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Droplet digital PCR for routine analysis of genetically modified foods (GMO) – A comparison with real-time quantitative PCR

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ABSTRACT

Droplet digital polymerase chain reaction (ddPCR) has seen increasing applications in recent times, also in the analysis of genetically modified (GM) food and feed samples. While quantitative real-time PCR (qPCR) methods have been traditional mainstays till now, the applicability of ddPCR in routine analysis of GM food and feed has not yet been widely demonstrated. In this work, we applied ddPCR on selected GM-food and feed samples that were recently analyzed on the qPCR platform in inter-laboratory proficiency tests and showed good performance of the ddPCR method. Sometimes GM DNA at different transgene levels, useful as reference material is not readily available. Applying ddPCR, different concentrations of GM material, specifically transgene DNA at different levels (0.1–10%) useful as reference DNA, were generated from 100% non GM material and 100% transgene plant material respectively, and key performance parameters of the ddPCR assay evaluated. DdPCR performed well, indicating its suitability for the production of reference GM materials. In an expanded analysis, DNA extracted from a 100% GM reference soy plant (CV127) was appropriately diluted to low copy numbers and the absolute $LOD_{95\%}$ determined at 2 copies (nominal value), comparing well with various published qPCR assays. In our inhibition studies, ddPCR showed a clear advantage over qPCR in SDS-inhibited samples, while its tolerance to other tested inhibitors was comparable with qPCR. Significantly, the qPCR assays demonstrated more asymmetrical amplification/inhibition with EDTA as inhibitor, with unequal inhibition in reference and transgene reactions, while inhibition was more symmetrical on the ddPCR platform. Finally, a panel of positive reference material with varying GM content from 0.1 to 10% were evaluated on the ddPCR platform and pertinent performance parameters assessed, namely, precision, accuracy and trueness of results, with good performance of the assay.

1. Introduction

The analysis of genetically modified (GM) foods and feed has seen an increased surge in recent times. While the list of EU-approved GMO foods continues to grow, food and feed analysts are constantly evolving new detection (screening) and control strategies to keep up with the increasing analytical demands. Currently the gold standard in the detection and quantification of GMO events in food and feed is the quantitative real-time polymerase chain reaction (qPCR). Indeed several qPCR assays for GM analysis, with or without quantification, have been published to date (Holst-Jensen, 2009; Köppel, Bucher, Meuwly, & Moor, 2014; Köppel, Zimmerli, & Breitenmoser, 2010, European Union Reference Laboratory for GM Food and Feed (EURL-GMFF). While the majority of these assays are validated and applied as singleplex reactions, an increasing number of multiplex assays, detecting and quantifying simultaneously several GM events, have been described in recent years (Köppel, Velsen, Felderer, & Bucher, 2012; Park, Kim, & Kim, 2015; Shin et al., 2013; Wang, Teng, Guan, Tian, & Wang, 2015). Although most qPCR approaches generate reasonably good results with acceptable levels of precision, they usually rely on the use of standard curves for the determination of unknown GMO content. With ddPCR on the other hand, GM content can be determined by measuring concomitantly the ratio of transgene relative to reference gene in the sample under investigation without using standard curves, either as two singleplex reactions in the same analytical run or in a combined duplex system.

While ddPCR in GMO analysis is gradually gaining popularity,

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also in a multiplexing context (Dobnik, Spilsberg, Bogozalec Kosir, Holst-Jensen, & Zel, 2015; Huggett & Whale, 2013; Köppel & Bucher, 2015; Köppel, Bucher, Frei, & Waiblinger, 2015; Morisset, Stebih, Milavec, Gruden, & Zel, 2013; Pinheiro et al., 2012), it remains to be conclusively demonstrated whether partitioning of a single PCR reaction into many individual reactions, is as analytically good and reliable as the validated qPCR approaches that have been overwhelmingly applied till now. The main advantages cited for ddPCR include: 1) it enables the determination of absolute copy numbers, precluding the use of standard curves (Corbisier, Bhat, Partis, Rui Dan Xie, & Emslie, 2010), 2) it allows more sensitivity in low copy number ranges (Whale et al., 2012) and 3) it is more tolerable of PCR inhibitors (Burns, Burrell, & Foy, 2010; Dingle, Sedlak, Cook, & Jerome, 2013; Nixon et al., 2014). But how does it compare with established qPCR assays? In this manuscript we present our experiences with ddPCR and show its suitability in GM food and feed analysis. Some samples recently quantified on the qPCR platform, within the scope of proficiency tests and European Reference Laboratory (EURL) validation studies (organized by the Joint Research Centre, JRC of the European Commission), were separately analyzed with ddPCR in our lab and results compared. Additionally, we compared the responses of both systems (ddPCR vs. qPCR) to the presence of inhibitory substances, such as sodium dodecyl sulfate (SDS), ethanol and ethylenediaminetetraacetic acid (EDTA). Lastly, ddPCR was employed in the generation of GM-standards with varying concentrations of the transgene event, namely in a 0.1–10% range (specifically 0.1%, 0.5%, 1% and 10%), employing 100% GM free and 100% GM plant material.

While most qPCR assays published to date for GM analysis have been validated on the qPCR platform, we sought to run our ddPCR assays along the lines of these already validated parameters. All assays were however run in a duplex format to increase handling and speed of the analysis (e.g. due to less pipetting steps), while reducing cost and potential error sources associated with the analysis of a greater number of samples when running singleplex assays.

2. Materials and methods

2.1. Samples

Certified reference materials of diverse GM events were either purchased from AOCS (Urbana, USA) or from IRMM (Geel, Belgium). Multi-target plasmids for MON 87701, MON 88017 and MON 89034 were designed in-house and subsequently synthesized by Eurofins-MWG (Ebersberg, Germany).

