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Research article

A digital PCR approach to assess the purity of oregano

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ABSTRACT

Herbs and spices are food categories known to be at high risk of adulteration. Presence of undeclared foreign plant species has often been reported in oregano and may have a direct impact on its organoleptic quality and potentially the safety of this aromatic herb. A droplet digital PCR approach was developed to assess the purity of oregano by quantifying the DNA copies of oregano versus the total plant DNA copies. Nuclear single-copy genes were selected by targeting the *terpene synthase 5* gene from oregano and the plant *phosphoenolpyruvate carboxylase 2* gene. The reactions were specific to the Origanum genus and plant materials respectively, whereas trueness and precision data confirmed the reliability of the method to quantify oregano. The applicability of the method was further verified on proficiency test samples before being applied on commercial oregano samples.

1. Introduction

The market for spices and seasonings was accounted at USD 36.9 billion in 2021 and is expected to nearly double by 2030 [1] with spices, herbs and salts representing approximately 60%, 30% and 10% of the shares, respectively. Oregano (*Origanum vulgare/onites*) is one the most popular aromatic herb being cultivated for its culinary taste when used as fresh or dried leaves, or for its health benefits when used as oregano extract or oregano essential oils [2].

Herbs and spices are often sold as powder which make them prone to adulteration with organic material by substituting them with cheaper ingredients, or with inorganic adulterants with filling agents such as sand or talc [3]. Several analytical tools may be applied to detect adulterants; they can be as simple as sensorial or visual methods, or advanced and complex laboratory technologies such as high-performance liquid chromatography or DNA-sequencing approaches [4]. Numerous studies have reported frequent adulteration of herbs and spices with undeclared foreign species, and more specifically in oregano [5–7]. DNA-based methods are regularly applied for species identification [8], and the evolution of DNA sequencing towards DNA metabarcoding with Next Generation Sequencing (NGS) has gained more and more interest. Indeed, several studies have applied DNA metabarcoding to identify species in herbs and spices [9–11], including the European Joint Research Center (JRC) who performed a massive survey on thousands of European samples [12]. To further complement the NGS findings, the JRC has applied a droplet digital PCR (ddPCR) approach to precisely assess the purity of the suspicious samples. This approach was based on the measurement of the DNA copies of the plant of interest by ddPCR, oregano for example, versus the theoretical amount of plant DNA copies added in the reaction. This theoretical amount was determined by dividing the sample DNA mass measured with QuBit fluorimetry and added in the ddPCR, by the published average 1C-value of the targeted plant species (oregano). However, critical measurement bias has already been reported for the determination of DNA concentration by spectroscopic or fluorometric measurements [13,14] while the plant 1C-values can vary drastically depending on the

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literature; the oregano 1C-value can typically vary from 0.70 to 0.89 pg per haploid genome [15,16] (KEW; Zonneveld, 2019).

In this study, a ddPCR approach was developed to assess the purity of oregano where oregano-specific and plant-specific sets of primers were designed, focusing on nuclear single-copy genes to enable a reliable and precise quantification. The development of the method was performed on fresh oregano leaves and specificity was verified against other common plant and animal species. The performance of the method was evaluated on oregano spiked with species frequently detected in oregano samples, typically parsley, olive, and bindweed [11,12]. Proficiency test samples for oregano authenticity were also analysed before applying the method on several commercial oregano powders and oregano dried leaves collected on local markets and internet.

2. Materials & methods

2.1. Materials

2.1.1. Collected samples

Leaves, seeds and tubers of plant species (Table 1) were obtained from local markets. To remove potential particles/contaminants from other species, each sample was washed with distilled water for a few seconds. When the identity of the plant was not certain, its identification was confirmed by DNA barcoding [17] before using the sample. Ground samples of aromatic herbs were obtained from

Table 1
List of pure plant and animal species used in this study, and their amplification with Plant-PEPC2 and Oreg-TPS5 primers. Samples were collected as pure leaves (L), grains/seeds (G), pure tubers (T) or pure meat (M).

