Comparison of Temperature Programmable Split/Splitless and Cool On-column Inlets for the Determination of Glycerol and Glycerides in Biodiesel by Gas Chromatography with Flame Ionization Detection*

Application Note

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Abstract

The European Standard EN 14105:2011-07 is an analysis method for quantifying free glycerol and residual mono-, di-, and tri-acylglycerides impurities in biodiesel by gas chromatography[1]. The method specifies an "on-column injector or equivalent device" as the means of sample introduction. Cool on-column (COC) would appear to be an ideal choice, particularly for quantifying triacylglycerides, as it provides high quantitative accuracy and precision with minimal mass discrimination. However, there are a few drawbacks in using COC for this application. The relatively high concentration of the biodiesel in the prepared samples impedes solvent focusing of early eluting compounds such as glycerol, causing band broadening and shifts in retention time compared to the external calibration standards. More problematic is method robustness when using a metal retention gap. Repeated injections onto the retention gap cause the method control specification to fail within a small number of injections. As an alternative, a temperature programmable split/splitless (TPSS) inlet was investigated for performance equivalency. The results demonstrate that the TPSS yields concentration measurements indistinguishable from the COC inlet. In addition, the robustness of the TPSS far exceeds that of the COC inlet by eliminating the performance control failure and providing solvent focusing for the early eluting peaks.

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Introduction

Biodiesel is a fuel produced from a variety of renewable plant and animal lipids. It consists primarily of monoalkyl esters of fatty acids prepared by transesterification of triacylglycerides. The reaction is carried out using a two-step process of acid-catalyzed pretreatment to convert free fatty acids to alkyl esters, followed by based-catalyzed esterification with methanol yielding monoalkyl esters in the form of fatty acid methyl esters (FAME) along with free glycerol as a main reaction byproduct[2]. Purification to remove glycerol and residual methanol is critical, as these impurities greatly reduce fuel quality by contributing to corrosion and engine deposits. Other impurities may include small amounts of unreacted and partially reacted acylglycerides, which also affect fuel quality by reducing low temperature operability[3].

The European Standard EN 14105:2011-07 is an analysis method for determining the concentration of free glycerol and residual mono-, di- and tri-acylglycerides to ensure compliance with EN 14213 and EN 14214 quality standards[3]. The method specifies the use of a gas chromatograph with flame ionization detection. Solutes containing free hydroxyls (that is, glycerol, monoglycerides, and diglycerides) are derivatized to their trimethylsilyl (TMS) analogs to increase volatility, reduce polarity, and improve chromatographic stability. Even with silylation, the separation encompasses a wide volatility range of analytes. To elute the triglycerides within approximately 30 minutes, the method specifies the use of a high-temperature column capable of programming up to 400 °C. For practical considerations, this necessitates the use of either metal or high-temperature polyimide-coated fused silica columns. The method recommends the use of cool on-column (COC) or equivalent to minimize mass discrimination and provide optimal quantitative accuracy, particularly for the high-boilers. The COC inlet is well suited for this analysis, however, there are inherent limitations including susceptibility to column fouling due to buildup of nonvolatile contaminants and the potential of sample degradation due to retention gap or column activity[4]. Temperature programmable split/splitless (TPSS) inlets, such as the multimode inlet (MMI), are more versatile and can be used in a variety of sample introduction modes. In cold-splitless mode, TPSS inlets can produce results similar to COC in that it is less susceptible to mass discrimination and thermal decomposition compared to hot split/splitless injections[5,6], and is easier to maintain, requiring liner replacement instead of column clipping when a contamination threshold is reached[7].

In this study, the use of the MMI was investigated as an equivalent alternative to the COC for the determination of free glycerol and acylglycerides in biodiesel. Two sets of data were collected, one with the COC inlet and one with the MMI using the same high-temperature metal column. The performance of the inlets was compared based upon the qualitative and quantitative analysis of a B100 biodiesel standard reference material within the guidelines of EN 14105:2011-07.

