commonly have protein-linked fat contents which are not easily extractable. Moreover, some infant formulae contain microencapsulated fat contingents, which are not easily accessible neither. Thus, the analysis of MCPD fatty acid esters in infant formula powder matrix combined with the low maximum levels for these compounds poses some analytical challenges. There are some indications that the presence of water may aid to overcome the extraction challenges, although the mechanism is not completely known. It should therefore be considered if addition of water to the CRM before or in the extraction step is beneficial. During the characterisation study, various validated extraction principles were used with or without addition of water, and provided acceptable results.

For general information on handling of reference materials, please consult ERM Application Note 6 [25].

## 9.4 Minimum sample size

The minimum sample size representative for all certified quantity values is 0.4 g.

## 9.5 Use of the certified values

The intended use of this material is to assess method performance, i.e. for checking accuracy of measurement results. Measurements results can be made metrologically traceable by calibrating a measurement with a suitable CRM that is metrologically traceable to the same reference. Using a CRM as quality control material in a measurement procedure serves to verify the metrological traceability. Certified values can be used for calibration and trueness controls.

It is not recommended to use this matrix material as calibrant. If used nevertheless, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1 [26]).

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_{meas}$).
- Combine the measurement uncertainty ($u_{meas}$) with the uncertainty of the certified value ($u_{CRM}$): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$

— Calculate the expanded uncertainty ($U_\Delta$) from the combined uncertainty ($u_\Delta$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.

— If $\Delta_{meas} \leq U_\Delta$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

The material(s) can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

Additional material information values are values that were obtained in the course of the study. They are values for which the reference material producer (RMP) is unable to provide an uncertainty as usually homogeneity and stability have not been assessed and insufficient data for characterisation are available. Consequently, an estimate of the true values is not possible and no uncertainty is given. Additional material information values cannot be used for calibration, demonstration of method or laboratory proficiency or method trueness. They can be used to e.g. anticipate possible interferences in measurement processes.

# 10 Conclusions

ERM-BD087 is a matrix material certified for the mass fraction of 3-MCPD fatty acid esters and 2-MCPD fatty acid esters. As all measurements of 3-MCPD and 2-MCPD were below LOQ, the mass fractions also cover the sum of 3-MCPD and 3-MCPD fatty acid esters and the sum of 2-MCPD and 2-MCPD fatty acid esters, as defined in Commission Regulation (EU) 2023/915 [16]. This material was produced and certified in accordance with ISO 17034:2016 [27] and ISO Guide 35:2017 [28]. ERM-BD087 was produced within the scope of ISO 17034:2016 accreditation.

ERM-BD087 is an infant formula produced with contaminated coconut oil to facilitate the compliance checks of infant formula according to the Commission Regulation (EU) 2023/915 [16].

The following values were assigned:

**Table 10.** Values assigned to ERM-BD087.

| Mass fraction | | |
|---|---|---|
| | Certified value [6] [µg/kg] | Uncertainty [7] [µg/kg] |
| 3-MCPD fatty acid esters [1,2] | 70 | 10 |
| 2-MCPD fatty acid esters [1,3] | 30 | 5 |
| Sum of 3-MCPD and 3-MCPD fatty acid esters [1,4] | 70 | 10 |
| Sum of 2-MCPD and 2-MCPD fatty acid esters [1,5] | 30 | 5 |

[1] As obtained by GC-MS and GC-MS/MS after hydrolysis.

[2] 3-monochloropropanediol fatty acid esters, expressed as 3-MCPD [CAS 96-24-2].

[3] 2-monochloropropanediol fatty acid esters, expressed as 2-MCPD [CAS 497-04-1].

[4] Sum of 3-monochloropropanediol (3-MCPD) and 3-MCPD fatty acid esters, expressed as 3-MCPD.

[5] Sum of 2-monochloropropanediol (2-MCPD) and 2-MCPD fatty acid esters, expressed as 2-MCPD.

[6] Certified values are values that fulfil the highest standards of accuracy. The given values represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and with a different method of determination. The certified value and its uncertainty are traceable to the International System of Units (SI).

[7] The uncertainty of the certified value is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of 95 %, estimated in accordance with ISO 17034:2016 and ISO Guide 35:2017.

The material is intended for the quality control and assessment of method performance.

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## List of abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| *b* | Slope in the equation of linear regression $y = a + bx$ |
| CEN | European Committee for Standardization |
| CFU | Colony-forming unit |
| CRM | Certified reference material |
| EC | European Commission |
| EI | Electron ionisation |
| EN | European norm (standard) |
| ERM® | Trademark owned by the European Commission; used by the JRC for reference materials |
| EU | European Union |
| GE | Glycidyl fatty acid esters |
| GC | Gas chromatography |
| GC-MS | Gas chromatography-mass spectrometry |
| GC-MS/MS | gas chromatography-triple quadrupole mass spectrometry |
| GUM | Guide to the Expression of Uncertainty in Measurement |
| ISO | International Organization for Standardization |
| IUPAC | International Union of Pure and Applied Chemistry |
| JRC | Joint Research Centre of the European Commission |
| *k* | Coverage factor |
| KFT | Karl Fischer titration |
| LOQ | Limit of quantification |
| MCPD | Monochloropropanediol |
| MCPDE | Monochloropropanediol fatty acid esters |
| MRL | Maximum residue level |
| MS | Mass spectrometry |
| $MS_{between}$ | Mean of squares between-unit from an ANOVA |
| $MS_{within}$ | Mean of squares within-unit from an ANOVA |
| *n* | Number of replicate analysis per unit |
| *N* | Number of units analysed |
| n.a. | Not applicable |
| n.c. | Not calculated |
| *p* | Number of technically valid datasets |
| PE | polyethylene |
| PLE | Pressurised liquid extraction (= accelerated solvent extraction) |
| PSA | Particle size analysis |
| PT | Proficiency testing |

