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**CREDIT AUTHOR STATEMENT**

To the attention of the editor-in-chief of the Scientific journal Food Control,
Dear editor
On behalf of my co-authors, I am declaring the authors contribution to the present paper:

Simona Cau: Data curation, Investigation, Writing - original draft, Writing - review & editing
Maria Giovanna Tilocca: Formal analysis, Investigation, Methodology, Writing - review & editing
Carlo Spanu: Data curation, Software, Data analysis, Writing - original draft, Writing - review & editing
Barbara Soro: Formal analysis, Investigation, Methodology, Writing - review & editing
Tiziana Tedde: Formal analysis, Investigation, Methodology, Writing - review & editing
Sara Salza: Formal analysis, Investigation, Methodology, Writing - review & editing
Rita Melillo: Formal analysis, Investigation, Methodology, Writing - review & editing
Gabriella Piras: Formal analysis, Investigation, Methodology, Writing - review & editing
Sebastiano Virgilio: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing
Bruna Vodret: Conceptualization, Funding acquisition, Resources, Writing - review & editing
Alessandro Mudadu: Conceptualization, Project administration, Writing - review & editing

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# Detection of celery (*Apium graveolens*) allergen in foods of animal and plant origin by droplet digital PCR assay

Cau S.[1], Tilocca M. G.[1], Spanu C.[2*], Soro B.[1], Tedde T.[1], Salza S.[1], Melillo R.[1], Piras G.[1], Virgilio S.[1], Vodret B.[1], Mudadu A. G.[1]

[1]Veterinary Public Health Institute of Sardinia, Complex Structure of Food Hygiene, Via Duca degli Abruzzi 8, Sassari, 07100, Italy

[2]Department of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100, Sassari, Italy

*Corresponding author. Tel.: +39 079 229425; fax: +39 079 229458. E-Mail address: cspanu@uniss.it (C. Spanu); Via Vienna 2, 07100, Sassari, Italy.



## Abstract

Celery is included among the allergenic foods that, under the EU 1169/2011 regulation, must be declared in the ingredient list. However, disposition covers only allergens that are voluntary used as ingredients and not the accidental presence of allergens in a food as consequence of cross contamination. To guarantee compliance with food allergen regulations and protect health of food-allergic consumers are needed specific and sensitive methods to detect the presence of allergens in foods. Detection of allergens relies of protein- and DNA-based methods. Real-time PCR (RT-PCR) targeting sequences from the mannitol dehydrogenase (*Mtd*) gene is currently the method of choice for detection and quantification of celery in foods. However, quantification by RT-PCR methods needs standard calibration curves of the target DNA. To overcome this limitation in the present study the use of a droplet digital PCR (dd-PCR) assay has been proposed for the quantification of celery in foods. A preliminarily optimization of the dd-PCR protocol was conducted using serial

DNA dilution extracted from celery powder. Ideal primer probe concentrations were 0.9 µM of both forward and reverse primers and 0.250 µM of probe. The optimal annealing temperature was at 60 °C. The limit of detection (LOD) was 0.20±0.12 Cp/µL while the limit of quantification (LOQ) was 0.83±0.20 Cp/µL. The dd-PCR assay showed no cross-reactivity with other vegetal species, indicating a good specificity. No effect of food matrix was observed on the dd-PCR performance. The method was able to quantify the presence of celery in commercial foods of animal and plant origin.

**Keywords:** Mannitol dehydrogenase gene; Limit of detection (LOD); Limit of Quantification (LOQ); Food labelling.

## 1. Introduction

The term food allergy is used to refer to an immune response directed toward foods (Sicherer, & Sampson, 2014). Food allergic reactions include a wide variety of symptoms involving the skin, the gastrointestinal and respiratory tract and a potentially life-threating anaphylactic shock (Renz et al., 2018). The onset of the symptoms is generally rapid, within a few minutes after the ingestion of the eliciting food leading, in extreme circumstances, to a fatal reaction (Ho, Wong, & Chang, 2014).