Experimental

Instrumentation

All experiments were performed on the Agilent 7890 Series GC with flame ionization detection. The Agilent MMI and COC inlet were installed on the front and back positions, respectively. To eliminate differences in results when comparing COC and MMI data, the same Select Biodiesel UltiMetal column from Agilent was used for the analysis of each sample set with dimensions of 15 m length, 0.32 mm diameter, and 0.10 µm film thickness. For COC experiments, a 2 m length by 0.53 mm diameter UltiMetal retention gap was installed to allow on-column injection without autosampler modification. The retention gap was connected to the analytical column using an Agilent Capillary Flow Technology Ultimate union. For MMI experiments, the column was connected directly to the inlet without installing the retention gap. For both COC and MMI experiments, the same 5 µL syringe needle was used for sample introduction. (Refer to Table 1 for details regarding instrument supplies.)

Table 1. Instrument supplies.

Component	Supplies
Column	Select Biodiesel for Glycerides, 15 m × 0.32 mm, 0.10 µm
COC	UltiMetal tubing, 2 m × 0.53 mm COC insert for UltiMetal columns Ultimate Union Kit Septa, BTO, 5 mm
MMI	Dimpled liner, 2 mm id, 200 µL Septa, BTO, 11 mm
Syringe	5 µL, 23-26s tapered needle
Tools	UltiMetal tubing cutter End straightener for UltiMetal tubing Reamer

Reagents

Standards and duplicate samples were prepared according to the procedures given in EN 14105:2011-07 using the reagents listed in Table 2. A soy-based B100 standard reference material (SRM 2772) from NIST (Gaithersburg, MD) was chosen as a biodiesel sample. The same standards and samples were used in the evaluation of both COC and MMI inlets to eliminate bias that could be introduced through variability in sample preparation.

Table 2. Reagents.

Reagent	Kit
Silylating reagent N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)	Biodiesel MSTFA kit
Glycerol standards at 4-levels with 1,2,4-butanetriol internal standard in pyridine	Glycerol Calibration Standards Kit
Glyceryl monononadecanoin (Mono C19), 1,3-glyceryl dinonadecanoin (Di C38), and glyceryl trinonadecanoate (Tri C57) in THF.	Standard Glycerides Stock Solution in THF
Monopalmitin, monostearin, monoolein in pyridine	Biodiesel Monoglyceride Kit
B100 Biodiesel (soy-based)	Standard Reference Material (NIST, 2772)

Instrumental methods

Table 3 lists the instrument conditions. All settings, except inlet conditions, were kept the same for data collected with the COC and MMI inlets. For the COC, the column and retention gap were configured as a composite column in the method settings with both the retention gap and column heated in the oven temperature zone.

Table 3. Instrument method.

Component	Parameter settings
Carrier gas	Helium, 5.6 mL/min constant flow
Oven	50 °C for 1 minutes 15 °C/min to 180 °C for 0 minutes 7 °C/min to 230 °C for 0 minutes 10 °C/min to 370 °C for 4 minutes
MMI	Mode: Splitless Temperature program: 88 °C for 0.1 minutes 250 °C/min to 370 °C Purge flow to split vent: 9.6 mL/min at 2.5 minutes Septum purge flow: 3 mL/min
COC	Mode: Track oven Septum purge flow: 1 mL/min
Injection volume	1 µL
FID	Heater: 380 °C H_2 flow: 30 mL/min Air flow: 400 mL/min Makeup flow: 25 mL/min

