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Research article

Development and validation of a ddPCR assay to detect and quantify tobacco DNA in smoke and smokeless tobacco and tobacco-free products

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ABSTRACT

The last decade, smoke and smokeless products claiming to be tobacco-free, including herbal cigarettes and herbal shisha, became available on the European market and gained popularity. This study proposes a new digital droplet PCR (ddPCR) method, designed based on a previously developed real-time PCR (qPCR) method being currently used by the U.S. Food and Drug Administration (FDA) to specifically detect the presence of tobacco DNA in targeting a sequence from the *Nicotiana tabacum* nia-1 gene. To ensure a harmonized and reliable control by enforcement laboratories, both of these qPCR and ddPCR methods were then evaluated and validated for their compliance to an international standard. First, the performance of these PCR-based methods was successfully assessed as specific and sensitive, and in line with minimum performance requirements from international standard. Secondly, the transferability to external laboratory was confirmed for these PCR-based methods. Finally, the applicability of these PCR-based methods was demonstrated using 7 ground tobacco reference materials from the Tobacco Research Center (TRC) Toronto University as well as 6 commercial smokeless and tobacco-free smoke and smokeless products. Based on this study, the previously developed qPCR method was confirmed as complying with international standard, ensuring a efficient and harmonize use by enforcement laboratories for tobacco control on the European market. Moreover, this study proposed to enforcement laboratories the possibility to use a ddPCR method, enabling the simultaneous detection and absolute quantification of tobacco DNA as well as a limited impact of PCR inhibitors.

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1. Introduction

In Europe, the determination of whether or not a product contains tobacco is the responsibility for the competent authorities [1]. Tobacco identification in commercial products is usually performed by enforcement laboratories through the detection of microscopic tobacco fragments. However, this method is not applicable for products composed of reconstituted tobacco, as the tobacco used to generate this paper-like material has been treated by chemicals and heat which is known to affect tobacco morphology. Alternatively, chemical screening for nicotine, neophytadiene and vitamin E are also performed although the presence of nicotine, neophytadiene and vitamin E is not specific for plants belonging to the *Nicotiana* genus. Therefore, in order to overcome these disadvantages, DNA-based methods were highlighted as a valuable alternative or option to strengthen the classical tobacco identification analysis [2–4].

In this context, it is pivotal to develop and validate robust DNA methodologies to target DNA from tobacco in smoke and smokeless tobacco and related products (e.g. herbal cigarettes). Currently, only a few methods targeting tobacco DNA are available in the scientific literature and they mainly make use of a qPCR systems targeting either a putrescine N-methyltransferase gene, an uridine 5′-monophosphate synthase gene, a cytochrome P450 monoxygenase CYP82E4 gene or a nitrate reductase nia-1 gene [2–4].

However, to the best of our knowledge, no digital droplet PCR (ddPCR) methodology, targeting tobacco DNA, is currently available. This PCR-based technology offers the advantage over the classical qPCR technology to simultaneously detect and absolutely quantify a target of interest without reference materials, standard curves and interpolations. Moreover, while qPCR raw data are expressed though C_q values which may vary from one platform to another, ddPCR raw data are directly expressed in target copy number. In addition, ddPCR technology has been shown to be more tolerant to many PCR inhibitors thanks to sample partitioning into thousands of droplets generated by the water-oil emulsion step. Therefore, especially at low target concentration, the measurement uncertainty is reduced [5–7]. In addition, none of these PCR-based methods were fully validated in line with European standards, such as the minimum performance requirements (MPR) for GMO analysis of the European Network of GMO Laboratories (ENGL) [8,9]. This is crucial to ensure a harmonized and reliable control by all enforcement laboratories.

In this study, both qPCR and ddPCR methods in line with international standard were proposed. More precisely, to simultaneously detect and absolutely quantify tobacco DNA in smoke and smokeless tobacco and related products, a ddPCR method was developed based on the qPCR method currently used by the U.S. Food and Drug Administration (FDA), being a reference agency, designed to target a sequence from the *Nicotiana tabacum* nia-1 gene [9–11]. Moreover, both of these NIA-1 PCR methods were assessed and validated in line with MPR [9,10]. The performance of the NIA-1 qPCR and ddPCR methods was also evaluated for their specificity and sensitivity. In addition, the transferability to an external laboratory of these NIA-1 PCR-based methods was investigated. Finally, the applicability of these PCR-based methods was assessed using ground tobacco reference materials as well as smoke and smokeless tobacco and tobacco-free products commercialized on the market. This study offers therefore to enforcement laboratories involved in market tobacco control the possibility to use qPCR or ddPCR methods in line with international standard.