2.2. DNA extraction

Genomic DNA was typically extracted from ground plant material with the Maxwell16 Instrument from Promega, (Mannheim, Germany), using a modified protocol (Guertler et al., 2013). For comparison of extraction methods, a few samples were simultaneously processed with the classical CTAB-protocol or with the Genespin DNA-Extraction kit (Eurofins-Genescan, Freiburg, Germany). DNA samples were further purified through cleaning columns (Eurofins-Genescan) prior to PCR.

2.3. Oligonucleotides

Oligonucleotide primers and probes, with appropriate quenchers, were synthesized by TIB MolBiol (Berlin, Germany) in HPLC grade. For oligonucleotide sequences for the GM events investigated in this work, the official EU method collection (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx) was consulted for sequence information and appropriate cycling parameters.

2.4. ddPCR

Amplification with ddPCR was performed with either a CFX96 or T100 PCR thermocycler with gradient function (Bio-Rad, Munich, Germany). The master-mix employed was the “ddPCR Supermix for Probes” (Cat. No. 186-3010, Bio-Rad), with a total reaction volume of 22 μl, comprising 1x master-mix, primers and probes at appropriate concentrations, and 5 μl sample DNA. Care was taken to employ primer and probe concentrations at previously validated concentrations, as deposited in the official EU method protocols, unless otherwise stated. 22 μl of the reaction mixture was loaded on eight-channel disposable droplet generator cartridges (Cat. No. 186-4008, gaskets Cat. No. 186-3009, Bio-Rad). Droplets were then generated with 70 μl of droplet generation oil (Cat. No. 186-3005, Bio-Rad) in the droplet generator of the QX100 system (Bio-Rad). Subsequently, the generated droplets were transferred to a 96-well PCR plate (TwinTec, Cat. No. 0030128.613, Eppendorf, Hamburg, Germany), strictly according to manufacturer's instructions. The transfer was carried out with an automatic 8-channel 50-μl-pipette (Rainin E8-50XLS+, filter tips Cat. No. 17002927, Mettler-Toledo, Giessen, Germany).

Following thermal sealing with pierceable foils in a plate sealer PXI (Bio-Rad, foil Cat. No. 181-4040), the following temperature profile was applied as standard for PCR cycling: 600 s at 95 °C, 45 cycles of 15 s at 95 °C, and 60 s at 60 °C. Following PCR, the sealed plates were processed in the droplet reader of the QX100 system (Bio-Rad), and the droplets analyzed according to manufacturer's recommendations (Droplet Reader Oil Cat. No. 186-3004).

2.4.1. Determination of GM content

In this work, all ddPCR assays were carried out in a duplex format, namely the concomitant generation of absolute copy numbers for both reference and transgene in the samples. Thresholds were manually adjusted when necessary and considered separately for both channels employed (FAM and HEX). Generally quantification was based on the formula outlined below, without zygosity correction:

$$x\,\% = \frac{DNA\ copy\ number_{transgene}}{DNA\ copy\ number_{reference\ gene}} \times 100\%$$

where x % denotes the proportion of the transgene in percentage (cp/cp), and DNA copy number$_{transgene}$ and DNA copy number$_{reference\ gene}$ denote the respective copy numbers of the transgene and reference gene as measured in the ddPCR assay.

2.5. qPCR

For the qPCR reactions, the MxPro real-time Cyler (Agilent Technologies, USA) and the ViiA7 PCR cycler (Applied Biosystems, USA) were applied. With the MxPro real-time PCR cycler, the following components were required for a 25 μl reaction volume: 2x Quantitect Multiplex PCR NoROX reagent (Qiagen, Hilden, Germany), 5 μl template DNA and validated primer and probe concentrations with the standard cycling protocol previously mentioned in Section 2.4. On the ViiA7 PCR cycler platform, 2 × universal mastermix (ROX, UNG) was employed at validated primer and probe concentrations (total reaction volume of 25 μl with 5 μl template DNA) standard cycling protocol.

2.6. Analysis of data

QuantaSoft software (Version 1.7.4.1118 from 01.01.15 and

Version 1.7.4.0917 from 08.07.2015, Bio-Rad) was used for data analysis of the ddPCR analyses. Each experiment was individually analyzed and the threshold manually adjusted on a well-by-well basis. For experiments conducted on the qPCR Cycler Mx3005P, the accompanying MxPro Software (Version 4.1) was used for data analysis, while the QuantStudio real-time PCR software (Version 1.1) was applied on the ViiA7 PCR cycler platform.

2.7. PCR-inhibition assays

Three known PCR-inhibitors were analyzed in this work, notably SDS, ethanol and EDTA. As DNA template, an artificially synthesized plasmid (p87701) was employed, with equal copy number for the plant-specific reference gene lectin and the soy transgene event MON 87701 (one copy each). Reactions were spiked with 1,000 nominal copies per reaction of the p87701 plasmid, yielding in uninhibited samples, equivalent proportions (1,000 copies) of the reference gene lectin and the transgene MON 87701.

Titrations of the inhibitor were added in increasing concentrations to the PCR reaction mix, replacing water, at the following end-concentrations: 0.001%–0.01% v/v for SDS, 0.1 mM–1 mM EDTA and 0.0625%–1% v/v for EtOH, and the samples analyzed, according to standard PCR cycling parameters (see 2.4). For the parallel qPCR assays, the Mx3005P cycler was employed, and a standard curve comprising five different DNA concentrations, ranging from 25,000 cp to ≈100 cp in triplicates was applied for relative quantification.

2.8. Verification of GM reference material

Several GM reference materials were analysed with the ddPCR assay to verify the declared GM content given in g/kg. The reference materials were either analysed in a dedicated ddPCR analytical assay or included as positive controls in the various ddPCR runs described in this work. Typical validation parameters were determined such as precision, accuracy and trueness of the results, according to the provisions specified in the ENGL Guidelines (2008).