| | |
|---|---|
| PTV | Programmable temperature vaporiser |
| QA | Quality assurance |
| QC | Quality control |
| rel | Index denoting relative figures (uncertainties etc.) |
| RM | Reference material |
| RMP | Reference material producer |
| *RSD* | Relative standard deviation |
| *RSE* | Relative standard error (=$RSD/\sqrt{n}$) |
| RT | Room temperature |
| $r^2$ | Coefficient of determination of the linear regression |
| $s$ | Standard deviation |
| $s_{bb}$ | Between-unit standard deviation; an additional index "rel" is added when appropriate; this parameter is linked to the homogeneity of the material |
| $s_{between}$ | Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| SI | International System of Units |
| $s_{meas}$ | Standard deviation of measurement data; an additional index "rel" is added as appropriate |
| SPE | Solid phase extraction |
| $s_{wb}$ | Within-unit standard deviation; this parameter is linked to the homogeneity of the material |
| $s_{within}$ | Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| $T$ | Temperature |
| $t$ | Time |
| $t_i$ | Time point for each replicate |
| $t_{sl}$ | Proposed shelf life |
| $t_{tt}$ | Proposed transport time |
| TPC | Total plate count |
| $u$ | Standard uncertainty |
| $U$ | Expanded uncertainty |
| $u^*_{bb}$ | Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate |
| $u_{bb}$ | Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate |
| $u_c$ | Combined standard uncertainty; an additional index "rel" is added as appropriate |
| $u_{cal}$ | Standard uncertainty of calibration |
| $u_{char}$ | Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate |
| $u_{CRM}$ | Combined standard uncertainty of the certified value; an additional index "rel" |

| | |
|---|---|
| | is added as appropriate |
| $U_{CRM}$ | Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate |
| $u_{\Delta}$ | Combined standard uncertainty of measurement result and certified value |
| $u_{lts}$ | Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate |
| $u_{meas}$ | Standard measurement uncertainty |
| $U_{meas}$ | Expanded measurement uncertainty |
| $u_{rec}$ | Standard uncertainty related to possible between-unit inhomogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate |
| $u_{sts}$ | Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate |
| $u_t$ | Standard uncertainty of trueness |
| $V$ | Volume |
| VIM | International Vocabulary of Metrology – Basic and General Concepts and Associated Terms |
| $\bar{x}$ | Arithmetic mean |
| $\bar{x}_{max}$ | Highest unit mean of the homogeneity study |
| $\bar{x}_{min}$ | Lowest unit mean of the homogeneity study |
| $\Delta_{meas}$ | Absolute difference between mean measured value and the certified value |
| $\nu_{s,meas}$ | Degrees of freedom for the determination of the standard deviation $s_{meas}$ |
| $\nu_{MSwithin}$ | Degrees of freedom of $MS_{within}$ |
| $\bar{y}$ | Mean of the mean values of the homogeneity study |

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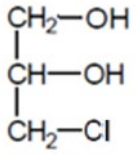
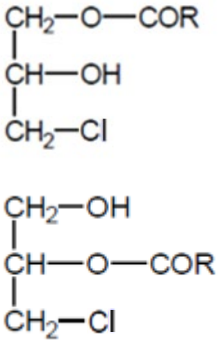
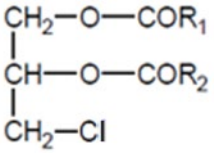
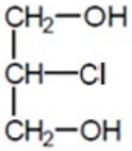
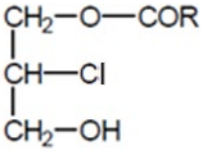
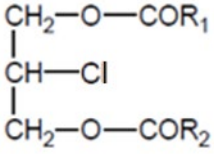
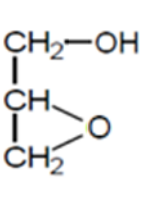
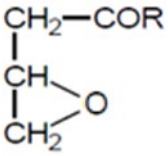
## Annexes

**Annex 1. Chemical structures of MCPD, MCPD fatty acid esters, glycidol and glycidyl fatty acid esters**

| Compound | Free form | Compound | Monoesters | Diesters |
|---|---|---|---|---|
| **3-MCPD** |  | **3-MCPD fatty acid esters** |  |  |
| **2-MCPD** |  | **2-MCPD fatty acid esters** |  |  |
| **Glycidol** |  | **Glycidyl fatty acid esters** |  | |