2. Materials and methods

2.1. PCR-based assays

For both PCR-based methods, primers (Forward: CAAACAATCCATCTCCCCTGAT; Reverse: GCAAGCCCAAGACTTGATCCT) and probe (TTCGATTTGCATTGCCCTT) from the NIA-1 qPCR method, previously developed by [11], were used. In this study, the probe was associated to the fluorophore HEX and the quencher BHQ1.

2.1.1. qPCR

For the NIA-1 qPCR method, the 25 µl standard reaction volume included 1X SsoAdvanced universal probes supermix (Bio-Rad, USA), 400 nM of each primer (Eurogentec, Belgium), 200 nM of the probe (Eurogentec, Belgium) and 5 µl of DNA. The qPCR program comprised a single cycle of DNA polymerase activation for 3 min at 95 °C followed by 45 amplification cycles of 15 s at 95 °C (denaturing step) and 30 s at 60 °C (annealing-extension step). All qPCR runs were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Each qPCR assay included a No Template Control (NTC). A qPCR reaction was considered as negative if no C_q value was observed.

2.1.2. ddPCR

For the NIA-1 ddPCR method, a standard 70 µl volume of Droplet Generation Oil for Probes (Bio-Rad, USA) and a standard 20 µl reaction volume, including 1X ddPCR Supermix for Probes (No dUTP) (Bio-Rad, USA), 900 nM of each primer (Eurogentec, Belgium), 250 nM of the probe (Eurogentec, Belgium) and 5 µl of DNA, were loaded into a QX200™ droplet generator (Bio-Rad, USA). The PCR amplification, using a standard 40 µl volume of the generated droplet solution, was performed on a T100™ Thermal Cycler (Bio-Rad, USA). The ddPCR program comprised (i) a single cycle at 25 °C for 3 min, (ii) a single cycle at 95 °C for 10 min (Taq polymerase activation), (iii) 40 cycles at 94 °C for 30 s (denaturation) and at 60 °C for 1 min (annealing-extension), and (iv) a single cycle at 98 °C for 10 min (Taq polymerase inactivation). Results, acquired using the HEX channel from the QX200 reader (Bio-Rad, USA), were analysed with the QuantaSoft software v1.April 7, 0917 (Bio-Rad, USA) in manually setting the threshold above the cluster of negative partitions. Only ddPCR reactions with at least 10,000 accepted droplets were included in the subsequent analysis. To consider a ddPCR reaction as positive, except for the sensitivity method assessment, a threshold of 3 positive droplets was used, as previously applied

[12,13]. Each ddPCR assay included an NTC.

2.2. Performance evaluation of the NIA-1 qPCR and ddPCR methods

2.2.1. Specificity assessment

The *in-silico* specificity of the NIA-1 PCR-based methods was assessed by performing a BLAST search, with default parameters, of the expected generated PCR amplicon sequence against all *Nicotiana* sp. genomes from the NCBI RefSeq Genome database as well as all non-*Nicotiana* sp. genomes from the NCBI RefSeq Representative Genome database (Table S1). In addition, by means of the Clustal Omega software (v1.2.4) with default parameters, the expected generated PCR amplicon sequence was compared to the one experimentally generated using the NIA-1 PCR-based methods applied on the control *N. tabacum* gDNA (PLG-1101 Amsbio, The Netherlands) (Tables 1, S2). The experimentally generated PCR products were purified and sequenced as previously described [6,14,15].

Next, the *in-vitro* specificity of the NIA-1 PCR-based methods was tested in duplicate on about 3000 estimated haploid genome copies from several materials (Tables 1, S3) [16,17]. For *Solanaceae* plant materials belonging to *Nicotiana* sp., it included DNA from *N. alata*, *N. langsdorffii*, *N. rustica, N. x sanderae*, *N. sylvestris* and *N. tabacum*, were used (Tables 1, S3A). For *Solanaceae* plant materials not belonging to *Nicotiana* sp., DNA from *Atropa belladonna, Capsicum annuum, Datura stramonium, Lycium barbarum, Physalis peruviana, Solanum aethiopica, S. betaceum, S. lycopersicum, S. melongena*, and *S. tuberosum* were tested (Tables 1, S3B). For non-*Solanaceae* plant materials, DNA from *Beta vulgaris, Brassica napus, Cannabis sativa, Curcuma longa, Glycine max, Glycyrrhiza glabra, Gossypium hirsutum, Humulus lupulus, Ilex paraguariensis, Oryza sativa, Syzygium aromaticum*, and *Zea mays* were used (Tables 1, S3C). In addition, non-plant materials were tested, including DNA from *Homo sapiens* (G3041, Promega, USA), *Bacillus licheniformis* (LMG 7558, BCCM, Belgium), *Aspergillus niger* (IHEM 2312, BCCM, Belgium) and *Pichia pastori* (MUCL 27793, BCCM, Belgium) (Table 1). For plant materials collected from the American Herbal Pharmacopoeia, Pharmaflore (FAGRON), National Research Council Canada and the botanical gardens of Meise (Belgium), UGent (Belgium) and Cambridge (UK), DNA was extracted using NucleoSpin® Food Kit (MACHEREY-NAGEL, Germany) according to manufacturer's instructions (Table S3). For plant materials collected from JRC Directorate F - Health and Food (JRC) and American oil chemists' society (AOCS), DNA was extracted using a CTAB-based procedure (Table S3) [18]. For microbial materials, DNA was extracted as previously described (Table 1) [19].