Tested species		ddPCR amplification	
		Plant-PEPC2	Oreg-TPS5
Oregano[L]	*Origanum vulgare/onites*	Yes	Yes
Marjoram[L]	*Origanum marjorana*	Yes	Partially
Rosemary[L]	*Rosmarinus officinalis*	Yes	No
Basil[L]	*Ocimum basilicum*	Yes	No
Thyme[L]	*Thymus vulgaris*	Yes	No
Parsley[L]	*Petroselinum crispum*	Yes	No
Chive[L]	*Allium schoenoprasum*	Yes	No
Dill[L]	*Anethum graveolens*	Yes	No
Fennel[L]	*Foeniculum vulgare*	Yes	No
Celery[L]	*Apium graveolens*	Yes	No
Chervil[L]	*Anthriscus cerefolium*	Yes	No
Coriander[L]	*Coriandrum sativum*	Yes	No
Lemon[L]	*Citrus limon*	Yes	No
Lettuce[L]	*Lactuca sativa*	Yes	No
Olive[L]	*Olea europaea*	Yes	No
Sage[L]	*Salvia officinalis*	Yes	No
Wild Sage[L]	*Lantana camara*	Yes	No
Mint[L]	*Mentha piperita*	Yes	No
Tarragon[L]	*Artemisia dracunculus*	Yes	No
Bindweed[L]	*Convolvulus arvensis*	Yes	No
Alfalfa[L]	*Medicago sativa*	Yes	No
Lemon bush[L]	*Lippia* sp.	Yes	No
Juniper berry[G]	*Juniperus communis*	Yes	No
Cumin[G]	*Cuminum cyminum*	Yes	No
Garlic[L]	*Allium sativum*	Yes	No
Rapeseed[G]	*Brassica* spp.	Yes	No
Black mustard[G]	*Brassica* spp.	Yes	No
Black pepper[G]	*Piper nigrum*	Yes	No
Turmeric[T]	*Curcuma longa*	Yes	No
Paprika/Chili[G]	*Capsicum* spp. *annuum*	Yes	No
Ginger[T]	*Zingiber officinale*	Yes	No
Maize[G]	*Zea mays*	Yes	No
Soya[G]	*Glycine max*	Yes	No
Wheat[G]	*Triticum aestivum*	Yes	No
Rice[G]	*Oryza sativa*	Yes	No
Rye[G]	*Secale cereal*	Yes	No
Barley[G]	*Hordeum vulgare*	Yes	No
Oat[G]	*Avena sativa*	Yes	No
Tomato[G]	*Solanum lycopersicum*	Yes	No
Beef[M]	*Bos taurus*	No	No
Pork[M]	*Sus scropa*	No	No
Chicken[M]	*Gallus gallus*	No	No
Salmon[M]	*Salmo salar*	No	No
Cod[M]	*Gadus morhua*	No	No
Pilchard[M]	*Sardina pilchardus*	No	No