2.9. Production of different concentrations of GM material

For the generation of different concentrations of GM material, particularly when these GM levels are not readily available, ddPCR was applied in this study. As published in the guidance document from ENGL on method verification (JRC-IHCP & ENGL, 2011), this is achieved by measuring the reference gene copy number of a GM positive and a GM negative DNA solution on the same plate, applying qPCR. The dilution factor for the two DNA solutions is then subsequently calculated and different concentrations of the GM positive material appropriately determined.

Using this approach, different concentrations (0.1%, 0.5%, 1% and 10%) of several GM events were prepared with ddPCR, notably for maize (MON 88017, MON 87460, MON 89034 and MIR162) and soya (CV127, MON 87701, and MON 87705), according to the above mentioned ENGL document.

In an expanded analysis, DNA from the applied CV127 (100% certified material) was appropriately diluted to yield 5,000, 2,500, 1,000, 500, 100, 50, 20, 10, 5, 2, 1 and 0.1 copies per ddPCR reaction. In three independent runs under repeatability conditions, the LOD_6 was determined, defined as the lowest dilution level (genome copy equivalent) where 6 from 6 replicates yielded a positive reaction.

The $LOD_{95\%}$ which is defined as the LOD at which the analytical assay detects the presence of the analyte at least 95% of the time (59/60 positive, with ≤5% false negative results) was also determined (ENGL 2008).

3. Results and discussion

3.1. ddPCR analysis of samples containing varying percentages of GM plant material

In this work, we analyzed GM samples arising from previous proficiency tests (EURL-GMFF, 2015a,b). The samples were previously analyzed with qPCR on the ViiA7 cycler and again with ddPCR on the Bio-Rad QX100. While the qPCR assay was carried out as two singleplex reactions, the ddPCR was run in a duplex format. The GM contents of two soya samples, positive for two transgene lines, DP356 and DAS68416, against the backdrop of the reference gene lectin (Fig. 1) were determined in a first analysis. Very little dispersion was observed with both methods in the determined GM components of the samples, with the precision of the results determined at 2.56–2.69% and 4.43–4.84% respectively for the two transgene events DP356 and DAS68416.

In another recent comparative test on the detection and quantification of GM events in instant soup and soybean flour EURL-GMFF 2015, two GM lines were investigated namely, the soy event DAS81419 and the oilseed rape event MON 88302 (EURL-GMFF, 2015a). In our lab, the samples were analyzed in parallel with ddPCR and qPCR, as previously outlined, and compared with the nominal value or the robust mean as published in the official JRC Technical Report (Fig. 1). The detected soybean event was in the range of 0.93–0.96% on both formats, against the robust mean of 0.99%, while the oilseed rape event was in the range of 1.06–1.2%, against the nominal value of 1.16%. The measured precision was between 3.03 and 6.80% across both transgene events, showing good performance of both methods.

3.2. Verification of the GM content of several GM reference materials

In order to assess the suitability of ddPCR assays for the accurate quantification of several transgene events in reference materials, appropriate reference transgene DNA covering the dynamic range of published PCR assays were quantified in this work. Table 1 summarizes the results of this assay. The precision, accuracy and trueness of the results lie well within the acceptance criteria of ±25% (ENGL, 2008).

3.3. Impact of inhibitors on PCR employing real-time and droplet digital platforms

For a PCR assay to perform optimally, the reaction must be reasonably free of inhibitors that may co-precipitate with DNA. In order to assess the tolerance of ddPCR to common PCR inhibitors, a panel of such inhibitors was investigated in this work, comprising SDS, ethanol and EDTA in varying concentrations. As DNA template, we employed an artificially synthesized plasmid (p87701), containing 1,000 nominal copies of the reference gene *lec* and the soya transgene event MON 87701. At the SDS concentrations tested, ddPCR appeared to be more stable in performance, compared to the real-time qPCR assay (Fig. 2, panel 3). The SDS concentrations tested were in the range of 0.001%–0.01% (end concentration), with the SDS-solution, directly added to the master mix in place of water (see 2.6). While on the ddPCR platform little inhibition was observed in this range, the qPCR assay showed significant instability (Fig. 2) starting with the 0.007% SDS concentration. That SDS impacts more negatively on qPCR assays, compared to ddPCR was previously reported in a few assays. In the work of Dingle et al. (2013) for example, the tolerance of ddPCR vs. real-time qPCR was investigated on a panel of inhibitors, including SDS. The authors observed more SDS-inhibition with qPCR, compared with