All DNA concentrations were measured by fluorometry using Qubit 3.0 Fluorometer (ThermoFisher, Belgium). The purity of

Table 1

Specificity assessment of the NIA-1 qPCR and ddPCR methods. The presence and absence of amplification are respectively symbolized by "+" and "-". For each result, the experiment was carried out in duplicate on 3000 estimated haploid genome copies. The means of the measured C_q values and copy number are indicated in brackets. For the ddPCR method, a minimum of 3 positive droplets is needed to consider the ddPCR reaction as positive.

Kingdom	Genus	Species	Common name	NIA-1 qPCR method	NIA-1 ddPCR method
Animalia	*Homo*	*sapiens*	Human	-	-
Bacteria	*Bacillus*	*licheniformis*	/	-	-
Fungi	*Aspergillus*	*niger*	/	-	-
	Pichia	*pastori*	/	-	-
Plantae	*Atropa*	*belladonna*	Belladonna	-	-
	Beta	*vulgaris*	Sugar beet	-	-
	Brassica	*napus*	Rapeseed	-	-
	Cannabis	*sativa*	Hemp	-	-
	Capsicum	*annuum*	Pepper	-	-
	Curcuma	*longa*	Turmeric	-	-
	Datura	*stramonium*	Thorn apple	-	-
	Glycine	*Max*	Soybean	-	-
	Glycyrrhiza	*glabra*	Liquorice	-	-
	Gossypium	*hirsutum*	Cotton	-	-
	Humulus	*lupulus*	Hop	-	-
	Lycium	*barbarum*	Chinese wolfberry	-	-
	Ilex	*paraguariensis*	Yerba mate	-	-
	Nicotiana	*alata*	Flowering tobacco	-	-
	Nicotiana	*langsdorffii*	Langsdorff's tobacco	+ (C_q: 26.4 ± 0.1)	+ (2770 ± 14.1 copies)
	Nicotiana	*rustica*	Aztec tobacco	+ (C_q: 26.7 ± 0.1)	+ (2810 ± 42.4 copies)
	Nicotiana	*x sanderae*	Sander's tobacco	–	-
	Nicotiana	*sylvestris*	Woodland tobacco	+ (C_q: 34.5 ± 0.6)	-
	Nicotiana	*tabacum*	Tobacco	+ (C_q: 26.9 ± 0.2)	+ (3090 ± 70.7 copies)
	Oryza	*sativa*	Rice	-	-
	Physalis	*peruviana*	Cape gooseberry	-	-
	Solanum	*aethiopica*	African eggplant	-	-
	Solanum	*betaceum*	Tamarillo	-	-
	Solanum	*lycopersicum*	Tomato	-	-
	Solanum	*melongena*	Eggplant	-	-
	Solanum	*tuberosum*	Potato	-	-
	Solanum	*tuberosum*	Potato	-	-
	Syzygium	*aromaticum*	Clove	-	-
	Zea	*mays*	Maize	-	-

extracted DNA was evaluated through the A260/A280 and A260/A230 ratios generated by spectrophotometry using Nanodrop® 2000 (ThermoFisher, Belgium).

2.2.2. Sensitivity assessment

The method sensitivity was assessed using the control *N. tabacum* gDNA (PLG-1101 Amsbio, The Netherlands). To this end, serial dilutions ranging from 20 to 0 estimated targeted copies were prepared according to the *N. tabacum* haploid genome size (4.6 pg) and tested in 12 replicates (Table 2, S4) [16]. The limit of detection $LOD_{95\ \%}$ was calculated as previously described (Table S5) [14,15]. The $LOD_{95\ \%}$ is defined as "the number of copies of the target DNA sequence required to ensure a 95 % probability of detection"[20,21].