Results and Discussion

Chromatographic comparison

Figure 1 shows a comparison of chromatograms generated with the COC inlet and MMI for the analysis of the B100 SRM. Indicated on the chromatograms are the *n*-heptane solvent peak, FAME elution range, glycerol, 1,2,4-butanetriol, Mono C19 internal standard (IS), Di C38 IS, and Tri C57 IS. The chromatograms are very similar in terms of retention and response with the exception of analytes eluting before the FAMEs. This is further illustrated in Figure 2, which shows expanded axis chromatograms of the target peak, glycerol, and internal standard (1,2,4-butanetriol) eluting before the FAME region for the COC inlet. The overlays demonstrate differences in retention and peak shape for the injection of a standard containing the target and internal standard in pure *n*-heptane compared to the injection of the B100 containing *n*-heptane in addition to a large fraction of FAMEs. For comparison, Figure 3 shows the overlays for the target and analyte peaks in pure *n*-heptane and B100 using the MMI.

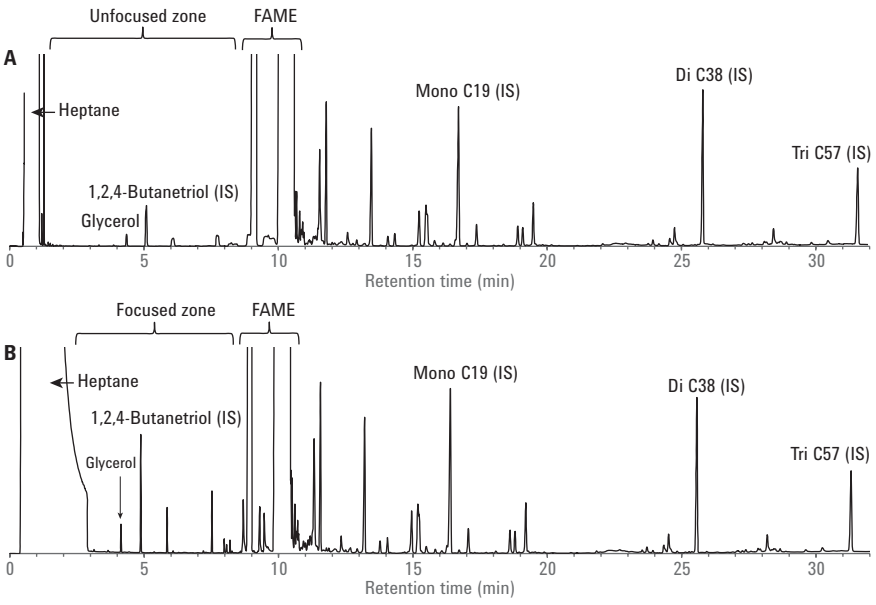

Figure 1. Comparison of chromatographs of B100 SRM using COC (A) and MMI (B).

Quantitative comparison

Figure 4 shows an overlay of calibration curves for glycerol obtained with the COC and MMI. As specified by EN 14105, the curves were generated using the relative response and relative concentration of glycerol with respect to 1,2,4-butanetriol in *n*-heptane. This curve is used to quantitate the amount of free glycerol in a sample of biodiesel. Table 4 lists the regression parameters. Quantitation of the remaining mono-, di- and tri-glycerides is based on the relative response of single-level standard spiked into the biodiesel sample. Total glycerol content is determined based on a weighted sum of mono-, di-, and tri-glyceride concentrations. Table 5 shows a comparison of the quantitation results of the B100 SRM using the COC and MMI.

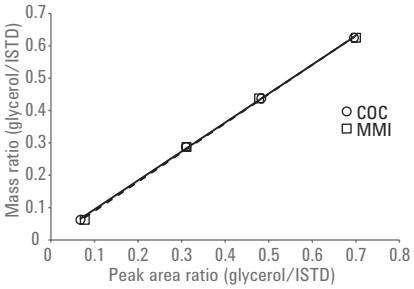

Figure 4. Comparison of TMS derivatized glycerol calibration curves using the COC inlet (solid line) and MMI (dashed line).