The frequency of food allergies has increased over the last 3 decades in both industrialized and developing countries. In Western Countries (i.e., Europe, North America and Australia) it is estimated that they affect up to 8% of young children and 2-3% of adults (Nwaru et al., 2014; Prescott, & Allen, 2011; Sicherer, & Sampson, 2014). In other geographical areas there is limited available information since until recently it has been perceived as uncommon issue (EFSA 2014; Loh, & Tang, 2018). Several factors such as difference in the exposure to environmental allergens (e.g., pollen), dietetic habits and individual sensitivity influence geographical variation in the

prevalence and in the spectrum of food allergens (EFSA 2014; Eriksson et al., 2004; Tang, & Mullins, 2017).

Despite almost a thousand of proteins have been recognized as food allergens by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) most of these food allergenic proteins belong to a few families, such as albumins, globulins, and other storage protein plants, muscle proteins in animal-related food (Faeste, Rønning, Christians, & Granum, 2011). The IgE-mediated food allergic reactions are generally accountable to 8 "major" food allergens including: milk, egg, peanut, tree nut, seafood, shellfish, soy and wheat (Burks et al., 2012). Even if the incidence of fatal cases is low (Umasunthar et al., 2013), food allergies represent a serious public health concern with additional burdens for health care, food safety authorities and the food industry (e.g., food recalls cost and reputational damage). It is generally accepted that a zero risk for food-allergic people is not a feasible food safety objective (Madsen et al., 2012). However, it is difficult to set allergen threshold in foods that would allow management control strategies to obtain a tolerable level of risk (Walker, Burns, Elliott, Gowland, & Mills, 2016). In fact, a food allergic reaction may occur as consequence of the direct ingestion of a small amount or even traces of an allergen as consequence of cross-contamination (e.g., foods processed on shared equipment) (Ho, Wong, & Chang, 2014; Monaci, Tregoat, van Hengel, & Anklam, 2006). A major complication here is the fact that the amount of protein necessary to induce an allergic reaction vary from protein to protein, from person to person and from time to time within one individual (Sathe, Teuber, & Roux, 2005). Any exposure to a food allergen can potentially trigger an allergic reaction and should be regarded as a potential threat to human health (Gendel, 2012).

Much effort has been put on the definition of "threshold", "action levels" or "reference doses" of major allergens in foods through challenge studies conducted in food-allergic individuals (Muraro et al., 2014; Taylor et al., 2014; Walker, Burns, Elliott, Gowland, & Mills, 2016). Despite the increasing availability of reference doses, to date several international public health authorities

defined no population threshold for any of the allergenic foods. The European Food Safety Authority (EFSA) declined to define threshold advocating the absence of allergens reference materials.

To date there is no effective cure or treatment for food allergies, therefore the most effective option to protect susceptible population is the permanent avoidance of ingestion of the eliciting foods (Arshad et al., 2007; Burks et al., 2012). A critical measure for the implementation of safe and effective avoidance diets is the correct labeling of the presence of allergenic foods (Costa, Mafra, Carrapatoso, & Oliveira, 2012; Sheth et al., 2010). For this reason, regulatory risk management strategies have focused on promoting consumers awareness on the presence of allergens in foods through label declarations. International public health authorities and regulatory bodies have taken different approaches in the identification of priority allergens, therefore food allergen labelling regulations differ significantly around the world.

Under the EU legislation (EU Regulation No. 1169/2011), among allergenic substances whose presence in food must be indicated on the label is included the celery (*Apium graveolens*). Celery is an important member of the *Apiaceae* family which is cultivated worldwide. Even small amounts of celery can immediately lead to allergic reactions in sensitive individuals. Cross reactivity between the major birch pollen allergen (Bet v 1) with the homologous PR10 allergen in celery (Api g 1), known as Pollen Food Syndrome (PFS), increase the risk of reaction to celery in people with birch pollen sensitization (Popescu, 2015; Skypala 2019; Wüthrich, Stäger, & Johansson, 1990). Other major identified celery antigens are: Api g 1, Api g 2, Api g 4, and Api g 6. Celery is generally eaten raw, as a spice mixture or as ingredient in various foods of animal or plant origin (e.g., meat products, sausages, soups and pureed vegetable).

