
# Biodiesel quality assessment: an automated approach for analysis of free and total glycerol content in biodiesel (B100), according to the EN 14105 and ASTM D6584 methods


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## Keywords

Automated sample preparation, TriPlus RSH SMART autosampler, glycerol, biodiesel, B100, gas chromatography, GC, flame ionization detection, FID, EN 14105, ASTM D6584


## Goal

The aim of this study is to demonstrate the suitability the Thermo Scientific™ TriPlus™ RSH SMART robotic autosampler coupled to the Thermo Scientific™ TRACE™ 1610 GC series for automated sample preparation and on-line analysis of biodiesel according to the EN 14105 and ASTM D6584 methods.

## Introduction

Fuel-grade biodiesel is made from vegetable or animal oils through a chemical process of transesterification with alcohol (typically methanol) whereby glycerin is separated from the resulted mixture of free fatty esters (biodiesel). Once separated from glycerin, biodiesel can be blended with petroleum diesel in various concentrations, labeled as BXX, where XX represents the percentage of biodiesel in the mineral diesel. Typically, it can be used at a concentration of 5% (B5) up to 20% (B20) in diesel engines with little or no modifications. Biodiesel quality is critical to ensure a safe and satisfactory engine operation; therefore, during the production process it is important that reaction conversion yield, removal of glycerol, absence of poly unsaturated fatty acids (PUFA), removal of alcohol, and absence of unreacted glycerides are monitored.

The American Society for Testing and Materials (ASTM) and the European Standards (EN) have published analytical methods that are widely adopted to characterize impurities in pure biodiesel (B100) by using gas-chromatography (GC) coupled to flame ionization detection (FID):

- ASTM D6584 for the determination of residual total monoglyceride, total diglyceride, total triglyceride, and free and total glycerin content[1]
- EN 14105 for the determination of residual free and total glycerol and mono-, di-, triglyceride contents[2]
- EN 14110 for the determination of residual methanol[3]
- EN 14103 for the determination of total FAMEs (fatty acid methyl esters) and linolenic acid methyl ester (C18:3) content[4]

This application note focuses on methods EN 14105/ASTM D6584. Refer to AN001898[5] for results on method EN 14110 and to AN001899[6] for results on method EN 14103.

### EN 14105 and ASTM D6584

The determination of residual unreacted glycerides and free glycerol in pure biodiesel (B100) is critical for the biodiesel quality before blending with mineral diesel, as high glycerin content can lead to injector clogging and formation of deposits in injector nozzles, pistons, and valves. In particular, the ASTM D6751[7] and the EN 14214[8] standards set the quality thresholds for total glycerin content to 0.24% and 0.25% by mass, respectively. Prior to GC analysis for this determination, a sample preparation is required, which is complex, time-consuming, and exposes the user to hazardous chemicals. The possibility to automate manual procedures through a robotic autosampler offers the opportunity to increase sample-throughput with unattended operations, to increase data precision and reliability by removing possible human errors, and to decrease exposure to toxic reagents.

The two methods differ only for the use of the internal standards and the quantitative calculation, as it is explained in the dedicated sections.

## Experimental

In this study, a TRACE 1610 GC equipped with a Thermo Scientific™ iConnect™ programmed temperature vaporizing injector (iC-PTV) working in simulated cold on column mode (COC) and a Thermo Scientific™ iConnect™ flame ionization detector (iC-FID) were used. The sample preparation procedure and the injection into the GC were performed with the TriPlus RSH SMART autosampler.

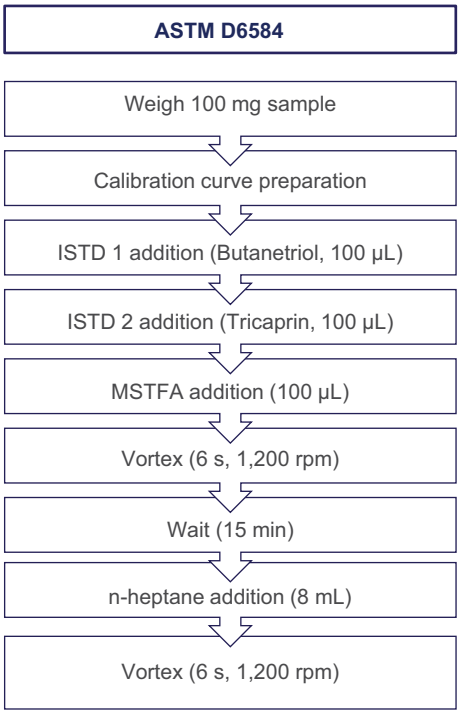
The GC analysis of free glycerol and glycerides in biodiesel requires a derivatization step with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) so that the target analytes are transformed into more volatile and stable silyl derivatives prior to injection. The TriPlus RSH SMART autosampler provides an advanced and built-in robotic solution that delivers high precision and accuracy, combined with the capability to fully automate simple sample handling operations up to more complex sample preparation workflows.[9] The autosampler also provides an additional layer of reliability and confidence in the analytical results thanks to the automatic SMART syringe identification and usage tracking capabilities.[10]

The sample preparation procedures described in the two official methods relevant for the determination of glycerol in biodiesel were automated by programming two dedicated prep cycles that include the full sequence of steps for both calibration curve preparation and sample derivatization. A schematic of the automated workflows for ASTM D6584 and EN14105 sample preparation is reported in Figure 1. A list of the required stock standard solutions and reagents is reported in Appendix 1. The TriPlus RSH SMART autosampler configuration required for calibration set up and sample derivatization is shown in Figure 2. A detailed description of the autosampler configuration, including a complete list of suggested consumables as well as standard and reagents, is reported in Appendix 2.

By using an automated approach to sample preparation, the analyst is only required to place the reagents, calibration stock solutions, and the required number of 10 mL headspace vials, each containing a 100 mg aliquot of biodiesel sample, in the autosampler tray. The automatic tool change (ATC) station available on the autosampler allows for automatic swapping between a dedicated 100 µL syringe, used for calibration curve preparation and derivatizing agent addition, and a 10 mL syringe used for heptane addition to quench the reaction. Once the samples are prepared, they can be automatically injected into the analytical system using a dedicated 10 µL syringe. The solvent station, with up to three 100 mL bottles, provides a reservoir for the addition of the required reagents. The vortex mixer allows for efficient mixing and derivatization of the samples. Dedicated vials are placed into the autosampler tray and used for syringe washing after the addition of each standard and reagent, thus minimizing the risk of carry-over and cross-contamination. The developed prep cycle allows for unattended preparation and injection of up to 30 samples.