2.2.3. Transferability assessment

The *in-house* sensitivity assessment described in section 2.2.2 was carried out by external laboratories (Tables 2, S5). For the NIA-1 qPCR method, the assays were performed by the Laboratoire SCL de Strasbourg (Strasbourg, France). Using oligonucleotides (Eurogentec, Belgium) and 1X SsoAdvanced universal probes supermix (Bio-Rad, USA), all qPCR runs were performed on a CFX96 Touch Real-Time PCR Detection System (BioRad, USA). For the NIA-1 ddPCR method, the assays were performed by the Unità Operativa Semplice a Valenza Direzionale - Ricerca e Controllo degli Organismi Geneticamente Modificati (CROGM) at the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M.Aleandri" (Rome, Italy). All reagents, including oligonucleotides (Metabion, Italy) and ddPCR reagents (Droplet Generation Oil for Probes (Bio-Rad, USA) and 1X ddPCR Supermix for Probes (Bio-Rad, USA)), were supplied by the external laboratory. All ddPCR assays were performed using QX200™ droplet generator (Bio-Rad, USA), T100™ Thermal Cycler (Bio-Rad, USA), QX200 reader (Biorad, USA) and the QX Manager software (Bio-Rad, USA).

2.2.4. Applicability assessment

Samples n°1–7 were collected from Tobacco Research Center (TRC), Toronto University (Tables 3, S6). These ground tobacco samples are reference materials for research purposes and contained either a single tobacco material or a mix of tobacco materials. The tobacco compositions of these samples included flue-cured (Virginia), oriental (Turkish), burley, unflavored cigar and/or dark air-cured tobacco materials. From 100 mg of each tobacco sample, DNA was extracted using the Quick-DNA™ HMW MagBead Kit (ZymoResearch, Germany) as previously described [22].

Samples n°8–13 are tobacco and tobacco-free smoke and smokeless products that were commercialized on the Belgian market (Tables 4, S6). These samples, including hookah tobacco product, dry herbal materials and cannabis cigarillo products, were provided by the Belgian Competent Authorities in the frame of their control plan. From 200 mg of each commercial sample, DNA was extracted using the NucleoSpin® Food kit (Macherey-Nagel, France) according manufacturer's instructions.

For all samples (n°1–13), DNA concentration was measured by fluorometry using Qubit 3.0 Fluorometer (ThermoFisher, Belgium). DNA purity was evaluated through the A260/A280 and A260/A230 ratios generated by spectrophotometry using Nanodrop® 2000 (ThermoFisher, Belgium). All extracted DNA were confirmed as amplifiable by PCR (Table S6). For each sample, 1 ng of DNA was tested in duplicate by qPCR and ddPCR assays (Tables 3–4).

Table 2

Sensitivity assessment of the NIA-1 qPCR and ddPCR methods for the in house validation and transferability assays. For each estimated target copy number, 12 replicates were tested. The presence and absence of amplification are respectively symbolized by "+" and "-". The number of positive replicate(s) out of the 12 replicates tested. The means of the measured C_q values and copy numbers are indicated in brackets.

		Estimated target copy number						
		20	10	5	2	1	0.1	0
NIA-1 qPCR method	In house validation assays	+ (12/12) (C_q: 35.4 ± 0.6)	+ (12/12) (C_q: 36.0 ± 0.6)	+ (12/12) (C_q: 36.8 ± 1.2)	+ (10/12) (C_q: 38.4 ± 0.8)	+ (7/12) (C_q: 39.0 ± 0.9)	+ (1/12) (C_q: 39.3 ± 0.0)	- (0/12)
	Transferability assays	+ (12/12) (C_q: 35.9 ± 0.4)	+ (12/12) (C_q: 36.7 ± 0.4)	+ (11/12) (C_q: 37.9 ± 1.0)	+ (11/12) (C_q: 39.6 ± 2.1)	+ (9/12) (C_q: 39.8 ± 1.7)	+ (2/12) (C_q: 41.7 ± 3.3)	- (0/12)
NIA-1 ddPCR method	In house validation assays	+ (12/12) (19.0 ± 3.6 copies)	+ (12/12) (11.0 ± 2.7 copies)	+ (12/12) (5.5 ± 1.6 copies)	+ (5/12) (0.8 ± 1.0 copies)	+ (3/12) (0.3 ± 0.6 copies)	- (0/12)	- (0/12)
	Transferability assays	+ (12/12) (13.4 ± 4.2 copies)	+ (12/12) (7.4 ± 3.3 copies)	+ (12/12) (3.6 ± 1.9 copies)	+ (8/12) (1.3 ± 1.1 copies)	+ (5/12) (0.8 ± 1.1 copies)	+ (1/12) (0.1 ± 0.0 copies)	- (0/12)