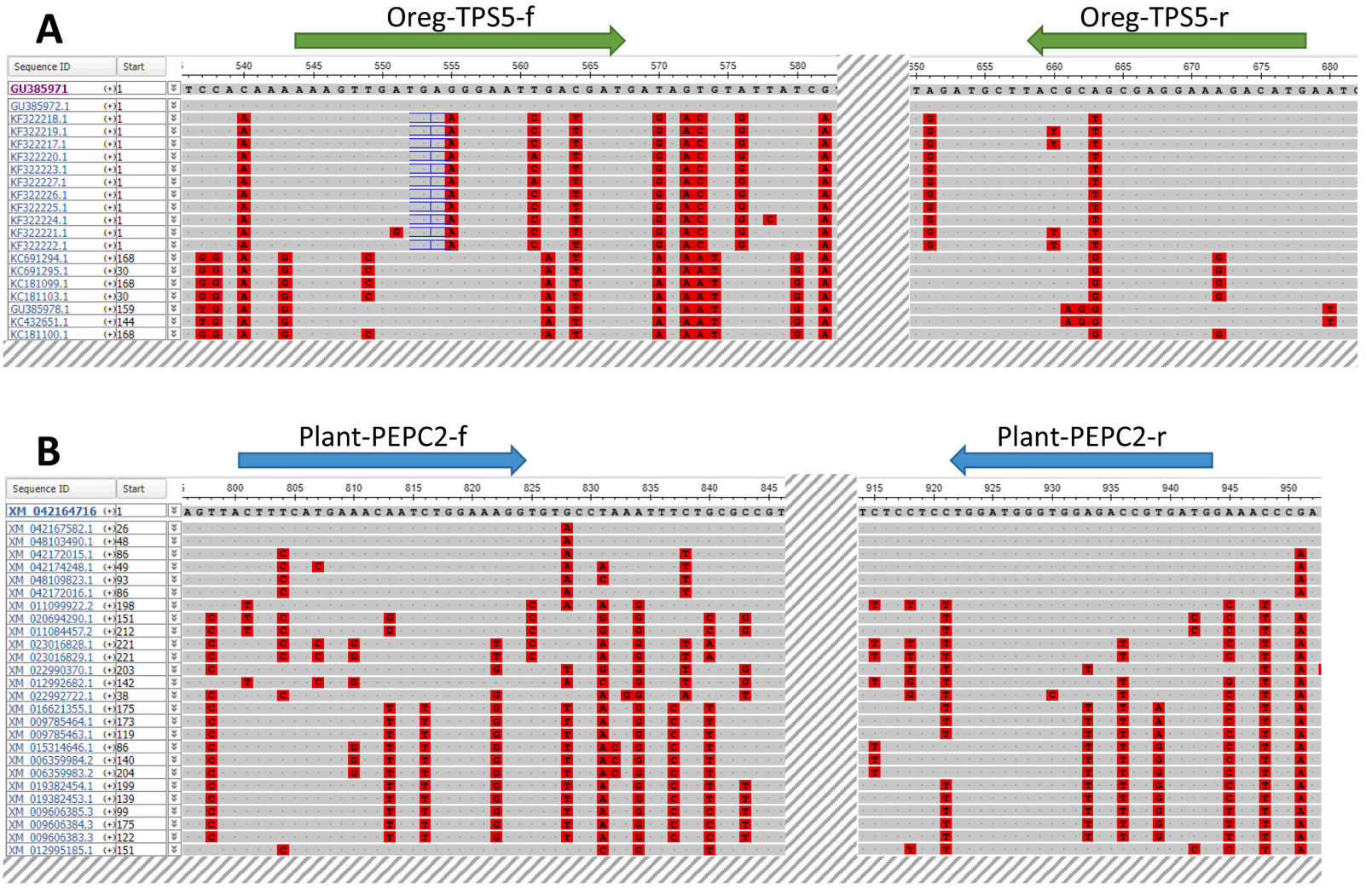

Fig. 1. Snapshots of nucleotide alignments from plant terpene synthase (**A**) and phosphoenolpyruvate carboxylase (**B**) genes obtained with the BLASTn MSA viewer when submitting *O. vulgare* TPS5 mRNA sequence (GenBank Accession no. GU385971) and *Salvia splendens* PEPC2 mRNA sequence (GenBank Accession no. XM_042164716), respectively. Conserved nucleotides are indicated with grey dots, whereas nucleotide polymorphisms are highlighted in red. Primers are indicated with arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

industrial suppliers and from local markets.

A total of 22 commercial oregano dried leaves, flakes and powders were purchased from local markets and from internet to have an insight of the current situation of adulteration in oregano products.

2.1.2. Spiked samples & proficiency test samples

To evaluate the performance of method to quantify oregano, spiked samples were prepared. To reduce potential microbiological hazard, it is well known that herbs and spices undergo various industrial treatments which degrade more or less extensively plant DNA [11,18] and have a direct impact on the quality and quantity of DNA extracted from these samples. To avoid biases linked to this variability of samples, spiking experiments were therefore not performed with powder mixtures, but with DNA mixtures extracted from these powders and standardized at 10 ng/μL. DNA extracted from oregano powder was spiked at 90%, 80%, 70% 50%, 25% and 10% (v/v) with DNA extracted from parsley, olive, and bindweed powders. These DNA mixtures were well vortexed to ensure homogeneity before being analysed.

To evaluate the performance of the method on real samples with known amount of adulterants, and not on DNA mixtures, proficiency test (p-test) samples for oregano authenticity from FAPAS (Fera Science Ltd., York, UK) were analysed. Although these p-test samples were qualitative, the approximative amount of spiked adulterant material(s) was described in their respective p-test reports.

2.2. Methods & instrumentation

2.2.1. DNA extraction

DNA from at least 200 mg of pure plant material (leaf, seed, tuber) was extracted according to Cottenet, Blancpain & Chuah (2019) [19], whereas DNA from 1 g of powder samples was extracted according to Cottenet et al. (2022) [11].

DNA concentration was measured with a NanoDrop (Thermo Fischer Scientific, Waltham, USA), and DNA extracts were diluted at 10 ng/μL in EB buffer (QIAgen). Extracted DNA and reconstituted DNA were stored at −20 °C until further use.

2.2.2. Design of PCR primers

No oregano-specific primers targeting nuclear single-copy gene(s) were identified in the literature. The JRC survey described primers designed on the *elongation factor 1* [12] but led to cross-amplifications on other *Lamiaceae* species (data not shown) and is known to be multicopy gene and have pseudogenes in plants [20]. Oregano is a diploid plant species [21] with some terpene synthase genes identified on one of the two alleles only. The oregano-specific PCR primers were designed on the *terpene synthase 5* gene (*tps5*) which has been described as a single-copy gene in oregano [22,23]. Sequences of *O. vulgare* TPS5 mRNA were retrieved from the NCBI nucleotide database (GenBank Accession no. GU385971 & GU385972) and similarities with sequences from other species were searched using the BLASTn tool [24] in order to identify discriminatory regions for the design of oregano-specific PCR primers (Fig. 1A). PCR primers were designed with Primer Express version 3.0 (Thermo Fisher Scientific) to amplify fragments shorter than 200 bp well adapted to degraded DNA in processed samples [25]. Selected Oreg-TPS5 forward and reverse primers (Oreg-TPS5-f: AAAGTTGATGAGGGAATTGACGAT, and Oreg-TPS5-r: CATGTCTTTCCTCGCTGCGT) amplified a fragment of 135 bp.