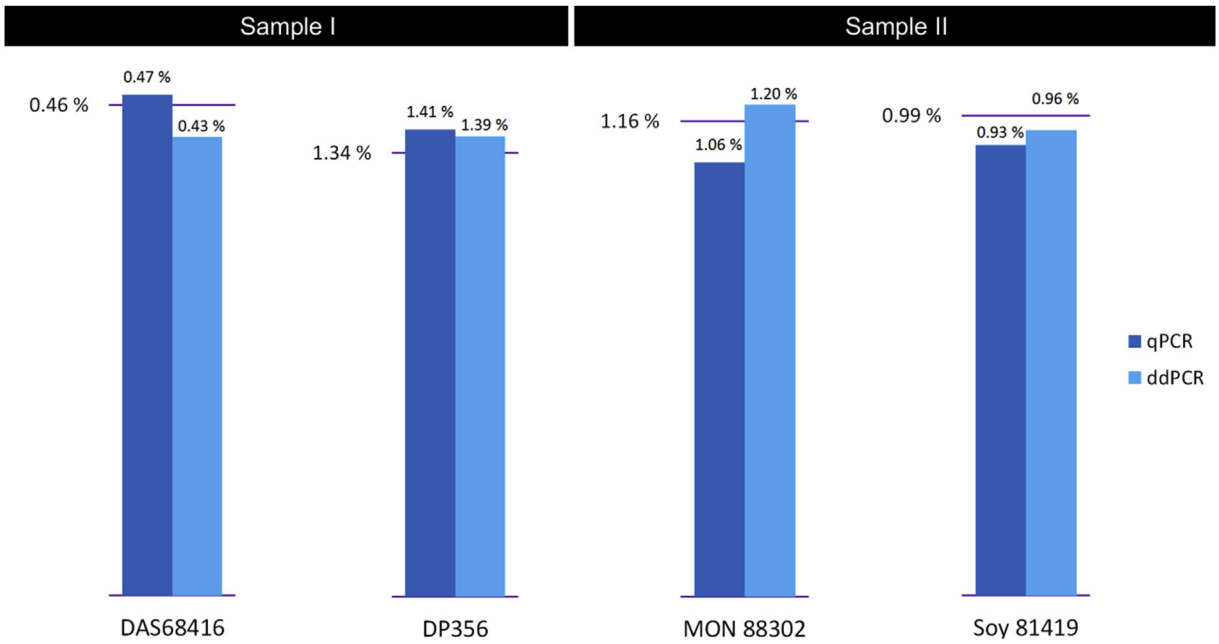

Fig. 1. Test sample I) containing the transgene events DP656043 and DAS 68416 and test sample II) containing MON 88302 and Soy 81419 in different matrices were independently quantified with 1) light blue bars: ddPCR on the QX100 and T100 Cycler, and 2) dark blue bars: qPCR on the ViiA7 PCR cycler. The horizontal bars at the tops of the diagrams represent robust means (or certified values) of the results arising from the internationally coordinated proficiency test. The measured GM quantities showed minimal dispersion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Precision, accuracy and trueness from analysis of GM reference material from different GM events containing 0.1–100% GM material as measured by ddPCR. Results were compiled from multiple runs with an average of at least 15 measurement points or test results.

Actual GM content (%)	Measured GM content (%)	Precision (%)	Accuracy (%)	Trueness (%)
100	100.80	5.14	5.03	0.57
10	10.34	4.22	4.52	2.42
1	0.94	7.18	7.61	3.99
0.5	0.49	6.15	6.13	1.28
0.1	0.10	5.18	5.02	2.39

ddPCR. While SDS impacts on the activity of DNA polymerase in the reaction mix, reaction partitioning through digitization and endpoint PCR may substantially decrease inhibition effects in this instance. Our data corroborates the assumption that sectioning the reaction mix into individual microreactions may lessen the impact of inhibitors on PCR amplification, by ensuring that discernible positive signals are detected in some reaction partitions, even in the global presence of inhibitors in the reaction mix. In contrast, while qPCR reactions are not partitioned into microreaction subsets, the end concentration of the inhibitor in the reaction mix will influence the overall PCR performance. At significant levels of inhibition, the amplification cycles required to attain a signal above a given threshold will increase, leading to inaccurate estimation of template in the sample, for example due to false negatives (Dingle et al., 2013).

Another useful tool of ddPCR on the QX platform is the visualization of the results as amplitude plots. When inhibition is suspected, a lower threshold can be applied close to the negative droplets to appropriately include a subset of partially inhibited samples (sometimes referred to as "rain"), while a higher threshold, placed close to the clustering of positive droplets would be more appropriate for uninhibited samples (Dingle et al., 2013). Interestingly, the degree of sensitivity to SDS of transgene and reference gene showed a slight asymmetry (Fig. 3), with more rain (intermediate signals between clearly positive and negative signals) visible with the transgene in the FAM channel, compared with the reference gene lectin in HEX.

With EDTA as inhibitor, this asymmetrical distribution of positive droplet populations in both channels was even more pronounced. Here we observed, an almost constant droplet population for both transgene and reference gene in samples with moderate EDTA-inhibition (0.1 mM – 1 mM EDTA) on the ddPCR platform, with the quantified GMO content between an acceptable range of 80–91% (Fig. 2, panel 1). With qPCR however, the reference gene content was not significantly affected by increasing EDTA concentration, and was relatively stable over the tested range (data not shown). In contrast, the transgene content was quite unstable with increasing EDTA concentration on the qPCR platform, with a marked overestimation observable (Fig. 2, panel 1, transgene content varied between 109% to a high 161%, compared with a nominal 100%). Interestingly, starting from an EDTA end-concentration of 2 mM, the PCR reaction was severely impaired on both platforms (data not shown). Our inhibition study with EDTA suggests that the often touted advantage of digital PCR over conventional real-time qPCR should be more rigorously investigated when sample inhibition is suspected. A useful approach is to apply at least two dilutions of the DNA samples to be analysed to the PCR reaction. While ddPCR was more resistant to SDS than qPCR (Fig. 2), qPCR showed more resistance to the deleterious effects of EDTA than ddPCR, although significant asymmetrical effects were observed