Table 3
Applicability assessment of the NIA-1 qPCR and ddPCR methods using ground tobacco reference materials (samples n°1–7). Available information on these samples is indicated, including the sample types (single tobacco material or mix of tobacco materials) and the sample compositions. The mix of tobacco materials is corresponding to ground unflavoured cigarette fillers. For each sample, 1 ng of extracted DNA was tested in duplicate. The presence or absence of PCR amplification is symbolized by "+" or "-", respectively. The means of the measured C_q values and copy numbers are indicated in brackets. For the ddPCR method, a minimum of 3 positive droplets is needed to consider the ddPCR reaction as positive.

Samples n°		Sample types	Sample compositions	NIA-1 qPCR method	NIA-1 ddPCR method
1		Single tobacco material	Flue-Cured (Virginia)	+ (C_q: 29.9 ± 0.1)	+ (511 ± 43.8 copies)
2		Single tobacco material	Oriental (Turkish)	+ (C_q: 29.2 ± 0.2)	+ (918 ± 76.4 copies)
3		Single tobacco material	Burley	+ (C_q: 30.7 ± 0.3)	+ (211 ± 9.9 copies)
4		Single tobacco material	Unflavored Cigar	+ (C_q: 30.2 ± 0.1)	+ (411 ± 29.7 copies)
5		Single tobacco material	Dark Air-cured	+ (C_q: 30.2 ± 0.1)	+ (354 ± 11.3 copies)
6		Mix of tobacco materials	Flue-Cured, Burley and oriental tobacco types, reconstituted tobacco sheets, expanded flue-cured, expanded burley, glycerin, isosweet (sugar), propylene glycol	+ (C_q: 30.2 ± 0.2)	+ (450 ± 14.1 copies)
7		Mix of tobacco materials	Flue-Cured, Flue-Cured puffed, Burley, Burley puffed and Turkish oriental tobacco types, reconstituted tobacco sheets, glycerin, inverted sugar	+ (C_q: 30.0 ± 0.1)	+ (422 ± 17.0 copies)

2.3. Plant material collection

The plant materials used in this study were collected from American Herbal Pharmacopoeia (*H. lupulus, I. paraguariensis, L. barbarum*), Amsbio (*N. tabacum, S. lycopersicum, S. tuberosum*), AOCS (*O. sativa*), Botanical garden of Cambridge, UK (*N. alata, N. langsdorffii, N. sylvestris, N. x sanderae*), Botanical garden of Meise, Belgium (*N. rustica*), Botanical Garden UGent, Belgium (*A. belladonna, C. annuum, D. stramonium, P. peruviana, S. aethiopica, S. betaceum, S. melongena*), JRC (*B. vulgaris, B. napus, G. max, G. hirsutum, S. tuberosum, Z. mays*), National Research Council Canada (*C. sativa*) and Pharmaflore, FAGRON (*C. longa, G. glabra, S. aromaticum*) (Table S3). The tobacco reference materials were collected from Tobacco Research Center (TRC), Toronto University and the commercial smokeless and tobacco-free smoke and smokeless products were collected from the Belgian Competent Authorities.

Table 4
Applicability assessment of the NIA-1 qPCR and ddPCR methods using tobacco and tobacco-free smoke and smokeless products commercialized on the Belgian market (samples n°8–13). Available information on these samples is indicated. For each sample, 1 ng of extracted DNA was tested in duplicate. The presence or absence of PCR amplification is symbolized by "+" or "-", respectively. The means of the measured C_q values and copies are indicated in brackets. For the ddPCR method, a minimum of 3 positive droplets is needed to consider the ddPCR reaction as positive.

Sample n°	Code	Sample description	NIA-1 qPCR method	NIA-1 ddPCR method
8	S23FD00805	Hookah tobacco product	+ (C_q: 30.6 ± 0.3)	+ (333 ± 9.9 copies)
9	S22FD00855	Dry herbal material - wrapping material	+ (C_q: 31.1 ± 0.1)	+ (178 ± 5.7 copies)
10	S23FD01605	Cannabis cigarillo product - roll paper	+ (C_q: 31.8 ± 0.2)	+ (113 ± 4.2 copies)
11	S22FD00856	Dry herbal material - wrapping material	+ (C_q: 32.1 ± 0.0)	+ (77 ± 7.1 copies)
12	S23FD01604	Cannabis cigarillo product - content material	+ (C_q: 36.1 ± 0.5)	–
13	S22FD02935	Dry herbal material - paste	–	–