Primers designed to amplify several candidates of nuclear single-copy genes for plants were retrieved from literature, such as *TOPO6* [26] or *LEAFY* [27] but all our attempts to amplify plant material failed using these primers. Other potential candidates such as *adh*, *actin*, *GAPDH* have been shown to be prone to frequent gene duplication and partial recombination [28,29] and were therefore not considered. Plant-specific primers were designed on the nuclear *phosphoenolpyruvate carboxylase 2* gene (*pepc2*) which has been described as a single-copy gene in the vast majority of plant species [28,30]. In absence of oregano *pepc2* sequences available in NCBI nucleotide database, the PEPC2 mRNA sequence from *Salvia splendens* (GenBank Accession no. XM_042164716), another *Lamiaceae* species, was compared to plant sequences using BLASTn. Sequences of *pepc2* and (putative) mRNA from over 1000 plant taxons were aligned with the BLASTn MSA viewer tool (Multiple Alignment Viewer 1.23.0) allowing the design of consensus plant primers (Fig. 1B). To ensure the amplification of a broad spectrum of plant species, wobble bases had to be introduced into the Plant-PEPC2 primers to cover nucleotide polymorphisms. Selected plant primers (Plant-PEPC2-f: CTTCCATGARACWATHTGGAARGG and Plant-PEPC2-r: CATCCGATCACCNCCCATCCA) allowed amplifying fragments of approximately 143 bp depending on plant species. The limited conserved regions between the primers didn't allow to design consensus TaqMan probes, thereby leading to the design of primers only and applying EvaGreen chemistry. Oligonucleotides were synthesized by MicroSynth AG (Geneva, Switzerland).

2.2.3. Droplet digital PCR

Determination of *tps5* and *pepc2* copy numbers was performed separately by ddPCR. The optimization of the ddPCR protocol was performed according to European guidelines [31], considering i) the amplification of a single target (unique population of positive droplets), ii) the resolution R_S of the separation of positive versus negative droplets and iii) the phenomenon of rain. The ddPCR analyses were performed on a QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories Inc., USA) according to the supplier's recommendations. The ddPCR mixture contained 10 μL of ddPCR EvaGreen Supermix (Bio-Rad), 250 nM of primer forward, 250 nM of primer reverse, and 2 μL of sample DNA. This sample input led to minimum 20% negative droplets which achieved the lowest technical ddPCR measurement uncertainty <5% [32]. Droplets were generated by loading 20 μL of this mixture and 70 μL of droplet oil (Bio-Rad) in a microfluidic cartridge (Bio-Rad) using the QX200™ Droplet Generator (Bio-Rad). Droplets were then transferred into a 96-well plate and the PCR was performed in a C1000 Touch™ thermal cycler (Bio-Rad) set at a ramp rate of 2 °C/s. The initial denaturation temperature was at 95 °C for 5 min, followed by 50 cycles consisting in a denaturation step at 95 °C for 30 s and an annealing/extension step at 58 °C for 1 min. During the method optimization, gradient experiments were performed with