**Annex 2. Results of the homogeneity measurements**

**Figure 2.1.** Homogeneity of ERM-BD087 for 3-MCPD fatty acid esters

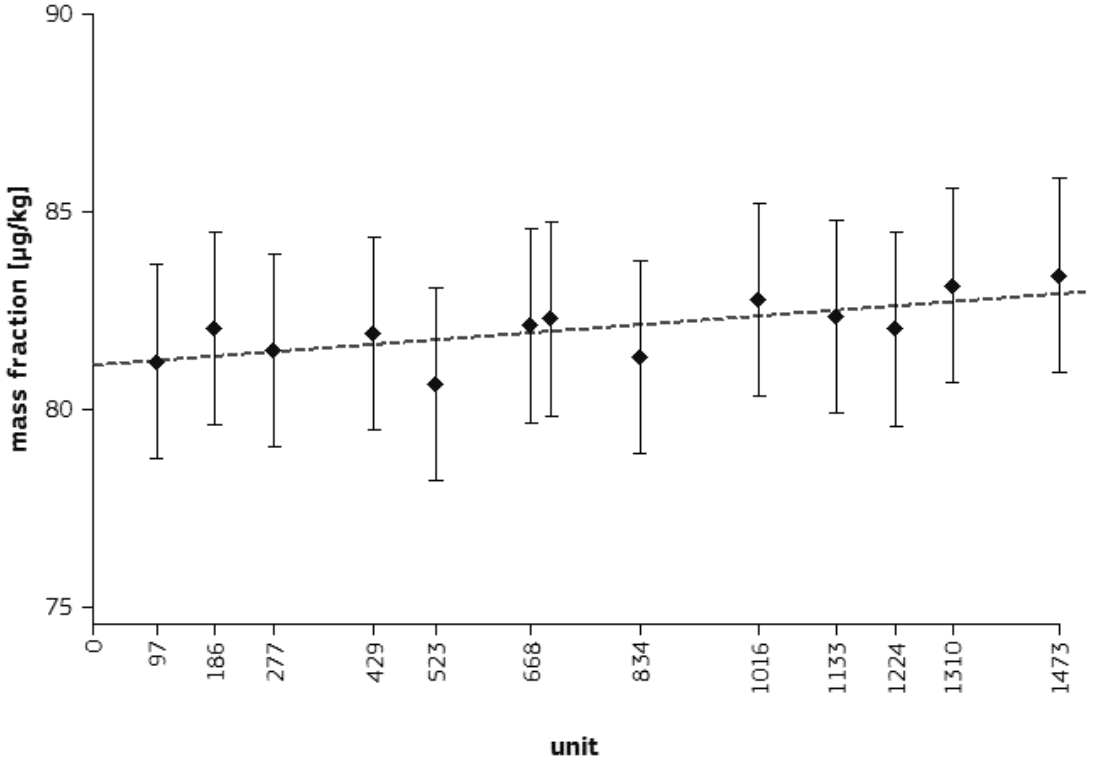
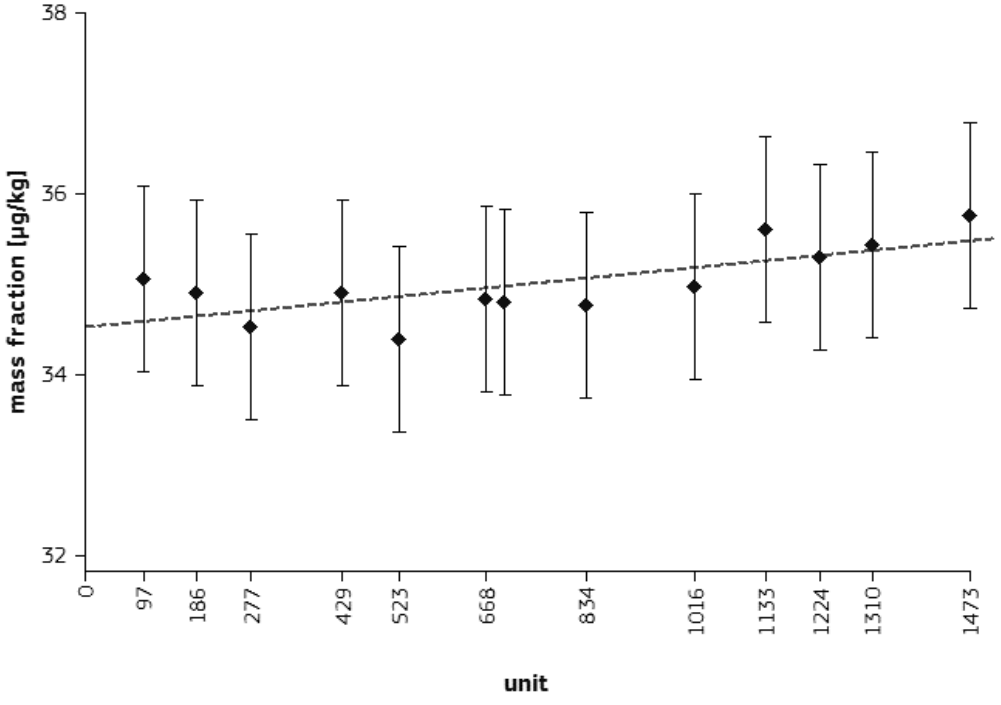



**Figure 2.2.** Homogeneity of ERM-BD087 for 2-MCPD fatty acid esters



## Annex 3. Results of the short-term stability measurements

**Figure 3.1.** Transport stability of ERM-BD087, 3-MCPD fatty acid esters assessed at 60 °C, over four weeks.

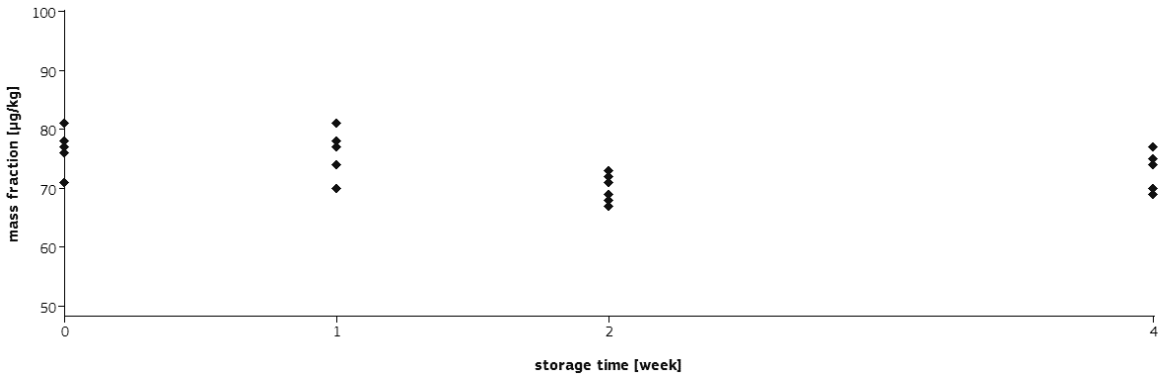
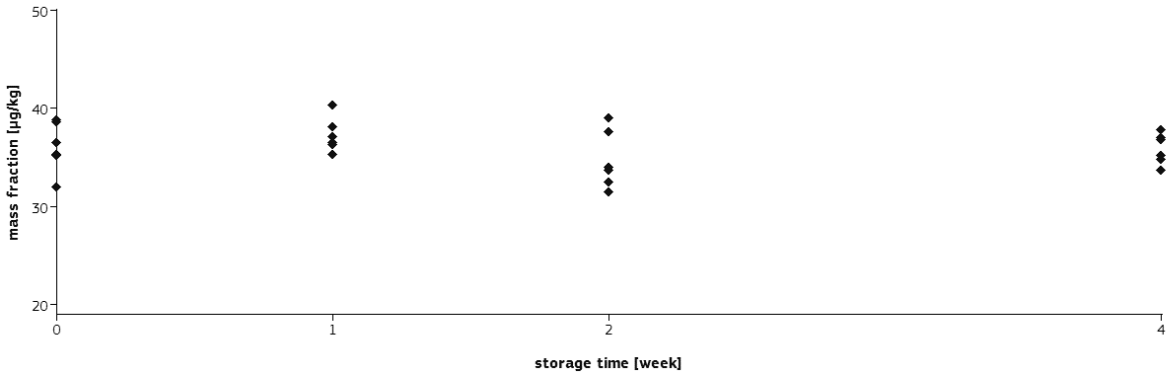