3. Results and discussion

The NIA-1 qPCR method, currently used by the FDA to detect the presence of tobacco DNA in smoke and smoke-free tobacco and related products, was selected [11]. The NIA-1 qPCR method includes a set of two primers and a probe designed to cover 74 bp of the *N. tabacum* nia-1 gene, a single-copy gene in a haploid *Nicotiana* genome encoding nitrate reductase. This qPCR method was initially developed within a triplex qPCR assay targeting also an exogenous internal control (IPC) and the *N. tabacum* cytochrome P450 monoxygenase CYP82E4 gene encoding the N-demethylation of nicotine to nornicotine [11]. In this study, the NIA-1 qPCR method was evaluated and validated to comply with MPR for GMO analysis defined by ENGL, an international standard ensuring a harmonized and reliable control by enforcement laboratories [9–11]. In addition, using the set of two primers and a probe from the NIA-1 qPCR method, a NIA-1 ddPCR method was designed, developed and validated according to MPR in this study [9–11]. This ddPCR method offers the opportunity to simultaneously detect and absolutely quantify tobacco DNA by targeting the single-copy nia-1 gene. The performance of these NIA-1 qPCR and ddPCR methods were assessed in term of specificity, sensitivity, transferability and applicability.

3.1. Specificity assessment of the NIA-1 qPCR and ddPCR methods

The performance of the NIA-1 qPCR and ddPCR methods was investigated in term of specificity.

At the *in-silico* level, the expected PCR amplicon generated from *N. tabacum* using the NIA-1 PCR-based methods was identified and analysed (Table S1). This PCR amplicon is expected to be identical for the NIA-1 qPCR and ddPCR methods as both use the same set of primers and probe that was previously designed by Korchinski *et al.* 2016 [11]. The sequence of this PCR amplicon presented a high similarity of coverage and identity to *Nicotiana* sp. sequences from the NCBI RefSeq Genome database (Table S1). However, one nucleotide mismatch located at the 3' end of the designed probe was observed in all of these *Nicotiana* sp. sequences. No additional nucleotide variation with the expected PCR amplicon sequence was detected for the sequences from *N. knightiana, N. otophora, N. paniculate, N. rustica, N. tabacum* and *N. tomentosiformis*. Nonetheless, for the other *Nicotiana* sp. sequences, few additional nucleotide variations were observed, including (i) one nucleotide mismatch located in the reverse primer for the *N. attenuata* sequence, (ii) one nucleotide mismatch located in the forward primer for the *N. benthamiana* and *N. undulata* sequences, and (iii) two nucleotide mismatches located in the forward primer for the *N. glauca, N. obtusifolia* and *N. sylvestris* sequences. Regarding the similarity to the non-*Nicotiana* sp genomes from the NCBI RefSeq Representative Genome database, no high similarity of coverage and identity were observed with the expected PCR amplicon sequence generated from *N. tabacum* using the NIA-1 PCR-based methods. The observed nucleotide variations included multiple nucleotide mismatches in the probe and primer sequences.

At the *in-vitro* level, several different materials were experimentally tested using the NIA-1 qPCR and ddPCR methods (Tables 1, S3). As expected, no amplification signal was detected for animal and microbial materials, including *Homo sapiens, B. licheniformis, A. niger* and *P. pastori*. Among the tested plant materials, no amplification signal was detected using the non-*Solanaceae* plant materials as well as the *Solanaceae* plant materials not belonging to *Nicotiana* sp. (Tables 1, S3). Regarding the *Solanaceae* plant materials belonging to *Nicotiana* sp., positive and negative signals were observed (Tables 1, S3). For *N. x sanderae* and *N. alata*, no amplification signal was observed, while an amplification signal was measured for *N. langsdorffii, N. rustica* and *N. tabacum* (Table 1). Although one nucleotide mismatch in the probe was identified by the *in-silico* specificity assessment of *N. rustica* and *N. tabacum*, the PCR reaction do not seem be impacted by such nucleotide variation (Table S1). For *N. sylvestris*, no amplification signal was detected using the NIA-1 ddPCR method while a non-optimal amplification signal was observed using the NIA-1 qPCR method (Table 1). This result is probably due to the presence of three nucleotide mismatches, two in the forward primer and one in the probe as showed by the *in-silico* specificity assessment (Table S1).

Based on all these results, the NIA-1 qPCR and ddPCR methods were demonstrated as being specific to *N. langsdorffii, N. rustica* and *N. tabacum*. As *N. tabacum* and, to a limited extent, *N. rustica,* are the predominant *Nicotiana* species used to generate tobacco, these NIA-1 PCR-based methods were therefore considered as being suitable for the specific detection of tobacco DNA in commercial products. *N. langsdorffii* on the other hand is not generally used in order to produce tobacco, but it is mainly used as an ornamental garden plant. Moreover, for other ingredients well-known to be used with tobacco, like *Canabis sativa* in "joints" and *Syzygium aromaticum* in "clove cigarettes", no amplification signal was reported [11,23–25].