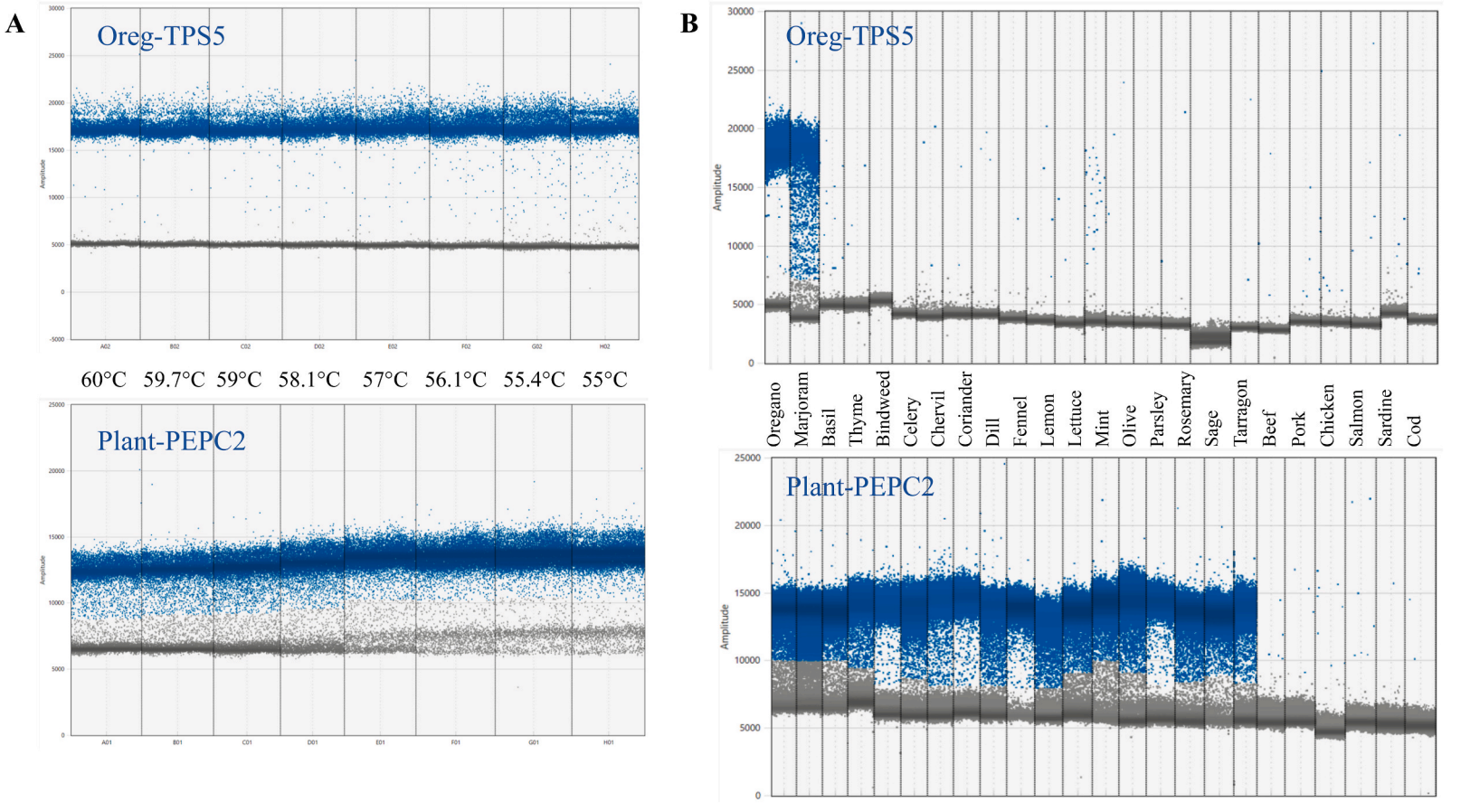

Fig. 2. Gradient ddPCR between 55 °C and 60 °C on pure oregano leaf DNA (**A**) and amplification at 58 °C on various plant species (**B**) using Oreg-TPS5 (upper graph) and Plant-PEPC2 (lower graph) primers. Positive and negative droplets are indicated in blue and grey, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

annealing/extension temperatures between 55 °C and 60 °C for 1 min. The run was completed by 5 min at 4 °C, then 5 min at 90 °C and back to 4 °C for minimum 1 min. The PCR plate was transferred into the QX200™ Droplet Reader (Bio-Rad) for fluorescence measurement. The QX Manager software version 1.2 (Bio-Rad) was used to analyze the data based on Poisson law statistics and delivered the results as target copies per reaction. Except when noted, samples were analysed in duplicate and the average target copies per reaction was calculated and considered. To calculate purity, the oregano *tps5* copy number was multiplied by 2 since *tps5* is present in one of the two alleles of oregano, and divided by the plant copy number; the sample purity was finally expressed in percentage.

2.2.4. Performance assessment of ddPCR

Specificity was evaluated by testing all the DNA samples from pure materials (Table 1) in duplicate [33]. Linearity and dynamic range of the quantification was performed by establishing linear regressions between the theoretical amount of *tps5* and *pepc2* copies added in the dPCR reaction and the ones measured by ddPCR; each concentration was analysed in triplicate. This theoretical amount was determined by dividing the sample DNA mass (pg) added in the ddPCR, by the oregano 1C-value (1C = 0.89 pg per haploid genome [16]).

Quantitative method performance, bias and coefficients of variability (CV) were determined according to ISO 5725 [34] by testing all spiked DNA samples in duplicates analysed at least on 6 different runs (2 different operators on 3 different days) leading to minimum 12 values per concentration. FAPAS proficiency test samples and the 22 commercial oregano samples were tested in triplicate.

2.2.5. DNA metabarcoding by Next Generation Sequencing

To confirm the results obtained by ddPCR on the 22 commercial samples of the survey, their DNA were also analysed by DNA metabarcoding using Next Generation Sequencing according to Cottenet et al. (2022) [11]. Briefly, the sample DNA was amplified with the SGS All Species ID Plant DNA Analyser Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the library was sequenced on the Ion GeneStudio S5 Food Protection sequencer (Thermo Fisher Scientific). The FASTQ file containing all the nucleotide sequences was uploaded in the SGS All Species ID software version 3.0.10 (SGS Molecular, Lisbon, Portugal) for species identification.

2.2.6. Statistical analyses

Each data point was analysed in triplicates minimum and expressed as the mean ± standard deviation. Bias, and precision (repeatability (r) and intermediate reproducibility (iR)) data were determined with an internal statistical software package QStat.net (version 4.9.2.2035), containing the formulas described in ISO 5725 [34]. Quantitative values obtained by ddPCR and by NGS on oregano survey samples were compared using a one-way analysis of variance (ANOVA) with a level of significance p of 0.05.