Chromatographic separation was achieved on a Thermo Scientific™ TraceGOLD™ TR-BIOD column (15 m × 0.32 mm × 0.10 µm, P/N 26MB9-1932) with integrated guard column. This column ensures reliable and reproducible performance with low bleed even at elevated temperatures, making this column ideal for the analysis of free and total glycerol content according to ASTM D6584 and EN 14105 methods. Hydrogen was used as carrier gas, providing efficient chromatographic separation combined with short GC run times.

Additional GC-FID and autosampler parameters applied for the two methods are detailed in Appendix 2.




**ASTM D6584 calibration curve preparation**

| Commercial standard solution | Calibration curve levels (stock standard, µL) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Glycerin solution in pyridine (0.5 mg/mL) | 10 | 30 | 50 | 70 | 100 |
| Monoolein solution in pyridine (5 mg/mL) | 20 | 50 | 100 | 150 | 200 |
| 1,3-Diolein solution in pyridine (5 mg/mL) | 10 | 20 | 40 | 70 | 100 |
| Triolein solution in pyridine (5 mg/mL) | 10 | 20 | 40 | 70 | 100 |

**ASTM D6584 calibration ranges**

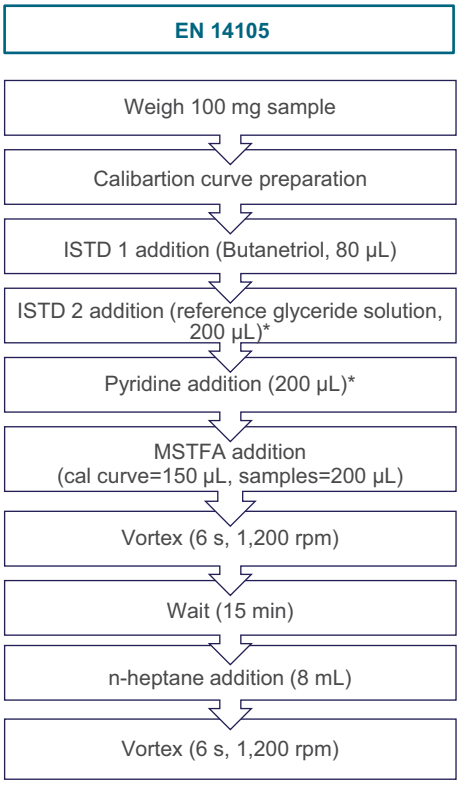
| Compound | Calibration curve levels (mg/mL) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Glycerin | 0.005 | 0.015 | 0.025 | 0.035 | 0.050 |
| Monoolein | 0.10 | 0.25 | 0.50 | 0.75 | 1.00 |
| 1,3-Diolein | 0.05 | 0.10 | 0.20 | 0.35 | 0.50 |
| Triolein | 0.05 | 0.10 | 0.20 | 0.35 | 0.50 |

**EN 14105 calibration curve preparation**

| Stock standard solution | Calibration curve levels (stock standard, µL) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Glycerin solution in pyridine (0.5 mg/mL) | 10 | 40 | 70 | 100 |

**EN 14105 calibration ranges**

| Compound | Calibration curve levels (mg/mL) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Glycerin | 0.005 | 0.02 | 0.035 | 0.050 |




* Reagent is added to samples only

**Figure 1. Schematic of automated sample preparation workflows for analysis of free and total glycerol content in biodiesel according to the ASTM D6584 and EN 14105 methods.** Calibration curves were prepared according to the tables.

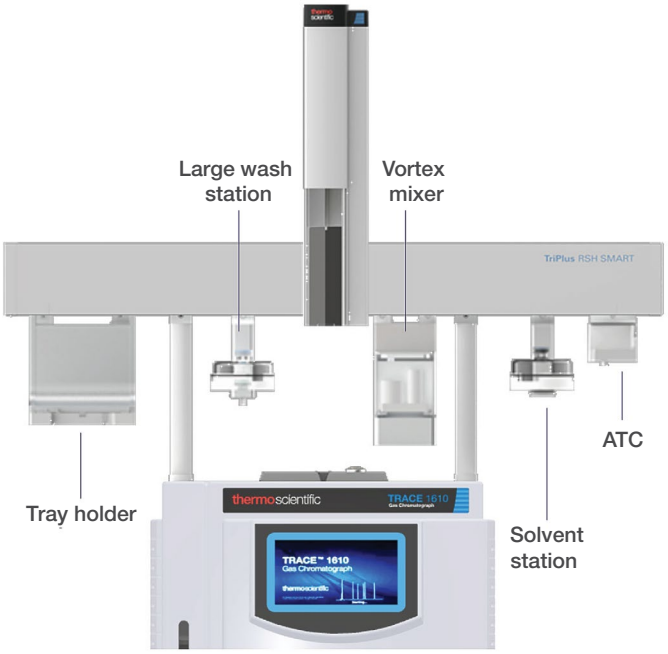



**Figure 2. TriPlus RSH SMART autosampler configuration for automated calibration curve and sample derivatization for assessment of free and total glycerol in biodiesel**

### Instrument control and data acquisition

For the experiments described here, Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS) was used. The instrument control is fully integrated in the CDS, ensuring a streamlined automated workflow from sample preparation to sequence set up, sample injection, and data acquisition with minimal user intervention. Moreover, with the ever-evolving compliance requirements for data integrity and data security, Chromeleon CDS provides a secure platform for analytical laboratories to comply with modern regulatory guidelines including FDA 21 CFR Part 11 and European Commission (EU) Annex 11.

## Results and discussion

### ASTM D6584-21 method

### Chromatography

The specific TG-BIOD metal column offers mechanical and chemical robustness for high oven temperature and low bleed, resulting in enhanced chromatographic performance with Gaussian peak shapes. The column is available with a pre-fitted guard column, ensuring a leak-free and seamless connection through a low volume connector and making the column installation a lot easier. The benefit of the guard column is to prevent any flooding effect and column contamination.