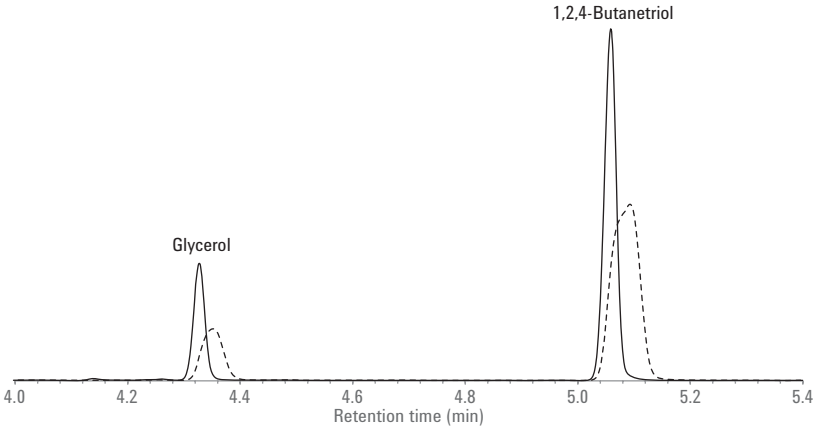

Figure 2. Comparison of derivatized glycerol and 1,2,4-butanetriol in pure heptane (solid line) and in B100 biodiesel (dashed line) using the COC inlet.

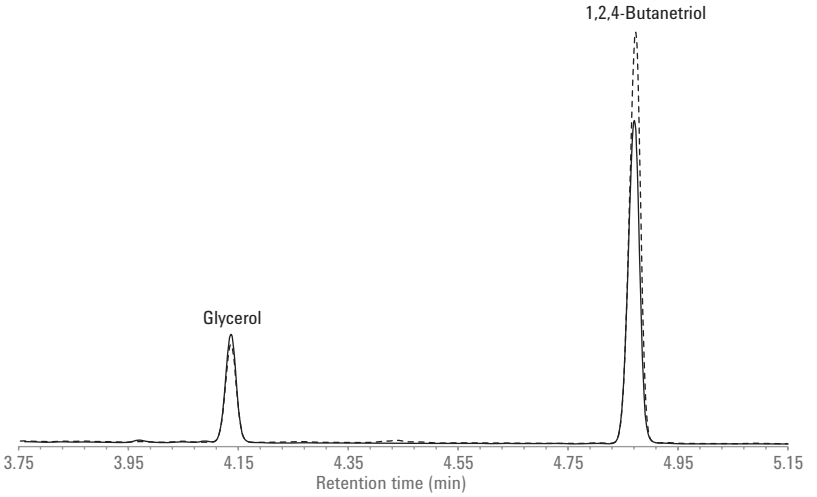

Figure 3. Comparison of derivatized glycerol and 1,2,4-butanetriol in pure heptane (solid line) and in B100 biodiesel (dashed line) using the MMI.

Table 4. Glycerol calibration parameters.

Inlet	Slope (± 95 % C.I.)	Intercept (± 95 % C.I.)	Standard error of regression	Correlation coefficient
COC	0.89 ± 0.04	0.01 ± 0.02	0.0045	0.99988
MMI	0.90 ± 0.09	0.00 ± 0.04	0.0097	0.99944

Table 5. Comparison of COC and MMI glycerol and glyceride composition in the B100 SRM

Analyte group	COC (wt%) ±95 % C.I.	MMI (wt%) ±95 % C.I.	Difference	EN 14105 Repeatability limit
Glycerol	0.015 ± 0.006	0.015 ± 0.006	0.000	0.003
Monoglycerides	0.24 ± 0.08	0.24 ± 0.08	0.00	0.03
Diglycerides	0.10 ± 0.03	0.11 ± 0.03	0.00*	0.01
Triglycerides	0.05 ± 0.05	0.05 ± 0.05	0.00	0.01
Total glycerol	0.096 ± 0.020	0.097 ± 0.020	0.001	0.007

* Subtracted before rounding.