**Figure 3.2** Transport stability of ERM-BD087, 2-MCPD fatty acid esters assessed at 60 °C, over four weeks.



**Annex 4 Results of the long-term stability measurements study**

**Figure 4.1.** Storage stability of ERM-BD087, 3-MCPD fatty acid esters assessed at -20 °C, over twenty-four months.

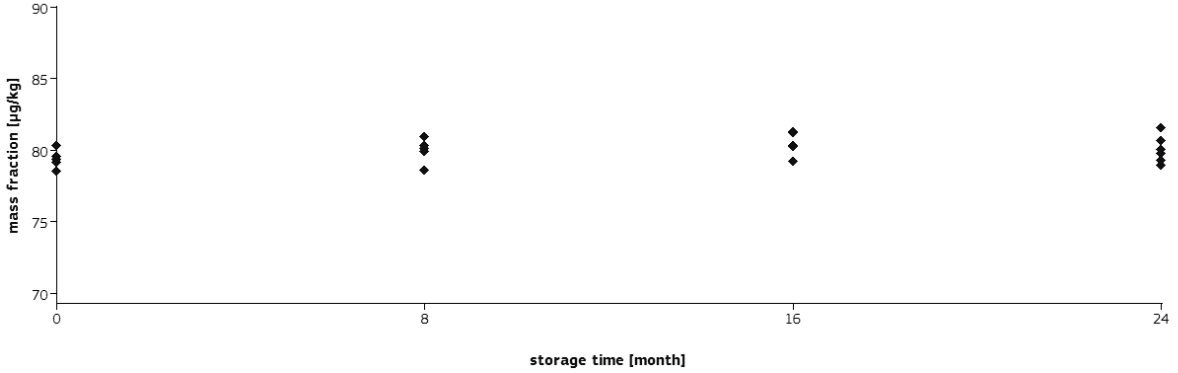



**Figure 4.2.** Storage stability of ERM-BD087, 2-MCPD fatty acid esters assessed at -20 °C, over twenty-four months.

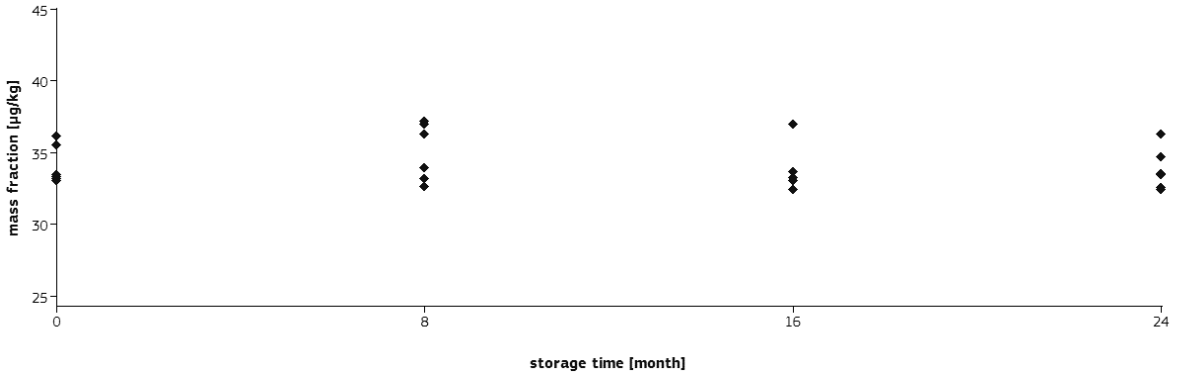
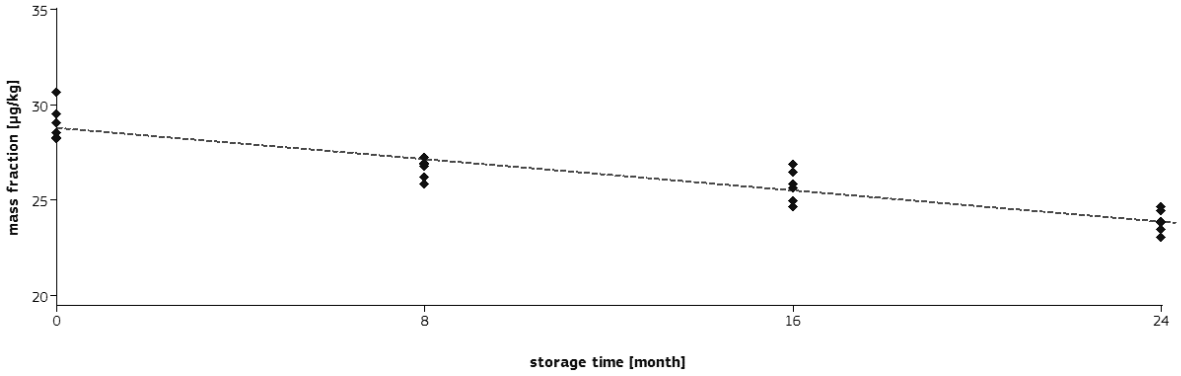