To verify the compliance with labelling requirements and avoid unintentional cross-contamination it is necessary to develop reliable, specific and sensitive methods for the detection and quantification of allergens in food products (Poms, Anklam, & Kuhn, 2004). The analytical

detection of allergens faces several issues related to the type of food matrix, insufficient extraction techniques, detection limit outside the dose of clinical significance, cross-reactivity among allergens, laboratory reproducibility (EFSA, 2004; Poms, Anklam, & Kuhn, 2004).

Currently for the detection of allergens in foods the most common techniques are based on the recognition of the allergen itself, generally a protein, such as the enzyme-linked immunosorbent assay (ELISA) or on specific DNA-based methods such as Real-Time quantitative PCR (qPCR) indicating the presence of the allergenic ingredient (Holzhauser, 2018). Advantages and disadvantages of both approaches have been reviewed (Prado et al., 2016). For the detection of celery, where the protein content is low, DNA-based methods may be a better choice as compared to ELISA methods that are generally non-specific due to cross-reactivity with other vegetal species (EFSA, 2014; van Hengel, 2007). The highly specific real-time PCR (RT-PCR) is currently the method of choice for detection and quantification of celery in food (Luber, Demmel, Pankofer, Busch, & Engel, 2015). Both conventional PCR and RT-PCR methods target the gene encoding for mannitol dehydrogenase (UNI EN 15634-2:2019). The main limitation of DNA-based methods for the quantification of allergens is the unavailability of certified reference materials (CRMs) for most target allergens which makes it very difficult to standardize analytical protocols. The use of droplet digital PCR (dd-PCR) is an alternative method to overcome RT-PCR limitation when determining allergenic ingredients in food (Sena-Torralba, Pallás-Tamarit, Morais, & Maquieira, 2020). The principle of dd-PCR is the random partitioning of target molecules into several thousands or millions of individual droplets in a water-oil emulsion, such that some droplets will contain the target DNA, while other will no contain the target molecules. The absolute number of target DNA contained in the original sample before partitioning can be calculated directly from the ratio of positive to total partitions, using binomial Poisson statistics (Pinheiro et al., 2012). Differently to RT-PCR for the quantification of the target DNA standard calibration curves are not required, making the method more accurate (Hindson et al., 2011; 2013).

The dd-PCR has already been used for various applications, such as the detection and quantification of genetically modified organisms (Demeke, & Dobnik, 2018), of meat species in raw and processed food (Ren, Deng, Huang, Chen, & Ge, 2017) and the food allergens peanut and soybean (Pierboni et al., 2018) and fish (Daga et al., 2018). To date no study has been conducted to evaluate the use of dd-PCR for the detection and quantification of the celery allergen in foods. The general aim of the present paper was to evaluate the feasibility of using a dd-PCR method for the detection and quantification of celery DNA in foods. Therefore, was conducted a preliminary optimization of the dd-PCR protocol. Successively were assessed the assay performances: limit of detection, limit of quantification, specificity, and selectivity. Finally, the method was tested on commercial samples of various foods of animal and plant origin.

## 2. Material and methods

### *2.1. Celery DNA extraction and quantification*

Fresh celery stalks (A. *graveolens* var. *dulce*) were manually grated using stainless steel cutter and dried at 60°C in a laboratory oven. Dried celery matrix was homogenized in a rotary blender (Sterilmixer, International PBI, Milan, Italy) for 10 min. to obtain a fine powder. DNA was extracted in duplicate (celery A and B) using the Sure Food® Prep Advanced kit (CONGEN, R-Biopharm, Germany) according to the manufacturer's instructions. The concentration and purity of the extracted DNA was determined by measuring the absorbance at 260 and 280 nm (A260 and A280) using a Sinergy 2 multi-mode microplate reader (BioTeK Instrument, Inc., Vermont, USA). The extracted celery DNA was diluted 1:5, 1:10 and 1:20 and successively used for optimization of the dd-PCR assay.