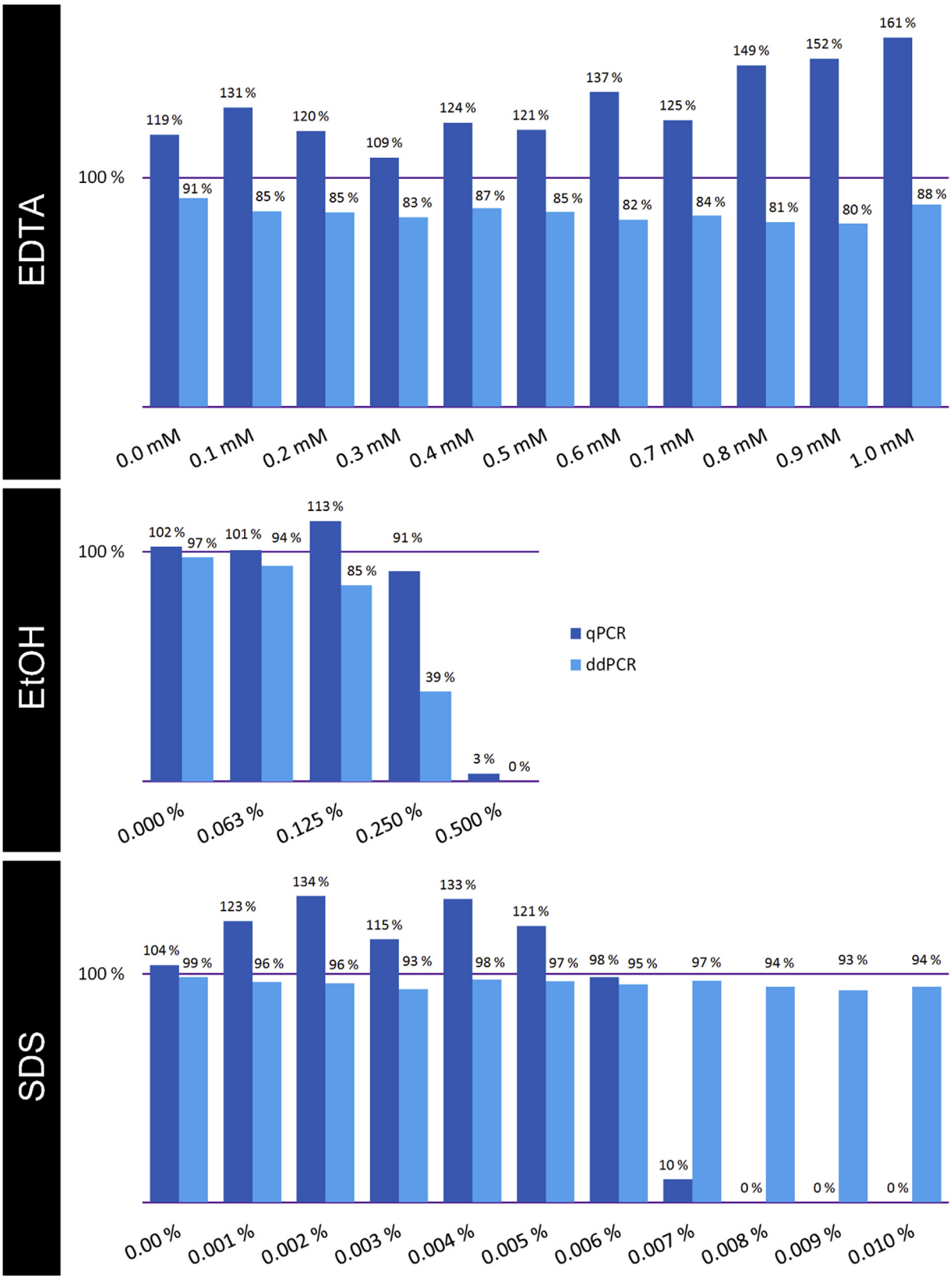

Fig. 2. Assessment of the impact of three inhibitors (EDTA, EtOH and SDS) on PCR performance on the real-time qPCR and ddPCR platforms. PCR assay was performed with a p87701 plasmid containing equivalent proportions (1000 copies each) of the MON 87701 transgene and the reference gene lectin.

with the latter at higher EDTA concentrations.

This finding corroborates the observation by Nixon et al. (2014) who carried out a comparative study of sensitivity, linearity, and resistance to inhibition of digital and qPCR for quantification of