**Figure 4.3.** Storage stability of ERM-BD087, glycidyl fatty acid esters assessed at -20 °C, over twenty-four months.



## Annex 5. Summary of methods used in the characterisation study

**Table 5.1** Method information as reported by the laboratories

| LABO-RATORY CODE | Sample intake (g) | Internal Standard | Extraction | Clean up |
|---|---|---|---|---|
| 1 | 5 | Carbon-13 labelled 1,2-dipalmitoyl-3-chloropropanediol(PP-3-MCPD-13C3) , Pentadeuterated glycidyl stearate (Gly-S-d5) | The extraction was be done by the Smedes extraction. Extraction with 2-pronanol, cyclo-hexane, sodium chloride-solution, water. In between samples are vigorously shaken. After centrifugation the upper layer will be removed and the organic layer will be evaporated for analysis. | ISO-18363-4:2021 Method using fast alkaline transesterification and measurement for 2-MCPD, 3-MCPD and glycidol by GC-MS/MS |
| 2a | 2 | | Sample mixed with water, fortified with IS and extracted with ethyl acetate. Ethyl acetate evaporated and residue dissolved in heptane, partitioned with ammonium sulphate (aq), heptane layer discarded. Aqueous layer extracted into ethyl acetate and derivatised with PBA. Ethyl acetate evaporated and residue re-suspended in trimethyl pentane. | |
| 2b | 0.5 | | Sample is mixed with water, fortified with IS and extracted with ethyl acetate. Ethyl acetate evaporated. Residue dissolved in hexane/ethyl acetate and passed through an amino propyl SPE column to remove mono and diacyl glycerides. Solvent evaporated and residue dissolved in THF. The glycidyl esters are converted to 3-monobromopropanediol monoesters in an acid solution containing a bromide salt. 3-MBPD esters, together with 2- and 3-MCPD esters, are then hydrolysed to their free (non-esterified) diol in an acid methanolic solution. The fatty acid methyl esters generated are removed and 2- and 3-MCPD, as well as 3-MBPD, are derivatised with phenylboronic acid prior to GC-MS analysis. Ethyl acetate evaporated and residue re-suspended in trimethyl pentane. | |
| 3 | 1 and 2 | Bound form 3MBPD-d5, 3MCPD-13C3, 3MCPD-d5, Free form 2MCPD-D5, 3MCPD-D5 | Ethanol, Diethylether, n-Hexane | |
| 4 | 2 | d5-3-MCPD, d5-2-MCPD, d5-3-MCPD-1,2-bis-oleate, d5-2-MCPD-1,3-bis-oleate, d5-glycidyloleate/d5-glycidylpalmitate | HUPsSE: methanol 15 min US tmin = 65 °C + 15 min methanol/tert-butylmethyl ether 1:1 (v,v) 15 min US tmin = 65 °C + 15 min tert-butylmethyl ether 1:1 (v,v) 15 min US tmin = 65 °C | combined extracts evaporated to dryness, l/l extraction for separation free analytes from lipid phase, lipid phase undergoes alkaline treatment for ester cleavage, matrix removal by l/l extraction. |
| 5 | 1.6 | 2-MCPD-d5 , 3-MCPD-d5, 2-MCPDE-d5, 3-MCPDE-d5, GE-d5 | Extraction with a combination of Sodiumsulfate solution and n-Hexane at room temperature in an IKA DT-50 Dispersing tube | Almost identical to ISO 18363-3 using acid transesterification but derivatization is done with N-Heptafluorbutyrylimidazole (CAS No: 32477-35-3) instead of PBA |
| 6 | 0.5 | | 2x water/Ethylacetate extraction. Ethyacetate/methanol extraction (free vs bound) alkaline hydrolyses of the evaporated ethylacetate phase (bound analytes). Cleanup of the hydrolysate with aminopropylphase. Adding of methanol phase (free analytes), taking of an aliquot and derivatisation with phenylboronic acid | LLE |
| 7a | 1 | 2-MPCD-d5, 3MCPD-d5 | 1g sample + 12 mL carrez 1 and 12 mL carrez 2. Vortex, centrifugation, Add 5 mL hexane/acetone 1:1 on the liquid extract, + internal std, vortex, centrifugation. Add 1mL d'$H_2O_2$ on the previous extract, vortex, centrifugation. Discard superior layer. Evaporate the other phase, and re-constitute with 1,8 mL ethyl acetate and $NaSO_4$ , + 150 uL PBA, vortex, evaporate solvent, re-dissolve with 300 uL isooctane | |
| 7b | 0.5 | 2-MPCD-d5, 3MCPD-d5, Glycidil oleate d5 | Fat extraction: 0,5 g sample +12 ethyl acetate+12 water, 50 °C 15 min, agitation, centrifugation, 10g $NaSO_4$, agitation, centrifugation, re-collect organic phase, and second extraction doing the same. Evaporate total re-collected organic phase to obtain the fat. Add 2 mL THF, add 30 µL reagent (NaBr + $H_2SO_4$), 50 °C 15 min, 3mL $NaHCO_3$, vortex, 2 mL n-hexane, vortex. Evaporate n-hexane, add 1 THF, add MeOH+$H_2SO_4$ overnight 40 °C. Add $NaHCO_3$, evaporate methanol, add $NH_4SO_4$. Add hexane. Above aquous solution add ethyl acetate. Derivatise with PBA. Evaporate and re-dissolved with 300 µL isooctane. | |