3. Results & discussion

3.1. Optimization of ddPCR & specificity

The annealing/extension temperatures play a key role for ddPCR experiments, especially with EvaGreen chemistry. When applying a gradient test with Oreg-TPS5 and Plant-PEPC2 primers on an oregano DNA, successful amplifications were observed for both targets on all temperatures (Fig. 2A). However, an unspecific amplification started to appear on PEPC2 target at 57 °C and below. With an efficient separation of PEPC2 droplets at 58 °C, this temperature was selected for future experiments. The profile of the droplets obtained with Oreg-TPS5 on oregano samples (Fig. 2A & B) led to a high ddPCR resolution $R_S = 5.5$, well above 2 as recommended by European dPCR guidelines [31]. In absence of rain, positive droplets were easily distinguishable from the background negative droplets, which enabled the setting of a reliable automatic threshold by the QX Manager software. In comparison, a random phenomenon of rain was observed in the profiles of Plant-PEPC2 droplets with some plant species (Fig. 2B), with a ddPCR resolution R_S varying from 4 to 1.9 depending on the species, either considered as high or moderate and linked to a lower PCR efficiency in more difficult species [31]. The rain was already decreased by applying 50 cycles PCR as suggested by the manufacturer and by Lievens et al. (2016) [35].

The specificity of the designed PCR primers was evaluated on DNA extracted from pure plant and animal species. The Oreg-TPS5 primers successfully amplified on oregano (Table 1 & Fig. 2B) and partially on marjoram which was originally considered as oregano before being differentiated [36]. With marjoram being more expensive than oregano [37], it is unlikely to have oregano samples adulterated with marjoram; it has never been reported either [11,12]. When tested on other plant species or animal species frequently used in food recipes, no cross-amplification was detected. The Plant-PEPC2 primers led to successful amplifications on all the plant samples, without any cross-amplification on animal species (Table 1 & Fig. 2B).

Oreg-TPS5 and Plant-PEPC2 primers were designed on more than 1000 plant taxons and the experiments successfully confirmed their amplification on *Origanum* sp. and plant materials, respectively. The optimization of the ddPCR protocol allowed an efficient differentiation between positive and negative droplets for both targets which plays a key role in the delivery of reliable quantitative results.

3.2. Quantitative behavior of the oregano ddPCR method

3.2.1. Linearity & dynamic range

When analyzing serial dilutions from a fresh oregano leaf DNA, both sets of primers led to linear correlations with $R^2 \geq 0.99$

(Fig. 3). Linear regression with Plant-PEPC2 and Oreg-TPS5 primers led to slopes of 1.94 and 0.92 respectively, which indicated the presence of 2 copies of *pepc2* and 1 copy of *tps5* in the diploid oregano genome, and confirmed that *tps5* is present on one allele only [22]. Testing 20 ng of fresh oregano DNA led to approximately 43,000 *pepc2* copies, well aligned with the theoretical amount of chromosomal DNA molecules (≈45,000) determined with the latest published 1C value of 0.89 pg per haploid genome [16].

These data showed a linear correlation and a stable behavior of both targets along the dynamic range. Together with the optimized ddPCR conditions, these linear characteristics participate to the reliability of the quantitative data delivered by the method.

3.2.2. Quantification in spiked samples and proficiency test samples

DNA of oregano powders was spiked with DNA from parsley, bindweed, and olive powders, and analysed in duplicates on minimum 6 different days (Table 2). Measurements performed by ddPCR led to results very close to the expected concentrations of oregano all along the dynamic range tested, with biases below ±25% as recommended by validation guidelines [33]. The coefficients of variation for repeatability (CVr) were below 25% for all the concentrations tested, whereas the coefficients of variation for intermediate reproducibility (CViR) were all below 35%. Trueness and precision data were thus aligned with validation guidelines, demonstrating a reliable behavior of the method to quantify oregano DNA.

To mimic real-life oregano samples, 9 FAPAS p-test materials made of oregano dried leaves as the main matrix were analysed by ddPCR (Table 3). Amongst them, three test samples were pure oregano (T2985_B, T2990_B and T2996_C) and were successfully quantified ≥94% (w/w) by ddPCR. The six other samples were voluntarily spiked in mass ratios with cistus, myrtle, olive, savory, sumac and/or hazel adulterants. Oregano content of samples T2990_C, T2996_A and T2996_B was correctly determined by ddPCR with quantitative results very close to the expected values, whereas the content of oregano in samples T2990_C, T2996_A and T2996_B was underestimated by ddPCR. FAPAS reported that the main oregano matrix of these three last samples was unexpectedly adulterated with significant amount of olive particles. The ddPCR method was thus successfully able to indicate the additional presence of undeclared adulterants in these samples by quantifying lower oregano content.

Results obtained by ddPCR on all these tested samples were all in agreement with the expected data, thus confirming the validity of the method to determine the purity of an oregano sample.

3.3. Survey on oregano commercial samples

Commercial samples of oregano powders and oregano dried leaves were collected on local markets and on internet. Amongst the 22 samples analysed, 13 led to an oregano content below 90 % (w/w) by ddPCR (Table 4), indicating that 56 % of the tested samples were impure. This proportion is aligned with other surveys previously published [5,11,12]. To identify the plant species present in each sample, especially the impure ones, all the samples were also analysed by DNA metabarcoding using Next Generation Sequencing according to Cottenet et al. (2022) [11]. The NGS results confirmed the ddPCR findings and showed that the most frequent foreign species were parsley, bindweed, and lettuce. In addition, 6 of these samples analysed by ddPCR were shown not to contain any oregano DNA suggesting a complete substitution with undeclared plant species; no oregano was found by NGS either in these samples, confirming substitution with other plant species, especially with wild sage. Although precise quantification by DNA metabarcoding has not been demonstrated yet, the number of sequencing reads obtained per species allowed to estimate their relative proportion in each sample (Table 4). Both ddPCR and NGS were found to deliver quantitative oregano values statistically similar ($p < 0.05$), with an average difference of 0.3%, which confirmed the reliability of this new ddPCR method to quantify oregano.