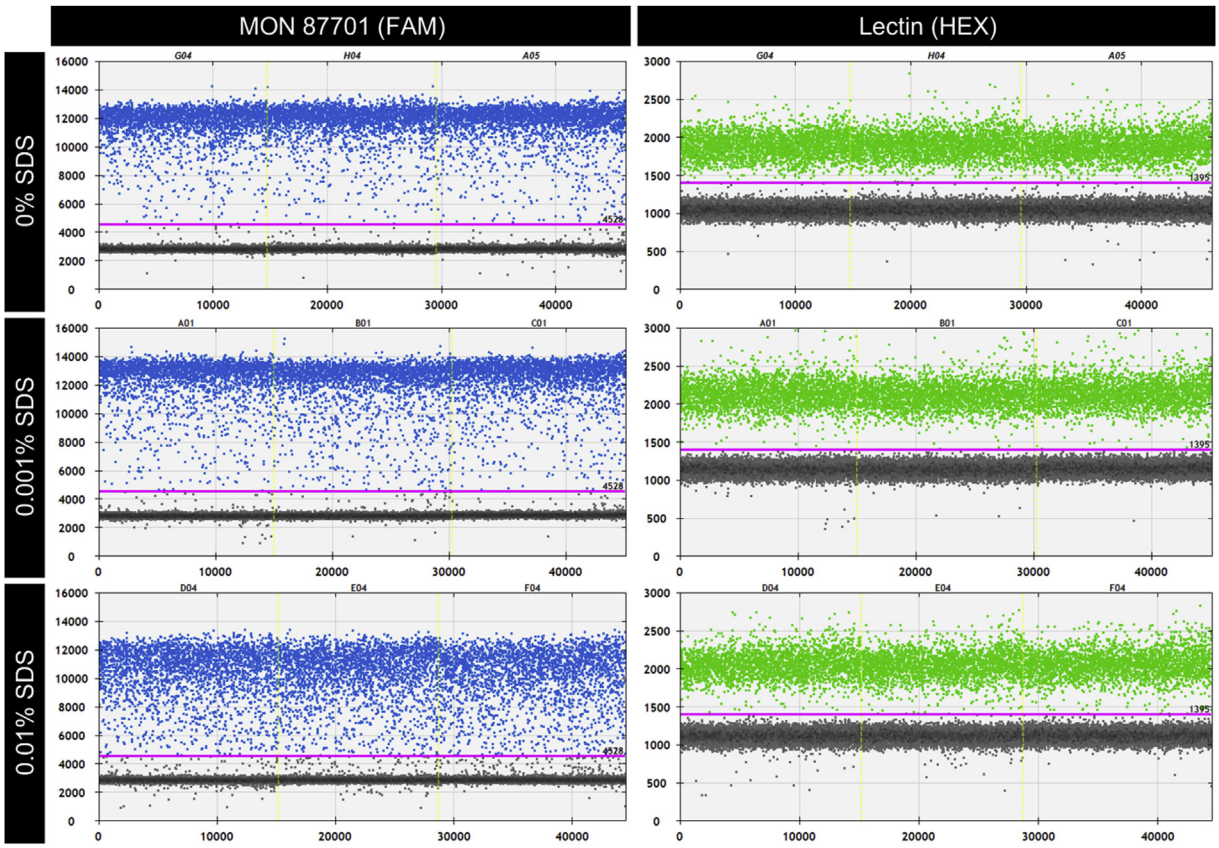

Fig. 3. 1D-Amplitude plots of a ddPCR PCR assay applied on the p87701 plasmid containing 1000 copies each of the transgene MON 87701 and the reference gene lectin. Samples were analyzed in triplicates containing varying proportions of SDS as PCR-inhibitor.

human cytomegalovirus. In their work, three common inhibitors in clinical samples were analyzed, namely human plasma, ethanol and EDTA. While ddPCR was more robust with ethanol as inhibitor, it was less resistant to EDTA, with both PCR platforms exhibiting similar sensitivities to plasma as inhibitor. The authors proposed that different inhibitors can exhibit different levels of inhibition on different nucleic acid amplification methods, and challenge the assumption that ddPCR is universally less susceptible to inhibitors than qPCR. They theorized that EDTA may likely inhibit digital PCR by increasing molecular dropout, arising from non-initiation of amplification in the presence of template, or insufficient generation of amplicon and reporter signal for adequate detection by the instrument. While EDTA acts as a calcium/magnesium (bivalent cations) chelator, increasing concentrations of the inhibitor will affect the processing activity of *Taq* polymerase and its probe cleaving activity, leading to molecular dropout (Nixon et al., 2014). As this mechanism of inhibition is similar with real-time qPCR and dPCR, it remains unclear why the real-time qPCR format is less resistant to dilute concentrations of EDTA. Interestingly in the work of Dingle et al. (2013), previously cited, the applied ddPCR assays were also not more tolerant to EDTA inhibition than the applied qPCR counterpart.

Additionally, the results of the EDTA-inhibition study reported here might have implications for the application of multiplex PCR reactions. With the qPCR platform, the transgene was significantly less resistant to inhibition, with the calculated copy numbers for transgene significantly exceeding the nominal copy with increasing EDTA concentration, while reference gene copy number remained reasonably stable. In the work of Huggett et al. (2008), an observed phenomenon was described as inhibition incompatibility, meaning differential susceptibility of PCR reactions to inhibitors. In their work, inhibition experiments were performed on DNA extracts from human urine samples, fresh urine and EDTA-inhibited samples (Huggett et al., 2008). Their results indicated that with increasing concentrations of inhibitor, it was possible for one PCR reaction to be unaffected by a potential inhibitor while another is completely suppressed. When comparing two different PCR reactions therefore, it is important that the two reactions are affected by potential inhibitors to the same extent (inhibition compatibility). Inhibition compatibility will have significant consequences in multiplex PCR assays, like the duplex assay described in this work where GM content is calculated in a relative context, relying on concomitant quantification of the reference gene and transgene. When the two reactions are not affected by potential inhibitors to the same extent, inhibition incompatibility occurs, leading to overestimation or underestimation of the event under investigation. With the qPCR-EDTA assay described in our work, the reference gene content remained relatively stable, while the transgene level was significantly exaggerated, leading to overestimation of the transgene event. The quantified GM content was however within an acceptable range with the applied ddPCR assay (Fig. 2).

A third inhibitor assessed in this work was ethanol (Fig. 2, panel 2). Here 0.25% ethanol in the reaction mix significantly impaired ddPCR-efficiency, while the qPCR assay was only moderately affected. With 5% ethanol however, both PCR systems were almost completely inhibited. Details of reference gene and transgene copy numbers for the inhibition tests are detailed in Table 1 in supplementary material.

When assessing the robustness of a PCR assay, susceptibility to inhibitors should be carefully considered, particularly when

comparing two different PCR reactions, as in GM analysis where GM content is calculated from reference gene and transgene quantities, over two dedicated PCR reactions. The ideal situation would be to minimize from the onset potential inhibitors. However, as noted by Huggett et al. (2008), it is difficult to elucidate factors or amplicon parameters that can be reliably or consistently associated with susceptibility to PCR-inhibition. Potential factors suggested in their work include amplicon GC content and primer melting temperature (Tm), and the authors suggested careful selection of thermostable DNA polymerase, and reduction in template DNA as factors that can reduce susceptibility to inhibition.

Our study suggests that although ddPCR may offer unique advantages due to partitioning of reaction components, the assumption that it is more resistant to inhibitors should be assessed in a case-by-case context, by considering inhibitory effects of substances individually. As noted in the work by Nixon et al. (2014), describing one nucleic acid amplification platform as more inhibitor-resistant than another, must be carefully accompanied by adequate empirical studies (Huggett et al., 2008).