### *2.2. Primers and probe*

Primers and probes were designed targeting the celery (*Apium graveolens*) specific sequences of the mannitol dehydrogenase (*Mtd*) gene (GenBank acc. no. AF067082). The following sequences:

forward primer (Cel-MDH-iF) 5′-CGATGAGCGTGTACTGAGTC-3′, reverse primer (Cel- MDH-iR) 5′-AATAGGAACTAACATTAATCATACCAAAC-3′ and Probe (Cel-MDH-probe) 5'-FAM-AACAGATAACGCTGACTCATCACACCG-TAMRA-3' (UNI EN 15634-2:2019) were synthesized by Sigma-Aldrich (Darmstadt, Germany).

*2.3. Droplet digital PCR assay*

The dd-PCR was carried out using a QX200$^{TM}$ Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. Preliminary optimization of dd-PCR assay parameters was conducted testing three mix containing different primers and probe concentrations (supplementary table 1). Each primer/probe mix was tested amplifying the undiluted and diluted (1:5, 1:10 and 1:20) DNA extracted from the celery powder. For each combination duplicate samples of both celery DNA extraction were tested. Results of the dd-PCR optimization assay are reported in supplementary figure 1. Based on the optimization protocol the following parameters were selected for dd-PCR reaction: DNA dilution 1:20, primers and probe concentrations respectively 0.9µM and 0.25 µM. To allow an optimal distinction between positive and negative droplets, PCR annealing temperature was optimized by thermal gradient from 55°C to 65°C. The optimal annealing temperature was at 60 °C, which resulted in the greatest fluorescence amplitude difference between positive and negative droplets (Figure 1 and Supplementary figure 2). After the optimization assay, the dd-PCR reaction was carried out using a 1:20 DNA dilution in a total volume of 20 µL dd-PCR supermix for Probes (Bio-Rad Laboratories, USA) containing: 10 µL supermix 2X, 4µl of DNA and the primers and probe at final concentrations of 0.9µM and 0.25 µM, respectively.

No template controls (NTC) were used for monitoring primer-dimer formation and contaminations. Twenty microliters dd-PCR mixture/sample were placed in each well of droplet generator DG8 cartridge (BioRad Laboratories, USA) with 70 µl of droplet generator oil (Bio-Rad, Hercules), emulsified in QX-200 Droplet Generator (BioRad Laboratories, USA) and randomly

partitioned into approximately 20,000 water in oil nanoliter-size droplet. Then, a volume of 40µl of emulsion/sample was transferred to a 96-well reaction plate (Eppendorf, Hauppauge, NY, USA), heat-sealed with pierceable foil sheets by the PX1™ PCR Plate Sealer (BioRad Laboratories, USA), and amplified in C1000 Touch™ Thermal Cycler (BioRad Laboratories, USA). The cycling conditions were: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, at 60°C for 60 sec, 1 cycle of 98°C for 10 min essential for droplet stabilization and infinite 12°C hold. It was used a 2.5°C/sec ramp rate to ensure each droplet reached the correct temperature for each step during the cycling. At the end of amplification, the PCR plates were read by the QuantaSoft Droplet Reader (BioRad Laboratories, USA) that measures the fluorescence intensity of each droplet and detects the size and shape as droplets pass detector. The discrimination between positive droplets (containing target DNA) and negative droplets (not containing target DNA) was based on a good separation between them expressed as amplitude in fluorescence unit. The absolute concentration of each sample was automatically reported as copy number CelMDH/µL by the dd-PCR QuantaSoft Software V.1.7.4.0917 (Bio-Rad, Hercules) by calculating the ratio of the positive droplets over the total droplets combined with Poisson distribution with 95% confidence interval.

*2.4. Performance of dd-PCR assay: dynamic range, linearity, limits of detection and quantification and repeatability*

The pure DNA extracted from powder celery, previously submitted to spectrophotometric quantification was diluted to obtain a 1:20 initial dilution (I). After quantification by dd-PCR, celery DNA concentration was adjusted to ca 200 expected copies per µL (Cp/µL). From the initial dilution were prepared twelve two-fold serial dilutions ($S_1$-$S_{12}$). Each of the 13 dilutions and a non-template control (NTC) were analyzed in ten replicates for DNA quantification by dd-PCR (table 1).