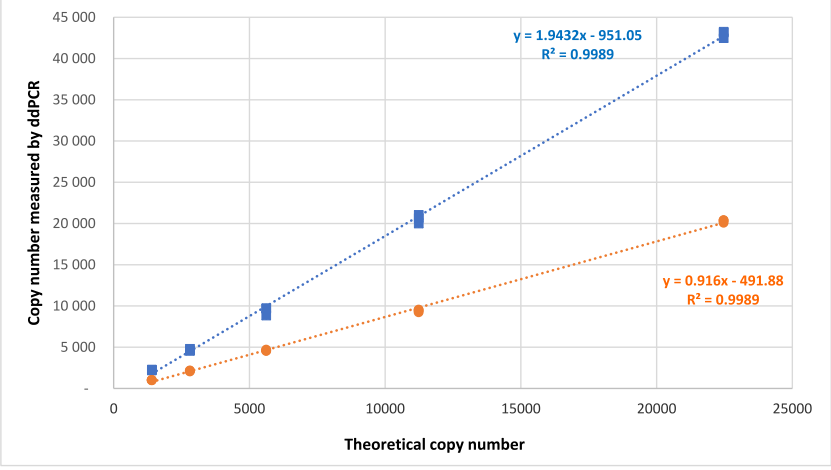

Fig. 3. Linear correlation between the theoretical number of plant *pepc2* and oregano *tps5* copies in oregano haploid genome (X-axis) and the number of copies measured by ddPCR (Y-axis). Plant *pepc2* and oregano *tps5* data are represented with blue squares and orange circles, respectively. All dilutions were tested in triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Trueness and precision data obtained on oregano DNA spiked with olive, parsley, and bindweed DNA at various concentrations. Each sample was analysed in duplicate on 6 different days leading to 12 values per concentration.

Adulterant	Oregano concentration % (w/w)	Average % (w/w)	Bias (%)	CVr (%)	CViR (%)
Olive	90	93.2	3.6	7.4	5.6
	80	80.1	0.1	4.6	7.5
	70	70.7	1.0	11.9	1.8
	50	50.8	1.6	7.3	13.0
	20	20.8	4.0	5.3	9.8
	10	11.5	15.0	4.9	27.9
Parsley	90	91.6	1.8	5.7	6.0
	80	78.7	−1.6	3.3	10.7
	70	68.9	−1.6	2.3	7.0
	50	44.3	−11.4	8.3	8.9
	20	15.7	−21.5	6.8	6.1
	10	7.6	−24.0	7.2	13.4
Bindweed	90	93.2	3.6	3.9	4.2
	80	85.2	6.5	17.1	11.5
	70	75.3	7.6	4.2	12.2
	50	53.3	6.6	20.0	18.5
	20	22.5	12.5	9.3	18.4
	10	12.2	22.0	8.7	18.5

Table 3
Content of FAPAS oregano proficiency test samples and results obtained by ddPCR. ddPCR data correspond to the average of triplicates of the target copies obtained per reaction for Oreg-TPS5 and Plant-PEPC2 targets.

Oregano samples	Measured by ddPCR			FAPAS information	
	Oreg-TPS5 (copies)	Plant-PEPC2 (copies)	Oregano (% w/w)	Adulterated? (Estimated oregano % w/w)	Spiked adulterant(s) (% w/w)
T2985_B	7894 ± 280	16,018 ± 73	99%	NO (100%)	–
T2990_B	11,212 ± 568	23,645 ± 822	95%	NO (100%)	–
T2996_C	6554 ± 50	12,550 ± 523	104%	NO (100%)	–
T2990_C	5854 ± 734	15,999 ± 130	73%	YES (75%)	Cistus (25%)
T2996_A	4976 ± 96	13,846 ± 180	72%	YES (71%)	Myrtle (17%) + Olive (12%)
T2996_B	4620 ± 54	13,000 ± 440	71%	YES (78%)	Hazel (11%) + Savory (11%)
T2985_A[a]	8543 ± 43	30,128 ± 100	57%	YES (71%)[a]	Myrtle (17%) + Savory (12%)
T2985_C[a]	12,389 ± 244	32,323 ± 1730	77%	YES (91%)[a]	Sumac (9%)
T2990_A[a]	11,597 ± 38	31,596 ± 356	73%	NO (100%)[a]	–

[a] Intrinsically adulterated with olive leaves.