Peaks in the sample were identified based on retention time comparison with the reference standards (glycerin, monoolein, diolein, and triolein). To properly define the correct group integration windows for monoglycerides, a commercial solution containing monopalmitin, monoolein, and monostearin was also injected. An example of typical chromatograms for the reference standard mix and unknown biodiesel sample is shown in Figure 3. The inset shows the overlaid chromatograms of the monoglycerides solution and the unknown biodiesel sample.

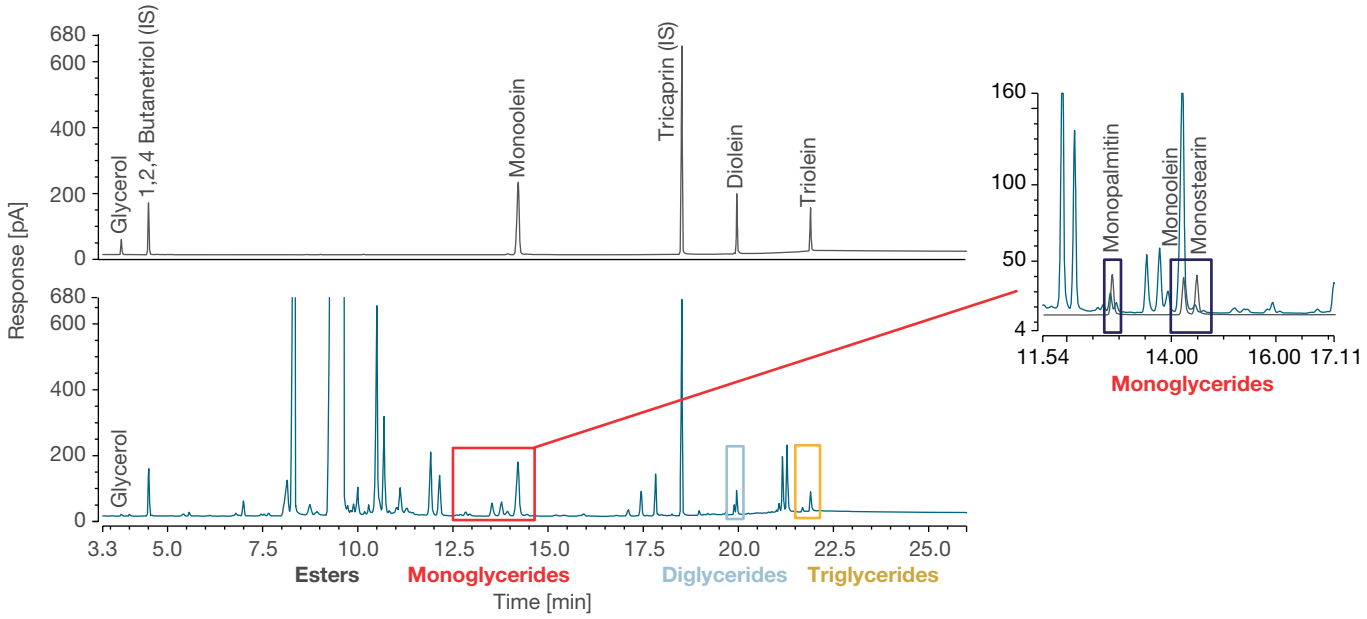



**Figure 3. Example of typical chromatograms for reference standard mix (gray trace) and unknown biodiesel sample (blue trace).** The inset shows the monoglycerides elution window with overlay of the monoglyceride standard solution (gray trace).

## Calibration

Linear response for glycerol, mono-, di-, and triglycerides was evaluated with individual calibration curves over five concentration levels in the range 0.005–1.00 mg/mL, using two ISTDs: 1,2,4-butanetriol for free glycerol and tricaprin for mono-, di-, and triglycerides. The automated preparation of the calibration curves included the derivatization step for each calibration level and resulted in a correlation coefficient ($r^2$) of 0.999 for single injections, exceeding the method requirement of 0.990, as shown in Figure 4. The correlation coefficient $r^2$ was calculated according to the Equation 1 reported in Appendix 4.

Quantitative analysis of free and total glycerol was assessed for an unknown biodiesel sample and for a certified reference material (CRM) and calculated by summing the free glycerin, the monoglyceride, the diglyceride, and the triglyceride portions, according to Equation 2 in Appendix 4. The results for the unknown sample and the CRM were within the limits of 0.24% required for B100, with values of 0.21% and 0.12%, respectively.

## Repeatability

The repeatability of the measurement of total glycerol along with free glycerol, monoglyceride, diglyceride, and triglyceride was assessed including fully automated derivatization and injection of n=21 samples. The repeatability results were calculated according to the definition reported in the method, which considers the difference between successive results, obtained by the same operator with the same apparatus under constant operating conditions on identical test material. The difference should not exceed, in more than one case on twenty, the average m% ± r, where r are the repeatability values calculated using the formula reported in the method[1] (Equation 3 in Appendix 4). The calculated repeatability met the acceptance requirements stated in the method as reported in the graphs and the tables in Figure 5. The overlaid chromatograms (n=21) for the analyzed samples are also shown.

## Carry-over

Carry-over was assessed by injecting a solvent blank at the beginning and at the end of a 30-sample sequence. The total absence of carry-over demonstrated that the complete elution of the heavy fraction of biodiesel was achieved in each sample run as shown in Figure 6.