| | | | | |
|---|---|---|---|---|
| 8 | 1 | d5-3-MCPD-1,2-bis-palmitoylester, d5-2-MCPD-1,3-bis-palmitoylester, d5-3-MCPD, d5-2-MCPD | 1. extraction with t-BME; 2. extraction with methanol/t-BME (hot extraction) | cleaning with hexane extraction, afterwards extraction with mixture of ethylacetate/diethylether. Derivatisation with PBA, evaporation and resolving with i-octane |
| 9 | 0.4 | 2-MCPD-d5, 3-MCPD-d5, 2-MCPD-dipalmitate-d5, 3-MCPD-dipalmitate-d5, Glycidyl palmitate-d5 | Add 15mL water and 15mL Ethyl Acetate, ultrasonicate for 15 mins at 50°C, vertical shake for 3 min at 1500 rpm, add 10 g of sodium sulfate anhydrous, vertical shake for 3 min at 1500 rpm again, centrifuge, then collect the upper layer. Second extraction with another 15 mL of Ethyl Acetate in the same procedure (without adding more sodium sulfate). Extracts are combined then dried under nitrogen stream. Sodium sulphate solution and n-hexane are then used to separate free MCPDs, and bound MCPDs + GEs in the dried extracts into two different phase: free MCPDs in aqueous phase, and bound MCPDs + GEs in n-hexane phase | In aqueous phase: Ethylacetate is used to extract free MCPDs then derivatized with PBA.In hexane phase: Aminopropyl Solid Phase Extraction (aminopropyl SPE) is used to clean-up the n-hexane phase. Glycidyl esters (GEs) in the clean-up are brominated to 3-monobromopropanediol (3-MBPD) monoesters in an acid solution containing sodium bromide. Bound 2- and 3-MCPD (monochloroproanediol esters), and 3-MBPD esters are converted to the non-esterified forms by acidic methanol transesterification. The fatty acid methyl esters formed by the reaction are then removed from the sample. 2- and 3-MCPD together with 3-MBPD are extracted from the sample by ethyl acetate then derivatized with phenylboronic acid. |
| 10a | | 3-MCPD-D5 | Extrelut is added, extracted with a mixture of n-hexane and diethyl ether to remove non-polar components. The 3-MCPD is eluted with diethyl ether and the eluate is concentrated and derivatized with phenylboronic acid solution (EN14573:2004) | |
| 10b | 5 | 3-MCPDe-13C3, 3-MBPDe-D5, | Soxhlet extraction (Ester bound) | |
| 11a | 1 | 3-MCPD D5 | The liquid sample is extracted with a heptane/acetone mixture (1/1) followed by a liquid-extraction with water. The aqueous phase is retained and then washed with heptane. The aqueous phase is evaporated to dryness with a concentrator-evaporator at a temperature not exceeding 50°C. For the derivation, acid acetone is added to the mixture and the latter is placed for 90 ± 10 minutes at 40 ± 2° C. in an oven. The diols are then transformed into 2,2-dimethyl-1,3 dioxolane. The mixture is then neutralized and applied simultaneously by filtration on a cartridge of basic alumina. | |
| 11b | 0.7 | d5 1,2-bis palmitoyl-3-chloropropanediol, d5 glycidyl palmitate | The analysis is carried out on fat. An extraction with ethyl acetate with a microwave is carried out. Diethyl ether is added and the whole is stored in the freezer at –24 °C. for 30 ± 5 minutes. To this mixture is then added a solution of methanolic sodium hydroxide. The mixture is placed in the freezer at –24 °C for at least 16 hours (20 hours at most). This solution makes it possible to transform 3-MCPD esters into 3-MCPD esters into 3-MCPD and glycidol esters into 3-MBPD. The mixture is acidified with an acid solution of sodium bromide to stop the reaction. The volume of the organic phase is reduced to 100 µL under a nitrogen stream. The remaining volume is extracted twice using heptane (the organic phase is discarded). This step removes the fat. The molecules of interest are recovered by adding three times an ethyl acetate/diethyl-ether (40/60) mixture. The organic phase is recovered and dried. The solution is then derivatized by adding PBA (phenylboronic acid) then evaporated to dryness under a stream of nitrogen. The residue is re-dissolved in 500 µL of iso-octane then analyzed by GC/MS. | |
| 12 | 1 | rac 1,2-bis-palmitoyl-3-chloropropanediol-d5, glycidyl palmitate-d5, 3-chloroporpanediol-d5 | An amount of 1g sample is spiked with ISTDs mix solution. MilliQ water and ethylacetate were added, the tube was vortexed, ultrasonicated and homogenised via a vertical homogenator. 10 g of sodium sulfate was added, the tube was homogenised and centrifuged. The upper layer was collected and the lower layer was extracted by using ethylacetate via vortex, ultrasonic and homogenisation. The two upper layers were joined and the solvent was evaporated and re-dissolved in 2mL heptane and 4 ml MilliQ water and 1 g KBr. The upper layer was collected for bound MCPD and GEs determination. The lower layer (aqueous layer) was extracted twice with ethyl acetate and was collected for the determination of the free 3-MCPD form (removal of the solvent and addition of isooctane as a new solvent for the injection into the GC-MS/MS). | An SPE clean-up was performed by using aminopropyl SPE cartridge only for the determination of the esters. After the clean-up, the eluate was brominated by NaBr (incubation 50 °C for 15 min) and in a further step was transesterificated by using a 1.8 % v/v sulfuric acid/methanol solution. After extraction and purification, the compounds were derivatized by PBA (4 % w/v in diethylether). After evaporation, the residue was redissolved in isooctane, centrifuged and injected in the GC-MS/MS equipment |