4. Conclusion

Herbs and spices are known to be prone to adulteration, especially with the addition or substitution with cheaper botanical species. Since oregano is regularly reported as the most at risk, a ddPCR method was here developed to assess the purity of oregano by quantifying nuclear single-copy genes specific to oregano and specific to plant materials. Spiking experiments with frequent adulterant species, namely parsley, olive, and bindweed, led to satisfactory performance characteristics, aligned with quantitative requirements. The successful analysis of proficiency test samples further confirmed the reliability of the method to quantify oregano in real samples and detect adulterated ones. This new ddPCR purity method was then applied on 22 commercial oregano samples and showed that 56% of them were suspicious, especially 6 where no oregano was detected. ddPCR results were successfully confirmed by DNA metabarcoding which allowed to identify plant species present in each sample.

Since ddPCR is cheaper and quicker to run than DNA metabarcoding, our ddPCR method can be used as a first screening step to flag impure oregano samples. These suspicious samples could then be tested by DNA metabarcoding to identify the composition of plant species present in the samples and confirm adulterated samples. This approach can also be extended to other species of herbs and spices in order to determine their purity provided a set of primers specific to the species of interest has been designed on a nuclear single-copy gene.

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Table 4
ddPCR and DNA metabarcoding results obtained on commercial samples of oregano. ddPCR data correspond to the average of triplicates of the target copies obtained per reaction for Oreg-TPS5 and Plant-PEPC2 targets, whereas NGS was performed according to Cottenet et al., 2022.

Commercial samples	ddPCR			DNA metabarcoding by NGS	
	Oreg-TPS5	Plant-PEPC2	% oregano	% oregano	Other species detected
Oregano Powder 01	279 ± 14	779 ± 50	72 %	74 %	+14% parsley +6% alfalfa +3% lettuce +3% others
Oregano Powder 02	3420 ± 179	7662 ± 327	89 %	92 %	+8% parsley
Oregano Powder 03	7881 ± 469	16,124 ± 842	98 %	95 %	+3% bindweed +2% thyme
Oregano Powder 04	2505 ± 172	5478 ± 227	91 %	89 %	+5% bindweed +3% basil +2% thyme
Oregano powder 05	2713 ± 14	6459 ± 93	84 %	74 %	+12% olive +6% bindweed +3% alfalfa +5% others
Oregano powder 06	39 ± 13	13,840 ± 1792	1 %	0 %	+62% wild sage +38% lemon bush
Oregano powder 07	58 ± 3	14,948 ± 519	1 %	0 %	+39% bindweed +32% lettuce +29% sage
Oregano powder 08	2547 ± 39	6560 ± 104	78 %	83 %	+5% cynodon +3% bindweed +2% alfalfa +7% others
Oregano dried leaves/flakes 01	15,245 ± 656	30,107 ± 1410	101 %	100 %	
Oregano dried leaves/flakes 02	4 ± 2	13,939 ± 989	0 %	0 %	+40% wild sage +35% lemon bush +25% fogfruit
Oregano dried leaves/flakes 03	15,505 ± 541	35,084 ± 5177	88 %	90 %	+3% amaranth +3% lettuce +4% others
Oregano dried leaves/flakes 04	6547 ± 805	22,616 ± 1555	58 %	59 %	+23% coriander +15% lettuce +3% others
Oregano dried leaves/flakes 05	4165 ± 566	8397 ± 598	99 %	98 %	+2% bindweed
Oregano dried leaves/flakes 06	3079 ± 397	6137 ± 146	100 %	96 %	+2% alfalfa +2% garlic
Oregano dried leaves/flakes 07	1 ± 0	2528 ± 115	0 %	0 %	+42% wild sage +35% lemon bush +23% fogfruit
Oregano dried leaves/flakes 08	1 ± 0	8942 ± 388	0 %	0 %	+39% wild sage +35% lemon bush +26% ragwort
Oregano dried leaves/flakes 09	14 ± 5	8320 ± 411	0 %	0 %	+35% hempweed +32% *Henricksonia mexicana* + 33% sage
Oregano dried leaves/flakes 10	5715 ± 376	11,395 ± 48	100 %	100 %	
Oregano dried leaves/flakes 11	3570 ± 520	8100 ± 138	88 %	67 %	+19% olive +6 % bindweed +4% bishop +4% garlic
Oregano dried leaves/flakes 12	22,955 ± 485	47,951 ± 596	96 %	98 %	+1% bindweed +1% parsley
Oregano dried leaves/flakes 13	22,008 ± 1657	43,684 ± 754	101 %	99 %	+1% amaranth
Oregano dried leaves/flakes 14	2483 ± 46	8943 ± 380	56 %	56 %	+30% mint +6% olive +5% parsley +3% others

Data availability statement

The data associated with our study has not been deposited into any publicly available repository. It will however be made available upon request.

Ethics statement

Informed consent was not required for this study because no human biological samples were analysed, only food-grade ingredients.

CRediT authorship contribution statement

Geoffrey Cottenet: Writing – original draft, Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization. **Carine Blancpain:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **James Holzwarth:** Software, Resources, Data curation.

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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