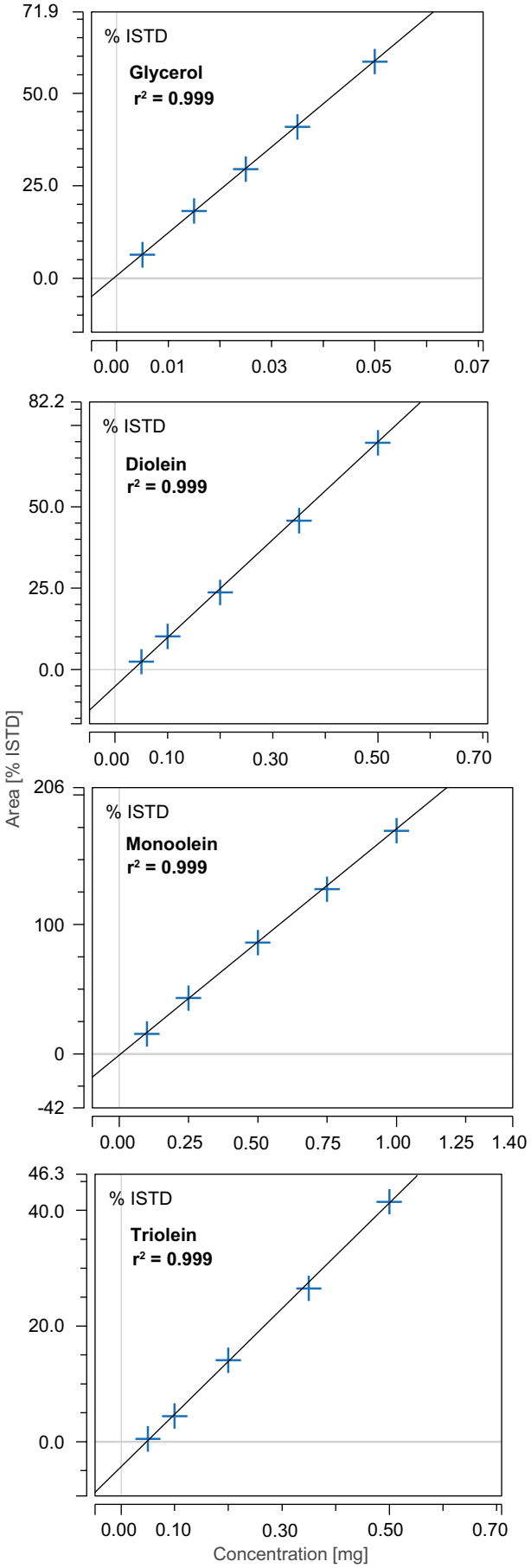
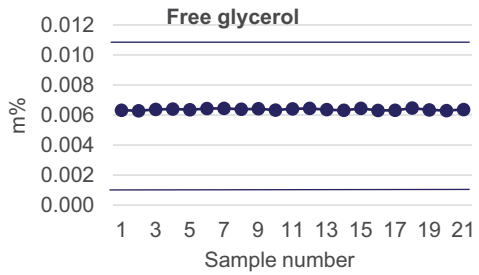
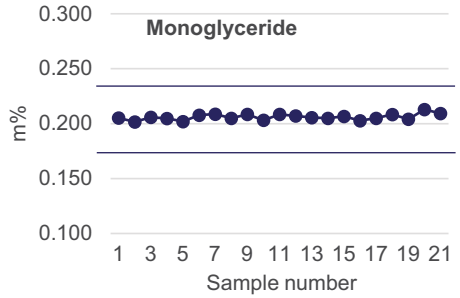
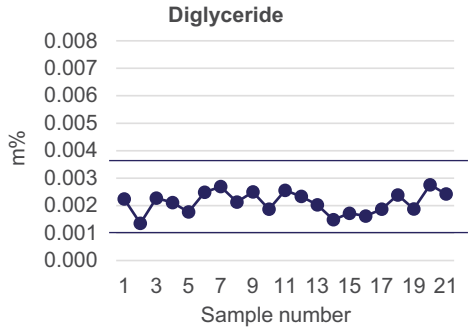


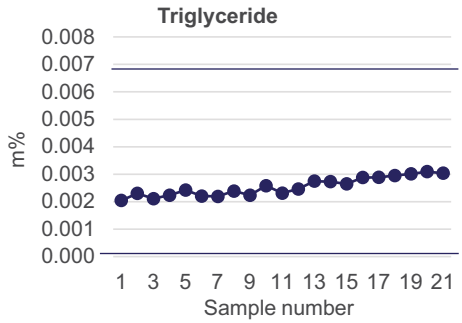

**Figure 4. Calibration curves obtained for glycerol, diolein, monoolein, and triolein after derivatization**

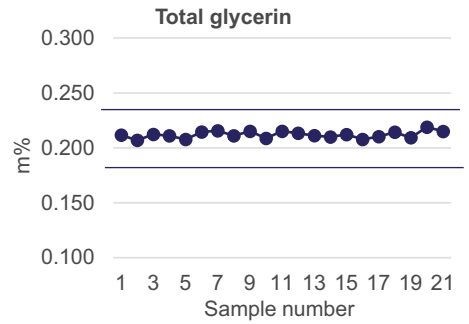
A




| Repeatability (n=21) | | | |
|---|---|---|---|
| **Component** | **Average m%** | **Average absolute difference** | **Calculated r** |
| Free glycerol | 0.006 | 0.0001 | 0.005 |
| Monoglycerides | 0.206 | 0.0034 | 0.029 |
| Diglycerides | 0.002 | 0.0005 | 0.001 |
| Triglycerides | 0.003 | 0.0001 | 0.004 |
| Total glycerin | 0.212 | 0.0041 | 0.024 |