**Table 5.2** Method information as reported by the laboratories

| LABO-RATORY CODE | Injection technique | Stationary phase and dimensions analytical column | Analytical column | Calibration | Ionisation technique | Mass analyser/ detector |
|---|---|---|---|---|---|---|
| 1 | split-splitless | (40mx0.18mmx0.07µm) | Rxi-PAH, Restek article number 49316 | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 2a | PTV | (30mx0.25mmx0.25µm) | DB-5MS UI | calibration curve | EI | Quadrupole, GC-MS |
| 2b | PTV | (30mx0.25mmx0.25µm) | DB-5MS UI | calibration curve | EI | Quadrupole, GC-MS |
| 3 | | (20mx0.18mmx0.18µm) | Restek Rxi-5Sil | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 4 | split-splitless | 50% diphenyl, 50% dimethylpolysiloxane (30mx0.25mmx0.25µm) | Restek Rxi 17 | single point | EI | Quadrupole, GC-MS |
| 5 | split-splitless | 35% phenyl, 65% dimethylpolysiloxane (60mx0.25mmx0.25µm) | Agilent J&W DB-35ms | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 6 | PTV | DB5 Ultra inert (30mx0.25mmx0.25µm) | HP-5 19091 S 431 Agilent | matrix matched | EI | Triple quadrupole, GC-MS/MS |
| 7a | split-splitless | 5 % phenyl metilpolisiloxane (30mx0.25mmx0.25µm) | HP-5MS | Calibration curve | EI | Quadrupole, GC-MS |
| 7b | split-splitless | 5 % phenyl metilpolisiloxane (30mx0.25mmx0.25µm) | HP-5MS | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 8 | split-splitless | phenylmethyl polysiloxane (15mx0.15mmx0.15µm) | Agilent VF-5ms | single point | EI | Triple quadrupole, GC-MS/MS |
| 9 | split-splitless | 100% poly(dimethyl siloxane (30 m x0.25 mm x0.25µm) | Equity-1 Capillary GC column, Supelco | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 10a | split-splitless | 5% diphenyl - 95% dimethylpolysolixane of similar polarity (20mx0.18mmx0.18µm) | Fused- silica-GC-column | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 10b | split-splitless | 5% diphenyl - 95% dimethylpolysolixane of similar polarity (20mx0.18mmx0.18µm) | Fused- silica-GC-column | calibration curve | EI | Triple quadrupole, GC-MS/MS |
| 11a | split-splitless | Polyethyleneglycol (PEG) (60mx0.25mmx0.25µm) | HP innowax | calibration curve | EI | Quadrupole, GC-MS |
| 11b | split-splitless | 5% diphenyl, 95% dimethyl polysiloxane (30 m x0.25 mm x 0.25µm) | HP 5MS | calibration curve | EI | Quadrupole, GC-MS |
| 12 | PTV | (30m x 0.25mm x 0.25µm) | DB-35ms | calibration curve | EI | Triple quadrupole, GC-MS/MS |

## Annex 6. Results of the characterisation measurements

**Table 6.1.** Data from the characterisation for 3-MCPD fatty acid esters

| Dataset code and method | Replicate 1 [µg/kg] | Replicate 2 [µg/kg] | Replicate 3 [µg/kg] | Replicate 4 [µg/kg] | Replicate 5 [µg/kg] | Replicate 6 [µg/kg] | Mean [µg/kg] | Expanded uncertainty [µg/kg] |
|---|---|---|---|---|---|---|---|---|
| D06 GC-MS/MS | 43.1 | 42.3 | 45.1 | 48.1 | 45.0 | 48.0 | 45.3 | 9.1 |
| D05 GC-MS/MS | 50 | 57 | 49 | 47 | 51 | 49 | 50.5 | 18.2 |
| D08 GC-MS/MS | 63 | 63 | 64 | 63 | 61 | 64 | 63.0 | 23.9 |
| D01 GC-MS/MS | 69.66 | 67.64 | 69.66 | 71.51 | 69 | 70.25 | 69.6 | 13.9 |
| D10 GC-MS/MS | 69.6 | 69.3 | 70 | 71.6 | 72.4 | 66.1 | 69.8 | 21.0 |
| D02 GC-MS | 75.5 | 73.2 | 75.4 | 75.1 | 75.4 | 74.2 | 74.8 | 5.2 |
| D09 GC-MS/MS | 79.9 | 78 | 74.4 | 88.2 | 79.3 | 72.6 | 78.7 | 11.0 |
| D04 GC-MS | 80.5 | 80.8 | 80 | 80.7 | 76.1 | 79.1 | 79.5 | 5.1 |
| D03 GC-MS/MS | 85.8 | 73.1 | 85.0 | 79.0 | 82.0 | 83.7 | 81.4 | 32.6 |
| D07 GC-MS/MS | 92 | 94 | 92 | 88 | 87 | 83 | 89.3 | 21.4 |