196 The dynamic range, defined as the range of concentration over which the method performs
197 in a linear manner with an acceptable level of trueness and precision, was obtained plotting the log
198 values of expected Cp/µL against the log Cp/µL measures by dd-PCR.

199 Linearity over the dynamic range was determined by the coefficient of correlation $R^2$ of the
200 linear regression line calculated on the average of the target copy numbers measured in the
201 replicated dilution series. Repeatability over the dynamic range was determined by the percentage
202 coefficient of variation (CV%) of the measured target copy number between the replicates of the
203 dilution series. The limit of detection (LOD) for dd-PCR assay was determined as the last serial
204 dilution detected in 95% of replicates, while the limit of quantification (LOQ) was set at the lowest
205 concentration showing a coefficient of variation percentage below the threshold (CV% = 25) for
206 acceptance criteria of quantitative methods (EURL-GMFF, 2015).

207 To determine intra-assay and inter-assay repeatability of the method for detection of celery DNA,
208 for each of three DNA dilutions were run ten replicates of dd-PCR assay which were all repeated in
209 two different days.

210 *2.4 Specificity and applicability*

211 To determine the specificity of celery DNA dd-PCR assay the following food matrices were also
212 tested: laurel (*Laurus nobilis*), sage (*Salvia officinalis), thyme (Thymus vulgaris), oregano*
213 *(Origanum vulgare),* black pepper *(Piper nigrum),* parsley (*Petroselinum crispum),* onion (*Allium*
214 *cepa),* cucumber (*Cucumis sativus),* carrot (*Daucus carota),* pea (*Pisum sativum),* potato (*Solanum*
215 *tuberosum*), mixed spices and peeled tomato. The DNA was extracted and tested using
216 primers/probe and dd-PCR conditions previously described. Duplicate reactions of each non-target
217 DNA were conducted to assess potential cross-reactivities with celery DNA.

218 *2.5. Selectivity*

219 The selectivity was defined as the performance of the dd-PCR assay in the identification and
220 quantification of celery allergens in the presence of interfering substances. Therefore, to determine

the matrix effect different types of food matrix spiked with celery were tested. Spiked samples were prepared at the Food Hygiene Laboratory (Veterinary Public Health Institute of Sardinia, Sassari, Italy) by mixing 2.5 g of celery powder with either 247.5 g of ground beef meat, pureed soup and tomato sauce (1% w/w). DNA extracted from spiked samples was quantified by both spectrophotometric and dd-PCR assay. Spiked samples were successively submitted to five two-fold serial dilutions which were prepared using the respective food matrix and then analyzed by dd-PCR. Celery DNA extracted from celery powder was also included in the assay (supplementary table 2). The matrix effect was evaluated comparing $R^2$ values obtained by plotting the log values of celery expected log Cp/µL and log Cp/µL measured by dd-PCR as previously described.

*2.6. Commercial samples*

To demonstrate the applicability of newly developed dd-PCR assay, twenty-one commercial packaged food samples were analyzed. Samples included different type of food matrices: composite foods and food of animal and plant origin (Table 3). Commercial food samples were selected based on the presence of celery in the ingredients label. The celery content (%), when declared on the label, was reported. Each sample was analyzed in duplicate.

## 3. Results

*3.1. Performance evaluation of dd-PCR assay: dynamic range, linearity, limits of detection and quantification and repeatability*