3.4. ddPCR on a duplex platform

When setting up quantitative PCR assays involving the detection of an event as in GM analysis, against an endogenous reference gene, the challenge is to design a method where both detection systems are equivalent in various performance requirements, such as PCR efficiency. When the reaction is carried out as two independent singleplex reactions, such considerations are minimized, however a more practical, less error-prone and cost effective method would be the application of a duplex endogene–transgene reaction. In the work of Morisset et al. (2013), an evaluation of duplex qPCR and ddPCR assays were performed and compared with singleplex assays for the endogene *hmg* and the transgene MON 810 systems. While the *hmg* system performed identically in duplex and singleplex qPCR reactions, MON 810 (transgene) specific amplification was significantly affected in the duplex reactions, with signal values exhibiting Cq values approximately 5.5 higher than in singleplex reactions (Morisset et al., 2013). Attempts at optimization of the duplex qPCR assays by varying primer and probe concentrations for example, yielded unsatisfactory results as MON 810 was either underestimated or the method suffered a loss of sensitivity (Morisset et al., 2013). This highlights a common problem inherent in duplexing efforts in qPCR GM analysis (Chaouchi et al., 2008; Heide, Drømtorp, Rudi, Heir, & Holck, 2008; Nadal, Coll, La Paz, Esteve, & Pla, 2006). The challenge is the selection of target sequences with comparable lengths, without compromising sequence specificity (Morisset et al., 2013). In our study with digital PCR, several assays run as singleplex and then as duplex reactions were compared, without significant differences observed in PCR performance criteria, and the quantified GM content (data not shown). This corroborates the data from Morisset et al. (2013) cited above, where no significant variation of the measured target copy number was observed between the singleplex and duplex ddPCR assays for both *hmg* and the tested MON 810 systems. While an exhaustive comparison between singleplex and duplex performance would be time-consuming for all possible events, our data suggest that at least some ddPCR assays can be reliably run on a duplex platform.

3.5. ddPCR for the generation of different GMO-concentrations

While some reference materials may only be commercially available in one or a few limited GM concentrations, it may be feasible to mix the positive material with non GM material to produce other GM concentrations. To achieve this, DNA extracted from appropriate certified 0% GM and 100% GM plant materials were analyzed with ddPCR and the content (copy numbers) of the reference gene determined. Applying the method cited in Section.

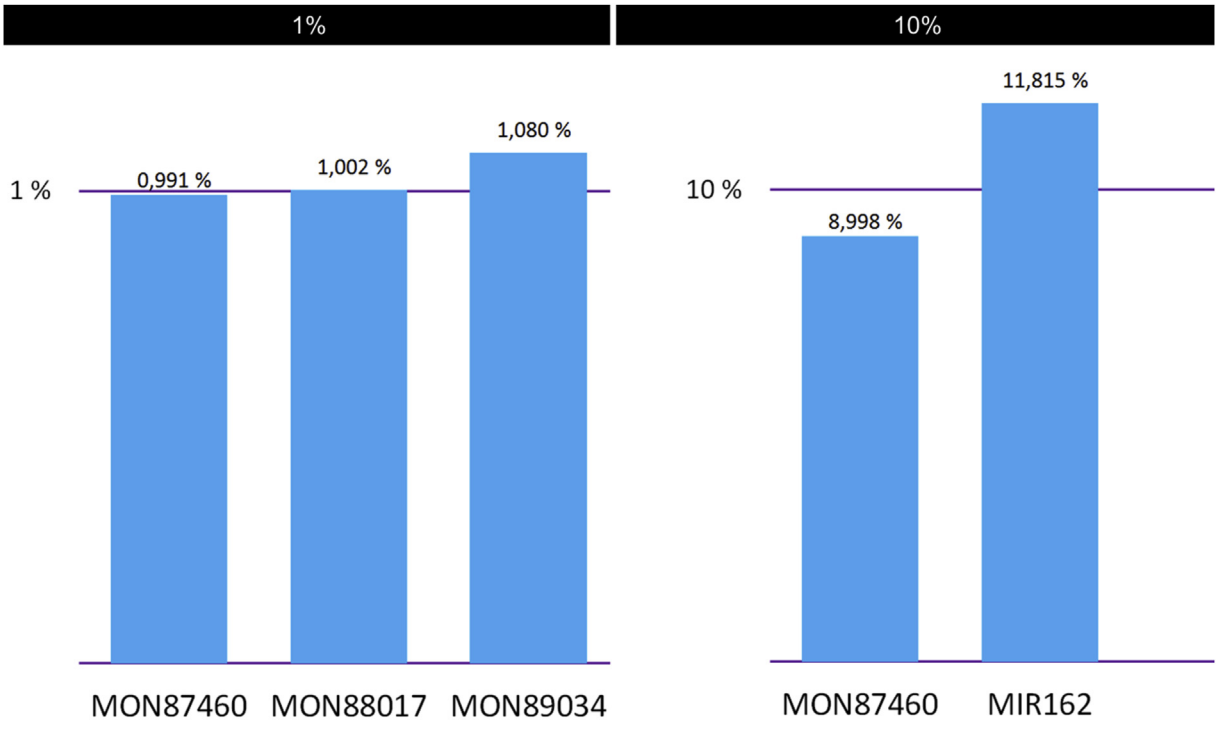

Fig. 4. ddPCR applied for the generation of commercially non-available intermediate concentrations of GM maize. Three maize events namely MON 87460, MON 88017 and MON 89034 were analyzed at 1% GM content and additionally MON 87460 and MIR162 at 10% GM content. The PCRs were set up as duplex reactions run in duplicates, and the content of the transgene calculated as a ratio of transgene copy number to reference gene copy numbers (with appropriate zygosity correction).