B

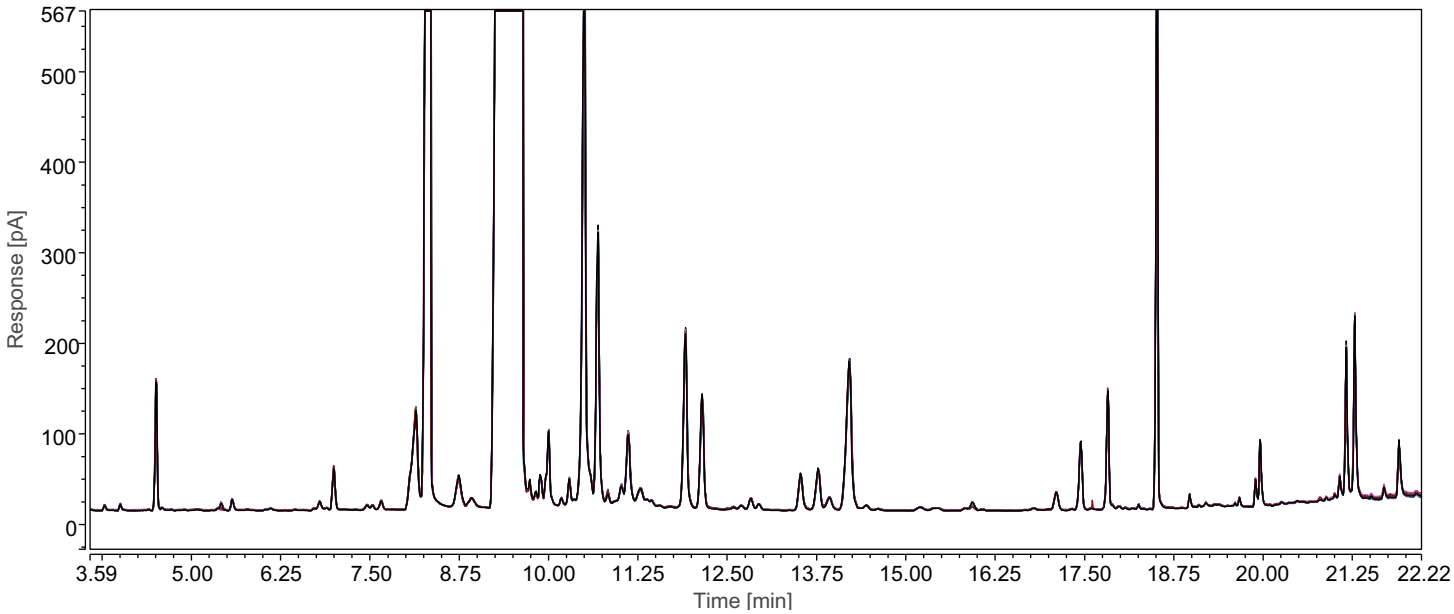



Figure 5. Repeatability assessed according to the ASTM D6584-21 standard by calculating the difference among n=21 replicates of the same sample (A); the overlaid chromatograms (n=21) for the analyzed samples are also shown (B).

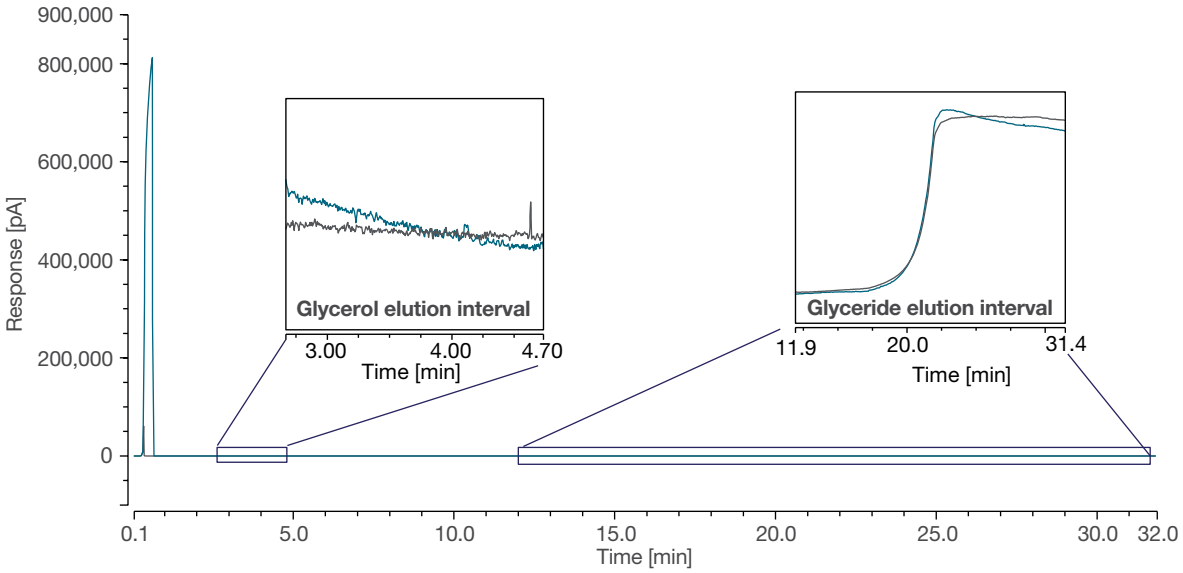



**Figure 6. Carry-over assessed by injecting a solvent blank at the beginning (gray trace) and at the end (blue trace) of a 30-sample sequence**

## EN 14105:2020 method

The EN 14105:2020 method differs from the ASTM method in the way the calibration is performed. It requires the preparation of a calibration curve for glycerol only using 1,2,4-butanetriol as ISTD, and it assumes that the detector response is linear within the considered concentration range for the other glycerides.[2] The individual glyceride content is calculated based on the response factor (RF) of a reference glyceride mix (1-glyceryl monononadecanoate (Mono C19), 1,3-glyceryl dinonadecanoate (Di C38), and glyceryl trinonadecanoate (Tri C57), 2.5 mg/mL each in tetrahydrofuran (THF)) used as internal standard. In this work a solution containing monoolein, diolein, and triolein was added to each sample instead of the glyceride internal standard solution, not immediately available for purchase, and used to evaluate the precision of the automated sample derivatization executed with the TriPlus RSH SMART autosampler.

### Chromatography

Typical chromatograms obtained for a biodiesel sample and a standard mixture of monoglycerides (monoplamitin, monoolein, and monostearin) including a glycerides solution (monoolein, diolein, and triolein) are reported in Figure 7.

### Calibration

As specified in the method, the calibration curve was generated for glycerol only. The automated preparation of the calibration curve included the derivatization for each level and resulted in a correlation factor r (calculated according Equation 1 in Appendix 5) of 0.999 for single injections, thus well exceeding the minimum requirement of 0.900 as reported in Figure 8.

The mass percentage (% m/m) of free glycerol was then calculated applying Equation 2 reported in Appendix 5.

### Repeatability

Repeatability for glycerol was assessed according to the definition reported in the method by preparing n=10 samples and calculating the absolute difference between two independent test results.[2] The repeatability (r) value was calculated by applying the formula specified in the method (Equation 3 in Appendix 5). The precision of the automated sample derivatization executed with the TriPlus RSH SMART autosampler was also evaluated by calculating the absolute peak area %RSD of the added glycerides solution. Absolute peak area %RSD was less than 2% for the target analytes, confirming that the advanced built-in robotics of the autosampler allows for reliable sample preparation and precise reagent dispensing. The calculated repeatability for glycerol and the absolute peak area %RSD obtained for the glyceride solution added to the sample are reported in Figure 9.