Robustness comparison

For each analysis, EN 14105 requires a column performance control measurement to ensure adequate detection of the triglycerides. The method sets a maximum relative response factor (RRF) of 1.8 for the triglyceride internal standard (Tri C57) relative to the diglyceride (Di C38). If the RRF is greater than 1.8, then the instrument is not suitable for analysis. Figure 5 shows the RRF for both the COC and MMI inlet over the course of multiple injections of samples prepared from the B100 SRM. For the COC inlet, the RRF increases as a function of injection number until the threshold value is reached at the 8th injection. System performance was restored at the 9th injection by removing 5 cm of the retention gap. In comparison, injections on the MMI show a more stable RRF, and do not reach the threshold limit over the course of 16 injections. In fact, Figure 6 shows a series of 50 injections on the MMI with little to no variation of RRF, indicating a high degree of system stability over the course of multiple injections.

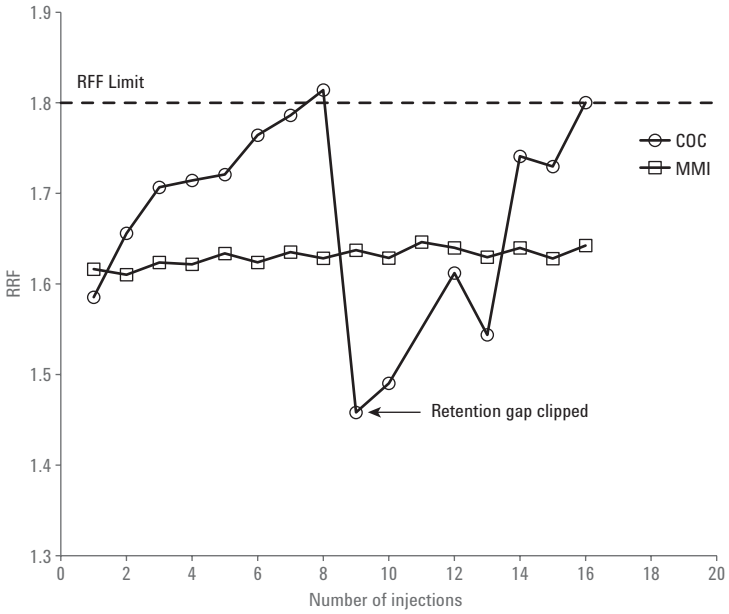

Figure 5. RFF as a function of injection number for COC and MMI.

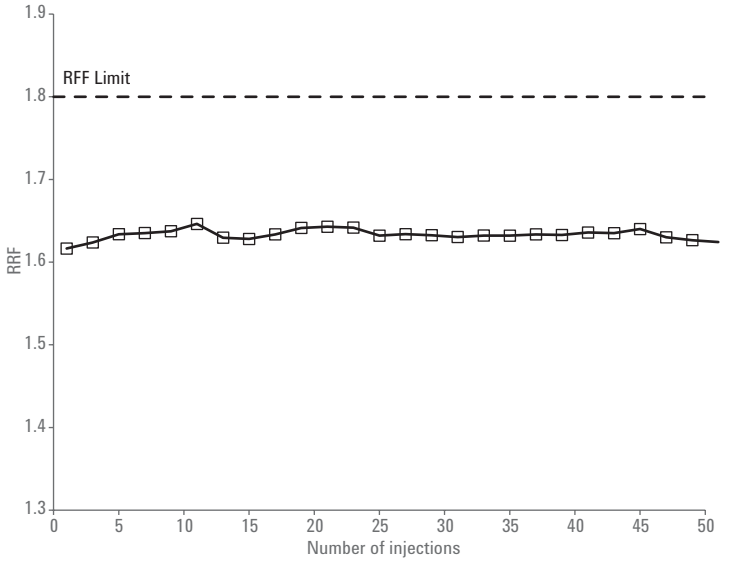

Figure 6. RFF as a function of injection number for MMI.