3.2. Sensitivity assessment of the NIA-1 qPCR and ddPCR methods

The performance of the NIA-1 qPCR and ddPCR methods was tested in term of sensitivity using different estimated copy numbers (20, 10, 5, 2, 1, 0.1 and 0) of the targeted sequence from the *N. tabacum* nia-1 gene (Tables 2, S2).

For the NIA-1 qPCR method, an amplification signal was observed as low as 5 estimated target copies for all 12 replicates and, according to modelling of the probability of detection (POD), the limit of detection ($LOD_{95\ \%}$) was calculated at ~4 estimated target copies ($LOD_{95\ \%}$ is 3.261 with a 95 % confidence interval of [2.055; 5.172]) (Tables 2, S5). For the NIA-1 ddPCR methods, all 12 replicates showed an amplification signal as low as 5 estimated target copies and the $LOD_{95\ \%}$ was determined at ~8 estimated target copies ($LOD_{95\ \%}$ is 7.225 with a 95 % confidence interval of [4.751; 10.984]) (Tables 2, S5).

Both NIA-1 qPCR and ddPCR methods showed equivalent method sensitivity performance since both of these PCR-based methods presented a positive signal for all 12 replicates as low as 5 estimated target copies. As presented an $LOD_{95\ \%}$ below 25 estimated target copies, these NIA-1 PCR -based methods were also both evaluated as sensitive and in line with MPR defined by ENGL [9,10].

3.3. Transferability assessment of the NIA-1 qPCR and ddPCR methods

Based on the same experimental set up employed for the in-house sensitivity evaluation, the performance of the NIA-1 qPCR and ddPCR methods was tested by external laboratories. The generated results were similar with those observed for the in-house validation (Tables 2, S5). For the NIA-1 qPCR method, as low as 10 estimated target copies, an amplification signal was observed for all 12 replicates. Moreover, the $LOD_{95\ \%}$ of the NIA-1 qPCR method was determined at ~3 estimated target copies ($LOD_{95\ \%}$ is 3.001 with a 95 % confidence interval of [1.888; 4.777]). For the NIA-1 ddPCR methods, an amplification signal for all 12 replicates was detected as low as 5 estimated target copies and the $LOD_{95\ \%}$ of the NIA-1 ddPCR method was calculated at ~5 estimated target copies ($LOD_{95\ \%}$ is 4.696 with a 95 % confidence interval of [3.027; 7.301]). As similar performance was observed between the in-house and external assays, these NIA-1 PCR -based methods were shown to be transferable.

3.4. Applicability assessment of the NIA-1 qPCR and ddPCR methods

The applicability of the NIA-1 qPCR and ddPCR methods was investigated using 7 ground tobacco reference materials (samples n°1–7) and (ii) 6 tobacco and tobacco free smoke and smokeless products present on the market (samples n°8–13) (Tables 3–4). The presence of plant DNA was detected in all samples (Table S6).

On the one hand, the ground tobacco reference materials (samples n°1–7) were composed of either a single tobacco material (samples n°1–5) or a mixture of various tobacco materials supplemented with additives (e.g., glycerin, sugar and propylene glycerol) (samples n°6–7) (Table 3). Among all these samples, several different types of tobacco were covered, including Virginia, Oriental and Burley representing the majority of the worldwide tobacco production [23,25]. As expected, an amplification signal was observed on all tested ground tobacco reference materials (sample n°1–7) using the NIA-1 qPCR method, with a C_q value of around 30 (Table 3). Similarly, an amplification signal was measured for all these tobacco samples using the NIA-1 ddPCR method, with a number of target copies varying between 211 and 918. In addition, a correlation between the recorded C_q values and copy numbers was observed. Based on these results, the relevance to use these NIA-1 PCR-based methods to control the presence of tobacco DNA in ground tobacco samples was confirmed.