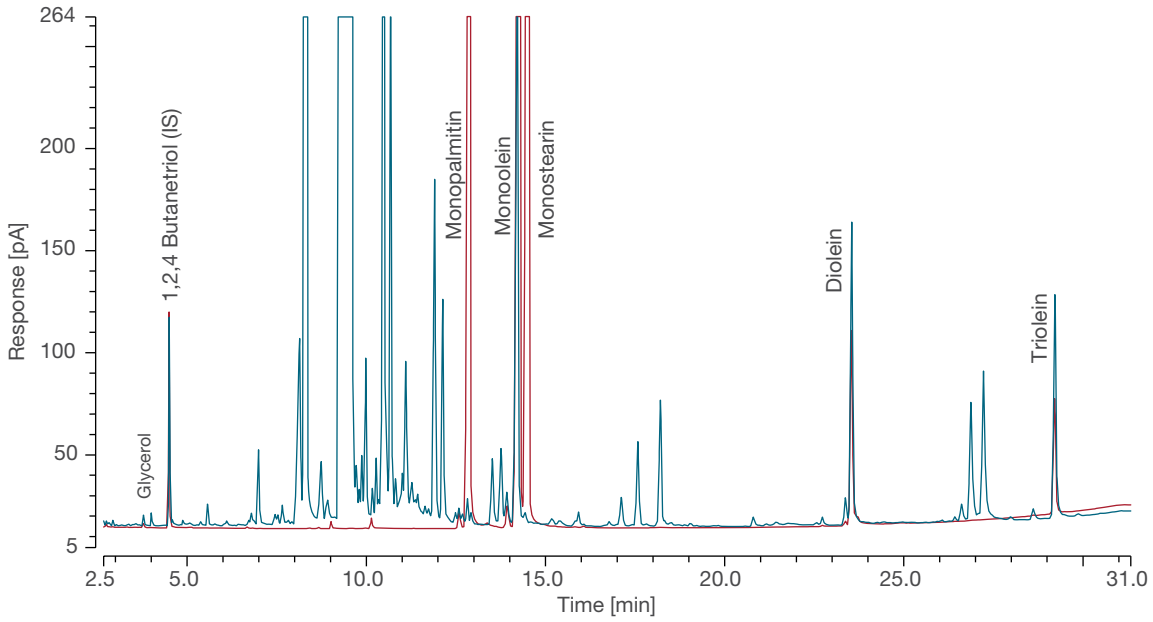



Figure 7. Overlaid chromatograms obtained for a biodiesel sample (blue trace) and glyceride-monoglyceride standard solution (red trace)

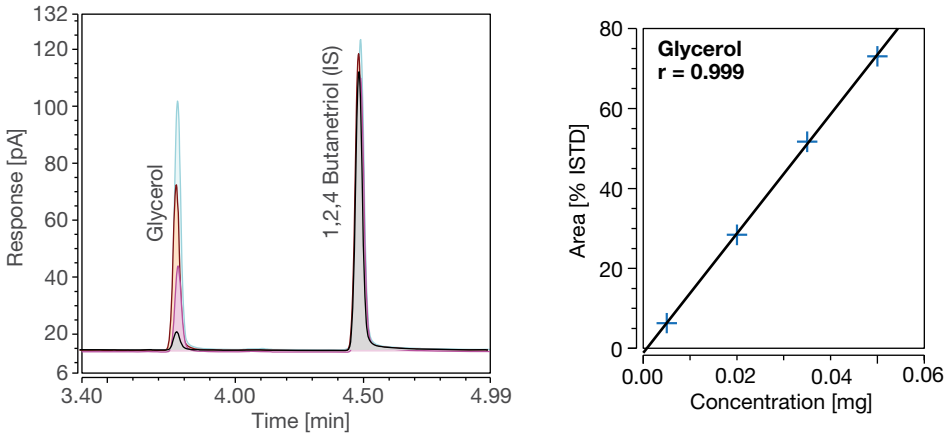



Figure 8. ISTD calibration curve for glycerol obtained with automated sample derivatization

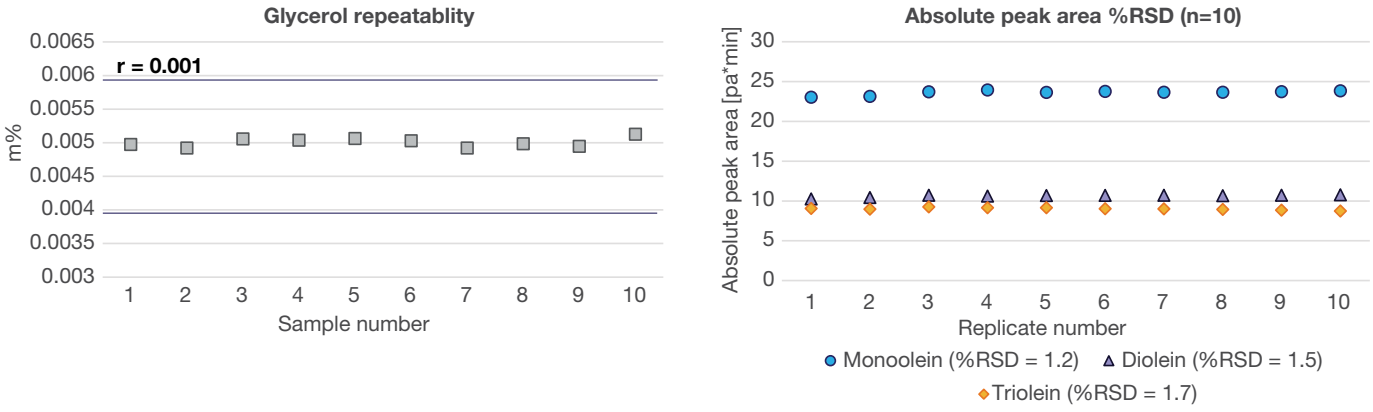



Figure 9. Repeatability calculated for glycerol by considering the absolute difference among n=10 repetitions of the same sample within a short interval as well as absolute peak area % RSD for glycerides solution added to the sample

## Conclusions

The results of these experiments demonstrate that the automated sample preparation capability of the TriPlus RSH SMART autosampler coupled to the TRACE 1610 GC provides a reliable and robust solution for laboratories performing quality control testing of biodiesel looking to improve productivity and deliver confident results.