TPSS Inlet optimization

The 2 mm internal diameter dimpled liner was selected based upon previous experience in our laboratory. This liner is generally preferred for cold splitless injections where the sample matrix may contain small amounts of nonvolatile contaminants. The offset dimples prevent a direct pathway from the needle tip to the column, and provide a large surface area for trapping nonvolatiles.

Settings for the temperature program listed in Table 3 were based upon first principles, and were not optimized experimentally. The inlet temperature was set to 88 °C, which is 10 °C below the boiling point of the heptane solvent, to allow the sample to be introduced as a condensed liquid. The starting temperature hold time of 0.1 minute was selected to ensure delivery of the sample into the liner and withdrawal of the syringe before heating the inlet. The inlet programming rate was chosen to heat the inlet to match the final column temperature of 370 °C before the oven temperature reached the boiling point of the heptane to provide solvent recondensation.

The optimal inlet purge time was determined experimentally. Four purge times were evaluated: 1, 1.2, 2, and 2.5 minutes. The purge time of 2.5 minutes was selected because it provided the least amount of discrimination as determined by the relative response factor of Di C38 versus Tri C57 while maintaining baseline separation between the heptane solvent peak and the first eluting glycerol peak.

Chromatographic comparison

For the early eluting compounds, the difference in chromatographic performance for the separation of biodiesel samples using the MMI and COC inlet as exemplified in Figures 1–3 are largely a result in the mechanism of sample introduction. The primary mechanism of solute focusing for early eluters using the COC inlet with a retention gap is based upon solvent focusing (that is, solute migration from area of low retention to high retention). For optimal solvent focusing, the condensed solvent should evenly coat the walls of the retention gap just after sample introduction but before temperature programming, forming what is essentially a pseudo-stationary phase. Components of the sample with a high partition coefficient in the condensed phase but with slightly lower volatility will be refocused onto the head of the analytical column during temperature programming. As column temperature increases, the solvent is gradually vaporized, and migrates with the entrained lower volatility solutes. This allows the solutes to concentrate into a narrow band[6,7,8]. In the case of the biodiesel, the high concentration of FAME in addition to the *n*-heptane forms a binary solvent mixture coating the walls of the retention gap. Early eluters are partially solvated by the *n*-heptane and partially solvated by the FAME. In this case, as the column is heated, the early eluting solute bands are retained by the less volatile FAME. This causes the solute band dispersion for peaks eluting before the FAME peaks. The hypothesis is supported by comparing the glycerol and 1,2,4-butanetriol in the biodiesel mixture to the calibration standard for the COC inlet (Figure 2). The calibration standard is diluted in *n*-heptane providing an ideal solvent for focusing both the glycerol and 1,2,4-butanetriol, producing sharp chromatographic peaks compared to the same compounds in the B100 biodiesel in which FAMEs are present.

The disruption in solvent focusing observed for the COC was not observed using the MMI due to the different mechanism of sample introduction. For the MMI, the sample is introduced to a cool inlet liner followed by a fast inlet temperature program allowing a slight but significant differentiation in sample introduction onto the column based on volatility. The higher boiling point FAMEs are likely to reside in the inlet liner for a longer period of time, while the *n*-heptane, glycerol, 1,2,4-butanetriol, and other early eluters are transferred to the column in spatial proximity. This effect is sufficient to preserve focusing of the early eluters, and is illustrated in Figure 3, comparing the peak shapes of glycerol and 1,2,4-butanetriol in the biodiesel mixture and the calibration standard using the MMI.