On the other hand, the commercial smoke and smokeless tobacco and tobacco-free products (samples n°8–13) included either hookah tobacco, dry herbal shisha or cannabis cigarillo's (Table 4). For samples n°8–11, an amplification signal for both the NIA-1 qPCR and ddPCR methods was detected, indicating the presence of tobacco DNA. A C_q value ranging from 30.6 to 32.1 was measured using the NIA-1 qPCR method and a number of target copies going from 333 to 77 was recorded using the NIA-1 ddPCR method. For sample n°12, an amplification signal was detected using the NIA-1 qPCR method while no amplification signal was measured using the NIA-1 ddPCR method. The observed C_q value from the NIA-1 qPCR method indicated a very low amount of tobacco DNA, being at the limit of detection determined for the NIA-1 PCR-based methods (Table 2). Therefore, such positive and negative signals are not contradictory. Additionally, only a trace amount of nicotine could be detected in the methanol extracts of these samples that were subjected to routine analysis by gas chromatography coupled to mass spectroscopy (GC-MS) [26], indicating that if tobacco is present in the sample, it must be present in trace amounts (Table S7). For sample n°13, no amplification signal was measured using both the NIA-1 qPCR and ddPCR methods, indicating the absence of tobacco DNA (Table 2). No nicotine was also detected by GC-MS for this sample (Table S7). In addition, as previously observed with ground tobacco reference materials (samples n°1–7) using the NIA-1 qPCR and ddPCR methods, a correlation between the recorded C_q values and copy numbers was observed for all tested commercial smoke and smokeless tobacco and tobacco-free products (samples n°8–13) (Table 2).

Taken together, these results clearly demonstrate the applicability of the NIA-1 qPCR and ddPCR methods on both ground tobacco reference material (samples n°1–7) and smoke and smokeless tobacco and tobacco-free products (samples n°8–13).

4. Conclusion

The newly developed NIA-1 ddPCR method as well as the previously developed NIA-1 qPCR method were evaluated and validated according the MPR for GMO analysis defined by ENGL. Their method performance in term of specificity and sensitivity was successfully assessed and deemed as suitable to target DNA from tobacco in line with the MPR defined by ENGL. These NIA-1 PCR-based methods were able to specifically detect *Nicotiana* species used for the manufacturing of commercial tobacco products, being,

predominantly, *N. tabacum* and, less frequently, *N. rustica*. Furthermore, no amplification signal for species well-known to be mixed with tobacco products, such as *C. sativa* in "joints" and *S. aromaticum* in "clove cigarettes", were observed [23,25]. These NIA-1 PCR-based methods showed also a comparable limit of detection, determined at ~4 estimated target copies for the NIA-1 qPCR method and at ~8 estimated target copies for the NIA-1 ddPCR method. With a limit of detection below 25 copies of the target, these NIA-1 PCR-based methods with equivalent sensitivity performance were consequently assessed as sensitive and fit for purpose. In addition, the transferability of the NIA-1 qPCR and ddPCR methods to an external laboratory was confirmed since similar performance between the in-house and external assays was observed. Finally, the applicability of these NIA-1 PCR-based methods was successfully demonstrated using 13 samples, including ground tobacco reference materials and commercial tobacco and tobacco-free smoke and smokeless products. For these 13 samples, comparable results were obtained using the NIA-1 qPCR and ddPCR methods. Consequently, these NIA-1 PCR-based methods were considered as appropriate to support the competent authorities in their control of tobacco on the market. In this study, both validated qPCR and ddPCR methods were also proposed, offering more flexibility to the enforcement laboratories for tobacco control according their competent authorities requirements and their in-house infrastructures. Although both NIA-1 qPCR and ddPCR methods are valuable for tobacco control on the European market, the newly developed NIA-1 ddPCR method offers however the additional advantage of simultaneously detecting and absolutely quantifying the tobacco DNA present in the tested sample without dependence on optimal reference materials and standard curves as well as a limited impact of PCR inhibitors.

On this basis, this study represents a step forward for the standardization of a reliable and efficient control of tobacco on the market using PCR-based methods validated in line with international standard. In the future, these NIA-1 PCR-based methods may be performed through an international collaborative ring trial involving a minimum of 12 independent enforcement laboratories in order to ensure their full validation at the European level [9,27].

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Data availability

Data will be made available upon request.

CRediT authorship contribution statement

Marie-Alice Fraiture: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Andrea Gobbo:** Writing – review & editing, Formal analysis. **Chloé Guillitte:** Writing – review & editing, Formal analysis. **Sophia Barhdadi:** Writing – review & editing, Formal analysis. **Céline Gau:** Writing – review & editing, Formal analysis. **Patrick Philipp:** Writing – review & editing, Formal analysis. **Lucas Marmin:** Writing – review & editing, Formal analysis. **Ugo Marchesi:** Writing – review & editing, Formal analysis. **Daniela Verginelli:** Writing – review & editing, Formal analysis. **Nina Papazova:** Writing – review & editing, Validation, Formal analysis. **Céline Vanhee:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Nancy H.C. Roosens:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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