- The flexibility of the developed prep cycle allows for automated sample preparation according to the ASTM D6584 and EN 14105 methods.
- The automated approach to calibration standards and sample derivatization allows for precise and reliable results exceeding the performance criteria stated in both methods and reducing the risk of errors and cross-contaminations and improving safety by limiting the user's exposure to toxic chemicals.
- Up to 30 raw biodiesel samples can be analyzed from sample preparation to sample injection with unattended operations, giving back to the user time for more valuable activities like data interpretation.

### References


1. ASTM D6584-21 Standard test method for determination of total monoglycerides, total diglycerides, total triglycerides, and free and total glycerin in B-100.
2. EN 14105 Fat and oil derivatives - Fatty acid methyl esters (FAMEs) - Determination of free and total glycerol and mono-, di-, triglyceride contents, September 2021.
3. EN 14110 Fat and oil derivatives - Fatty acid methyl esters (FAMEs) - Determination of methanol content, June 2020.
4. EN 14103 Fat and oil derivatives - Fatty acid methyl esters (FAMEs) - Determination of ester and linolenic acid methyl ester contents, December 2020.
5. Thermo Fisher Scientific, Application Note 001898: Biodiesel quality assessment: characterization of residual methanol in finished biodiesel (B100) by headspace sampling according to EN 14110 standard.
6. Thermo Fisher Scientific, Application Note 001899: Biodiesel quality assessment: determination of esters and linolenic acid methyl ester content in biodiesel (B100) by GC-FID, according to EN 14103.
7. ASTM D6751-20a Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels.
8. EN 14214:2012, A2:2019 Liquid petroleum products - Fatty acid methyl esters (FAME) for use in diesel engines and heating applications - Requirements and test methods, October 2021
9. Thermo Fisher Scientific, Guide to automated sample preparation for GC and GC-MS: EB000396
10. Thermo Fisher Scientific, TriPlus RSH SMART robotic sampling system brochure: BR52235-EN 0921C


## Appendix 1. List of standard stock solutions and reagents

| Standards and reagents | Supplier | Part Number |
|---|---|---|
| Standards and reagents | Supplier | Part Number |
| Biodiesel (B100) blank CRM | LGC | VHG-B100-BLK-500 |
| Tricaprin (1,2,3-tricaprinoylglycerol) | Restek | 33025 |
| (s)-(-)-1,2,4-Butanetriol | Restek | 33024 |
| MSTFA (N-metil-N-trimetilsililtrifluoroacetamide) | Restek | 35600 |
| Monoglyceride stock solution | Sigma-Aldrich | 49446-U |
| ASTM D6584 Monoolein solution | Sigma-Aldrich | CRM44893 |
| ASTM D6584 1,3-Diolein solution | Sigma-Aldrich | CRM44894 |
| ASTM D6584 Triolein solution | Sigma-Aldrich | CRM44895 |
| ASTM D6584 Glycerin solution | Sigma-Aldrich | 44892-U |
| Pyridine (>99%) | Fisher Scientific | 10795253 |
| Heptane (99%) | Fisher Scientific | 11438727 |
| Hexane (99%) | Fisher Scientific | 10677201 |

## Appendix 2. TriPlus RSH SMART autosampler configuration and suggested consumables for automated sample preparation of free and total glycerol in biodiesel

| Part number | TriPlus RSH SMART configuration for ASTM D6584 and EN 14105 | Qty |
|---|---|---|
| 1R77010-2003 | TriPlus RSH SMART Advanced Autosampler for liquid injections, regular rail(*) including:<br>- one universal liquid syringe tool, for syringes of 0.5, 1.0, 5, 10, 25, 50, or 100 µL with a 57 mm needle length (P/N 1R77010-1007)<br>- two 10 µL SMART syringes, 57 mm needle length, 26S gauge, cone needle type (P/N 365D0291-SM)<br>- one tray holder (P/N 1R77010-1021)<br>- three VT54 trays, for 54 vials/tray (P/N 1R77010-1023)<br>- one standard washing station with 5 x 10 mL vials (P/N 1R77010-1029)<br>(*) or equivalent TriPlus RSH base liquid / headspace configuration, in case of an existing instrument | 1 |
| 1R77010-1019 | Automatic Tool Change Station (ATC) Station<br>Up to two ATC stations can be configured on each TriPlus RSH SMART Advanced autosampler | 1 |
| 1R77010-1031 | Solvent Station 3 × 100 mL solvent bottles<br>Bottles with seal and caps are included | 1 |
| 1R77010-1030 | Large Wash Station for 2 × 100 mL solvent bottles and one waste position<br>Bottles with seal and caps are included | 1 |
| 1R77010-1033 | Vortexer Module<br>Suitable for 2-, 10-, or 20-mL vial | 1 |
| 1R77010-1022 | VT15 Vial Tray for 10/20 mL vials<br>Sample tray for 15 vials of 10- 20-mL. Vials are not included | 3 |
| 1R77010-1008 | Universal liquid syringe tool, for syringes of 5 µL, 10 µL, 25 µL, 50 µL, or 100 µL with a needle length of 85 mm<br>SMART syringes not included | 1 |
| 1R77010-1011 | Universal Liquid Syringe Tool for a 10,000 µL syringe with a needle length of 57 mm<br>SMART syringes not included | 1 |

For more details about orders and quotations, please contact your Thermo Fisher Scientific sales representative.

| Suggested consumables | Part number |
|---|---|
| 5 µL Fixed Needle SMART syringe<br>85 mm needle length, 26S gauge<br>**The on-column injection requires the use of a 26Ga needle syringe.** | 365C0241-SM |
| 100 µL Fixed Needle SMART syringe<br>57 mm needle length, 26S gauge, cone needle type | 365H2141-SM |
| 10000 µL Fixed Needle Gas-Tight SMART syringe<br>57 mm needle length, 19S Gauge | 365N2721-SM |
| Thermo Scientific™ SureSTART™ 10 mL Glass Screw Top Headspace Vials, Level 2 High-Throughput Applications | 6ASV10-1 |
| Thermo Scientific™ SureSTART™ 18 mm Precision Screw Caps, Level 3 High Performance Applications | 6PMSC18-ST2 |
| TraceGOLD TG-BIOD column, 15 m × 0.32 mm × 0.10 µm w/guard | 26MB9-1932 |
| PTV Silcosteel Liner for on-column injection | 45322052 |