Quantitative comparison

EN 14105 requires the calculation of free glycerol in a sample of biodiesel by means of a response curve. The response curve is constructed by plotting the concentration ratio of glycerol to internal standard versus the peak area ratio of glycerol to internal standard for four concentration levels. The data are fitted using an ordinary linear least squares regression model to determine slope and intercept parameters. According to the method, linearity of the curve is established if the correlation coefficient (R) is greater than or equal to 0.9. Figure 4 shows the calibration curves for both the COC inlet and MMI, and Table 4 lists the fitted model parameters slope, intercept, and correlation coefficient. Although not specified by the method, Table 4 also includes calculated confidence intervals for the slope, intercept, and the standard regression error for each data set. Visually, the response curves shown in Figure 4 for both data sets are virtually indistinguishable, and confidence intervals for the model parameters in Table 4 overlap considerably. Linearity for both data sets was established in excess of the minimum requirement of 0.9 for the correlation coefficient. However, the precision determined by the confidence interval in model parameters and standard regression error suggest that the COC inlet is more precise than the MMI. This is not surprising considering the mechanism of sample introduction of the COC inlet, which is considered the most accurate and precise injection technique[6].

In addition to glycerol, EN 14105 specifies the determination of mono-, di-, and tri-glyceride, and total glycerol concentration. As opposed to a response curve, calculations are based upon a single RRF for internal standards of each glyceride type. The total glycerol is reported as a percent concentration derived from a weighted sum of each constituent: free glycerol (*G*), mono- (*M*), di- (*D*), and tri-glycerides (*T*) as given by Equation 1.

$$GT = G + 0.255M + 0.146D + 0.103T$$

Equation 1.

The method also includes an estimation of measurement precision at the 95 % confidence limit in accordance with the statistical methods described in EN ISO 425911. Two precision metrics are assigned:

- **One for repeatability:** Single operator, single laboratory, and single instrument measurements
- **One for reproducibility:** Multiple operators, multiple laboratories, and multiple instrument measurements

For both metrics, the precision limit is obtained by calculating the expected precision based upon the results of a 16 laboratory study in which the variance in repeatability and reproducibility was determined. The calculated repeatability and reproducibility limits are compared to the differences in results of two measurements. In the absence of systematic error, it is expected that the differences in results are less than the precision limit. The repeatability metric was selected to compare the results of the COC and MMI as it provides a more rigorous comparison and higher precision threshold to achieve. Table 5 shows the results, along with the associated uncertainties at the 95 % confidence level. Uncertainties were calculated using the method specified in section 7.2.3 of EN ISO 4259[9]. Interestingly, the confidence intervals are greater than the repeatability precision interval. This is because the calculations of confidence intervals as specified in EN ISO 4259 incorporate both repeatability and reproducibility, where reproducibility is the dominant term. For each analyte group, the differences in COC and MMI results are less than the precision limit, indicating no discernable discrepancy in inlet performance. Based on the data analyzed, the results produced by the MMI appear indistinguishable from the COC in this study.

Robustness comparison

As demonstrated in Figure 5, the column performance control metric for the COC based on the RRF of triglyceride internal standard (Tri C57) relative to the diglyceride (Di C38) exceeds the 1.8 limit at the 8th injection, with performance being restored with the 9th injection by clipping the retention gap. It was hypothesized that the surface deactivation was removed by abrasion of the syringe needle during insertion into the metal retention gap. This was supported by the fact that only a small portion of the retention gap needed to be removed to restore performance, which was equal to the approximate depth of needle insertion into the gap (approximately 5 cm). Even with careful straightening of the retention gap, this effect could not be eliminated. This effect was not observed with the MMI (Figure 6), supporting the hypothesis that the loss in column performance was a result of the physical mechanism of sample introduction with the COC and not due to matrix fowling.

Conclusion

EN 14105 specifies the use of a COC inlet or equivalent device as a means of sample introduction for the analysis of glycerol and glycerides in biodiesel. The results presented in this Application Note comparing the MMI and COC provide compelling evidence that the threshold of equivalency is achieved with the MMI. In addition, the MMI provides better resolution for the early eluting compounds, and better robustness compared to the COC.

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