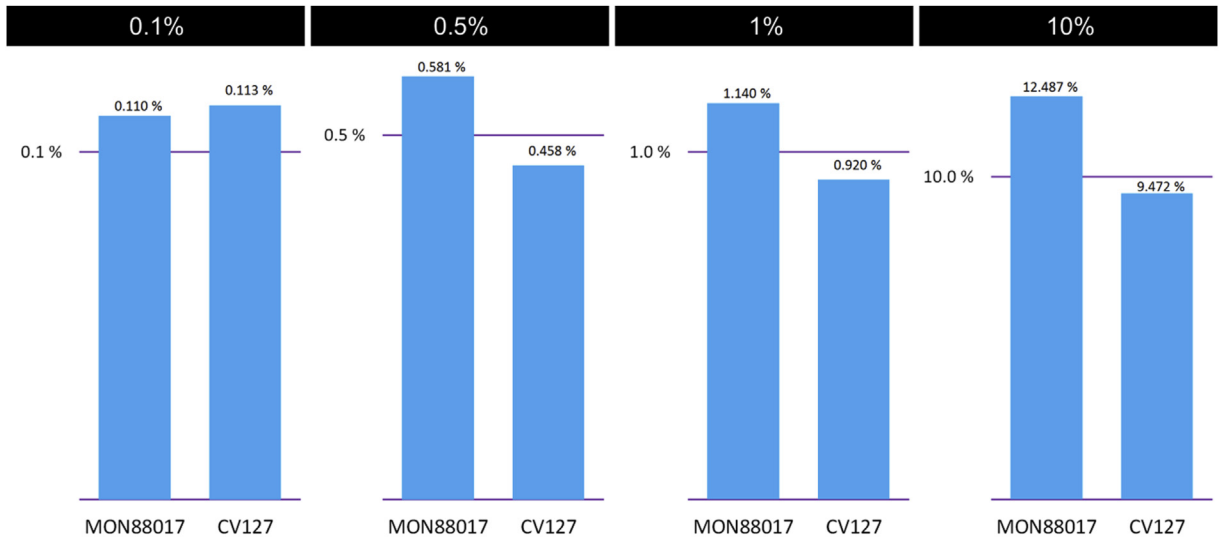

Fig. 5. ddPCR applied for the generation of different concentrations of GM maize (MON 88017) and soy (CV127). The transgene events were analyzed at 10%, 1%, 0.5% and 0.1% GM content. The PCR was set up as duplex reactions in eight replicates and the content of the transgene calculated as a ratio of transgene copy number to reference gene copy number.

Table 2
Precision, "accuracy" and "trueness" from analysis of ddPCR-generated intermediate concentrations (0.1–10%) of the soy event CV127. Results were compiled from at least 2 different runs with an average of 16 measurement points or test results.

Actual GM content (%)	Measured GM content (%)	Precision (%)	Accuracy (%)	Trueness (%)
10	9.75	3.21	3.65	1.78
1	0.91	9.19	9.40	11.58
0.5	0.47	8.98	9.82	4.61
0.1	0.10	11.24	0.66	11.21

2.9 in materials and methods, different contents (cp/cp %) of maize and soya transgene events were generated. Fig. 4 shows a summary of the runs employing this analytical approach on four maize events for the respective generation of 1% and 10% transgene reference material. All tested lines analyzed yielded good results, with the quantified transgene lying within the acceptable range of ±25% (ENGL, 2008).

In a parallel assay, different concentrations of the GM CV127 soya event were also prepared with ddPCR, and the results, together with a parallel assay for the MON 88017 maize event, are depicted in Fig. 5. The calculated GMO contents were well within the acceptable range of ±25% (ENGL, 2008). All samples were analyzed in triplicate and the mean values calculated from all measurement points. While 0.1% GM content was reliably quantified for all tested GM events, more dispersion or variability in measured precision was observed for this level. This is not surprising as the challenge is to quantify low concentrations of transgene against a much abundant endogene background (0.1% vs. 99.9%). Scaling up the DNA volumes applied in the assay did not significantly improve results, as this disparity or asymmetry in concentration between reference and transgene remains. We recommend therefore that when quantifying low concentrations of targets, as is often necessary in GM analysis, at least 5 or 6 replicates are considered for a reasonably representative analytical coverage.

The accuracy, trueness, and precision of the generated different concentrations for CV127 were calculated (Table 2). Trueness represents the closeness of agreement between the average value obtained from a series of test results and an accepted reference value, and accuracy is the closeness between a measurement point and the true and accepted reference value. Thus the results depicted in Table 2 are not true in the absolute sense, in the absence of a DNA reference material certified for absolute copy number concentration. Nevertheless, the results indicate that ddPCR can be employed for the generation of different concentrations (e.g. relevant to GMO analysis) as demonstrated in this study. Interestingly, the qPCR platform was applied in parallel for this study and confirmed the ddPCR results outlined here (data not shown).

For the visualization of the 1D-amplitude plots of the generated intermediate concentrations of CV127 please see Fig. 1 in supplementary material.

In an expanded assay, the assessment of the limit of detection of the applied 100% GM reference soy plant CV127 showed an LOD_6 of 2 copies, confirmed by the applied $LOD_{95\%}$ assay.

4. Conclusion

This study demonstrates the applicability of ddPCR assays for the routine analysis of GM food and feed. The examination of reference material with ddPCR, certified at varying levels of GM content, showed good performance, with the measured GM content lying well within acceptable boundaries of trueness, accuracy and precision. In inhibition tests, ddPCR showed more tolerance to SDS as inhibitor, with comparable tolerance to ethanol observed for both PCR systems. Finally, applying ddPCR, different concentrations of GM DNA from 100% GM and non-GM DNA solutions were generated, with good results. Our study indicates that ddPCR can be a powerful tool in the routine identification and quantification of genetically modified foods.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2016.04.048.

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