## Appendix 3. GC and autosampler operating conditions for analysis of free and total glycerol in biodiesel

| TriPlus RSH SMART Autosampler parameters | |
|---|---|
| Injection volume (µL) | 0.5 |
| Fill strokes count | 10 |
| Pre-injection dwell time (s) | 0 |
| Post-injection dwell time (s) | 0 |
| Injection depth (mm) | 73 |
| Penetration speed (µL/s) | 10 |
| Injection speed (µL/s) | 50 |
| Pre-injection wash cycles | 0 |
| Post-injection wash cycles | 5 |
| Post-injection wash solvent volume (hexane, µL) | 2 |
| Sample rinse cycles: | 3 |
| Sample rinse volume (µL) | 2 |
| Syringe | 5 µL, 85 mm, 26 gauge* (P/N 365C0241-SM) |

* The OC injection mode requires the use of a 26 gauge needle syringe.

| TRACE 1610 GC parameters | | |
|---|---|---|
| **iC-PTV simulated cold on-column parameters** | | |
| Injection temperature (°C): | 50 with oven tracking | |
| Liner: | PTV Silcosteel liner for OC (P/N 45322052) | |
| Inlet module and mode: | PTV, simulated OC mode | |
| Septum purge flow (mL/min): | 1, constant | |
| Carrier gas, flow (mL/min): | $H_2$, 3.0 | |
| **Oven temperature program** | | |
| | ASTM D6584 | EN 14105 |
| Temperature (°C) | 50 | 50 |
| Hold time (min) | 1 | 1 |
| Rate (°C/min) | 15 | 15 |
| Temperature 2 (°C) | 180 | 180 |
| Rate (°C/min) | 7 | 7 |
| Temperature 3 (°C) | 230 | 230 |
| Rate (°C/min) | 30 | 10 |
| Temperature 4 (°C) | 380 | 370 |
| Hold time (min) | 10 | 15 |
| GC run time (min) | 31.81 | 45.81 |
| **FID** | | |
| Temperature (°C): | 380 | |
| Air flow (mL/min): | 350 | |
| $H_2$ flow (mL/min): | 35 | |
| $N_2$ flow (mL/min): | 40 | |
| **Analytical column** | | |
| TraceGOLD TG-BIOD with guard | 15 m × 0.32 mm × 0.10 µm (P/N 26MB9-1932) | |

## Appendix 4. Formulae applied for calculations according to the ASTM D6584-21 method

| Equation | Calculated parameter | Formula | Formula components |
|---|---|---|---|
| (1) | Correlation coefficient, $r^2$ | $(\sum xy)^2 / (\sum x)^2 (\sum y)^2$ | $x = Xi - \bar{x}$, where Xi = (Wi / Ws) and $\bar{x}$ is the average for all data points<br>$y = Yi - \bar{y}$, where Yi = (Ai / As) and $\bar{y}$ is the average for all data points<br>Wi = mass of compound, Ws = mass of internal standard<br>Ai = area of compound, As = area of internal standard |
| (2) | Total glycerin, TG | Free glycerin + $\sum GI_M$, $GI_D$, $GI_T$ | $GI_M$ = 0.2591 * ∑monoglyceride, mass %<br>$GI_D$ = 0.1488 * ∑diglyceride, mass %<br>$GI_T$ = 0.1044 * ∑diglyceride, mass % |
| | Free glycerin, G | $[W_{is1}/a_g]$ $([A_g/A_{is}] - b_g)$ [100/W] | G = %m of glycerin in sample<br>$W_{is1}$ = weight of 1,2,4-butanetriol, mg<br>$a_g$ = slope of the calibration function<br>$A_g$ = peak area of glycerin<br>$A_{is}$ = peak area of 1,2,4-butanetriol<br>$b_g$ = intercept of calibration function<br>W = weight of the sample, mg |
| | Individual glyceride, $GI_j$ | $[W_{is2}/a_{ol}]$ $([A_{glj}/A_{is2}] - b_{ol})$ [100/W] | $GI_j$ = %m of individual glyceride in sample<br>$W_{is2}$ = weight of tricaprin, mg<br>$a_{ol}$ = slope of the calibration function for mono-, di-, or triolein<br>$A_{glj}$ = peak area of glycerin<br>$A_{is2}$ = peak area of tricaprin<br>$b_{ol}$ = intercept of calibration function for mono-, di-, or triolein<br>W = weight of the sample, mg |
| (3) | Total glycerin repeatability, r | $0.76e^{-01} * TG^{0.73}$ | TG = total glycerin, mass % |
| (3) | Free glycerin repeatability, r | $0.195e^{-01} * (G+0.0001)^{0.27}$ | G = free glycerin, mass % |
| (3) | Monoglyceride repeatability, r | $0.78e^{-01} * GI_M^{0.62}$ | $GI_M$ = monoglyceride, mass % |
| (3) | Diglyceride repeatability, r | $0.344 * GI_D^{0.93}$ | $GI_D$ = diglyceride, mass % |
| (3) | Triglyceride repeatability, r | $0.12 * GI_T^{0.687}$ | $GI_T$ = triglyceride, mass % |

## Appendix 5. Formulae applied for calculations according to the EN 14105:2020 method

| Equation | Calculated parameter | Formula | |
|---|---|---|---|
| (1) | Correlation coefficient, r | $\frac{(N * \sum xy) - (\sum x * \sum y)}{\sqrt{((N * \sum x^2) - (\sum x)^2) * ((N * \sum y^2) - (\sum y)^2)}}$ | $x = (A_g / A_s)$<br>$y = (W_g / W_s)$<br>$A_g$ = peak area of glycerol,<br>$A_{is}$ = peak area of 1,2,4-butanetriol<br>$W_g$ = mass of glycerol, mg;<br>$W_s$ = mass of 1,2,4-butanetriol, mg<br>N = number of measures |
| (2) | Free glycerol, G | $[a_g (A_g / A_{ei}) + b_g] * (M_{ei} / m) * 100$ | $a_g$, $b_g$ = regression coefficients of the calibration function for glycerol<br>$A_{ei}$ = peak area of 1,2,4-butanetriol<br>$M_{ei}$ = weight of 1,2,4-butanetriol, in mg<br>m = weight of the sample, in mg |
| (3) | Free glycerol repeatability, r | 0.1615 * X + 0.0003 | X = average of two test results being compared |

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