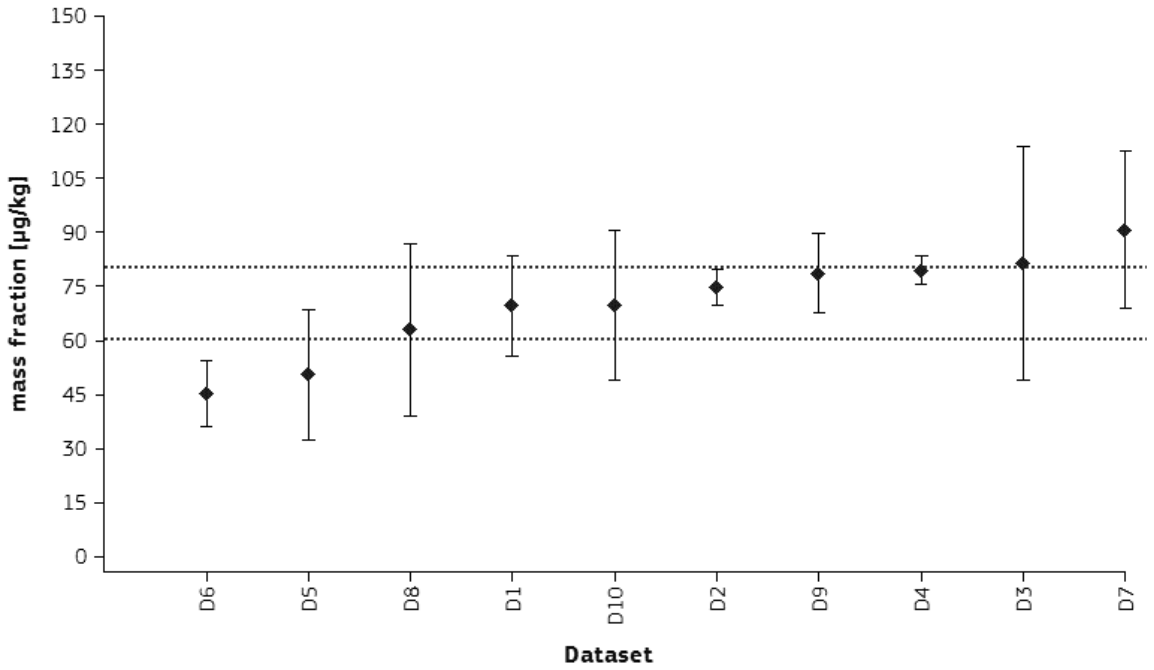
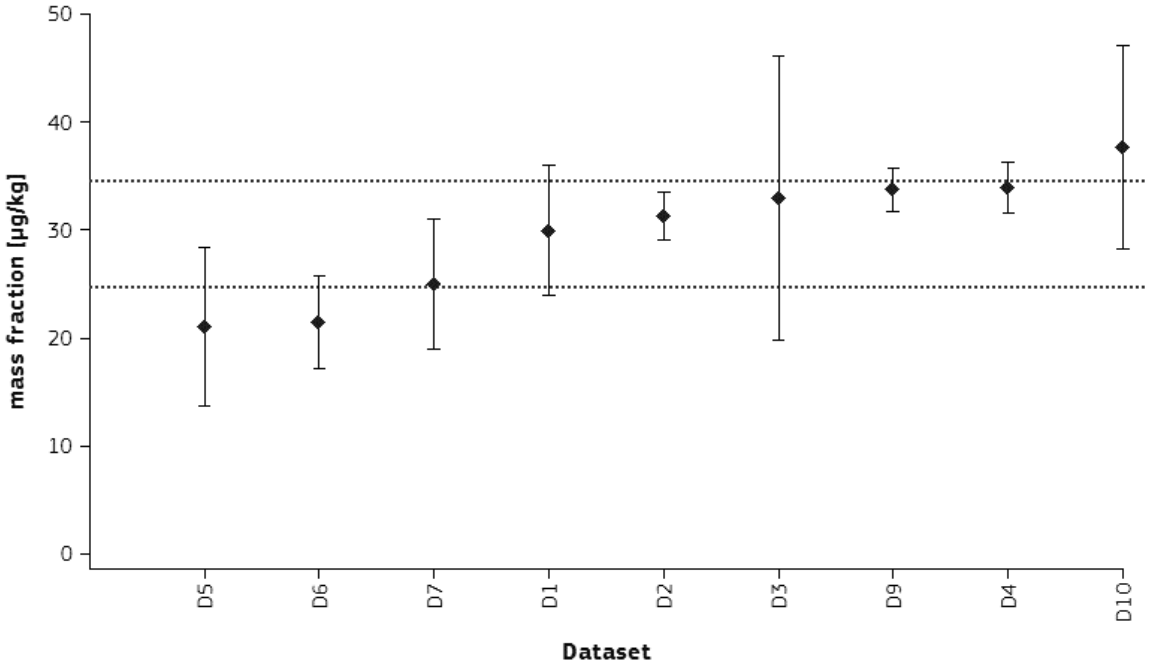


**Table 6.2.** Data from the characterisation for 2-MCPD fatty acid esters

| Dataset code and method | Replicate 1 [µg/kg] | Replicate 2 [µg/kg] | Replicate 3 [µg/kg] | Replicate 4 [µg/kg] | Replicate 5 [µg/kg] | Replicate 6 [µg/kg] | Mean [µg/kg] | Expanded uncertainty [µg/kg] |
|---|---|---|---|---|---|---|---|---|
| D05 GC-MS/MS | 19 | 21 | 20 | 23 | 21 | 22 | 21.0 | 7.4 |
| D06 GC-MS/MS | 21.3 | 21.1 | 20.8 | 21.9 | 21.8 | 21.9 | 21.5 | 4.3 |
| D07 GC-MS/MS | 28.4 | 28.3 | 28.5 | 25.2 | 17.3 | 22.3 | 25.0 | 6.0 |
| D01 GC-MS/MS | 27.92 | 22.72 | 36.85 | 24.83 | 31.11 | 36.38 | 30.0 | 6.0 |
| D02 GC-MS | 31.9 | 30.8 | 31 | 31.9 | 31.5 | 30.8 | 31.3 | 2.1 |
| D03 GC-MS/MS | 28.2 | 34.3 | 36.4 | 29 | 36.4 | 33.3 | 32.9 | 13.2 |
| D04 GC-MS | 33.7 | 34.1 | 34.2 | 34.4 | 33 | 33.9 | 33.9 | 2.6 |
| D09 GC-MS/MS | 33.3 | 34.9 | 34.1 | 34.6 | 32.3 | 33.2 | 33.7 | 2.0 |
| D10 GC-MS/MS | 36.1 | 37.3 | 40.2 | 40.5 | 37.4 | 34.7 | 37.7 | 9.4 |



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