In the optimization protocol of the dd-PCR assay celery DNA concentration ranged between 5.0 ng/µL and 0.001 ng/µL which corresponded to a number of expected copies per µL (Cp/µL) in the range between 200 and 0.05 Cp/µL. The average celery DNA content (Cp/µL±SD) by dd-PCR ranged between 178.21±30.60 and 0.06±0.06 (Table 1). The dynamic range and linearity are

showed in the regression line obtained by plotting the log values of expected Cp/µL and Cp/µL obtained by dd-PCR (Figure 2). The results showed that a good degree of linearity ($R^2 \geq 0.999$). The LOD of dd-PCR was as low as 0.005 ng/µL corresponding to approximately 0.20±0.12 Cp/µL of celery DNA with a detection rate of 100%. The LOQ of dd-PCR was 0.02 ng/µL corresponding to approximately 0.83±0.20 Cp/µL of celery DNA with an average droplet number of 16,696 (Table 1). Intra- and inter-assay variability showed a CV% of variation below the threshold (CV%= 25) for acceptance criteria of quantitative methods indicating a good repeatability of dd-PCR (Table 2).

*3.2. Cross-reactivity*

The two pairs of species-specific primers and the probe used in the dd-PCR assay amplified only CelMDH. Supplementary figure 3 shows a clear separation of positive droplets (celery DNA) from negative droplets (other food matrix DNA) showing no cross-reaction with non-target DNA. These results confirmed the specificity of the dd-PCR qualitative and quantitative method for celery DNA.

*3.3. Matrix effect*

The matrix effect was tested on six different DNA dilutions of celery spiked ground meat, pureed soup and tomato sauce. As showed in figure 3, all celery-spiked food samples revealed a good linearity with $R^2$ >0. 990 indicating no matrix effect on the performance of the method.

*3.4. Commercial samples*

Table 3 and figure 4 report the results of the dd-PCR assay conducted on different food matrices collected from the market. The celery DNA was detectable in 18 out of 19 samples where celery was stated in the ingredient list while was not detectable in the two samples where label stated traces content. For all the positive sample the CV% was below the 25.0% threshold for acceptance criteria of quantitative methods.

These results showed that dd-PCR can reliably quantify the presence of celery when stated on the ingredient label even at concentration as low as 0.03%. The dd-PCR assay was also able to quantify the presence of celery DNA in food samples in which celery was declared in the label but with no indication of the % content.

**Discussion**

Food allergen labelling is a fundamental strategy in the protection of food allergic consumers safety and health. However, food allergen labelling covers only allergenic ingredients voluntary used in food preparation but not the unintentional presence due to cross-contamination. To limit the risk of allergy as consequence of accidental presence of an allergen in the food, voluntary precautionary statements have been implemented by food industry (Gendel, 2012). Hence, reliable and rapid food allergen test methods are needed by both official control and food industry laboratories to ensure compliance with regulatory requirements and protect consumers health. To date the gold standard for the determination of celery food allergen is real-time (i.e., quantitative) PCR (qPCR) assay targeting sequences from the mannitol dehydrogenase (*Mtd*) gene due to the lower risk of cross-reactivity with sequences of closely related species (Hupfer, Waiblinger, & Busch, 2007). The dd-PCR is a molecular method with the advantage over qPCR to provide an absolute quantification of the nucleic acid with no need of standard curves (Hindson et al., 2011; 2013). In the dd-PCR the reaction is randomly distributed into thousands of droplets, then individual PCR reactions are conducted in each droplet to determine whether they contain or not one or more copies of the template DNA (Pinheiro et al., 2012).

To our knowledge the present is the first study aimed to investigate the feasibility of using a dd-PCR assay for the determination of celery allergens in foods. With this aim it was conducted a preliminary optimization of the dd-PCR protocol. The optimization was conducted on serial twofold dilution of celery DNA extracted from celery powder. The average ratio of absorbance at 260 nm

291 to 280 nm was between 1.7 to 2.0 indicating that the commercial kit used for the extraction
292 provided a high-quality DNA. Primers and probes were designed to target the celery (*Apium*
293 *graveolens*) specific sequences of the mannitol dehydrogenase (*Mtd*) gene. The performance of the
294 dd-PCR was optimized testing three different primer and probe concentration and the annealing
295 temperature. The optimal primer and probe concentrations were at 0.9 µM of both forward and
296 reverse primers and 0.250 µM of probe in the final concentration of PCR reaction. The optimal
297 annealing temperature was at 60 °C. The different amplitude in fluorescens observed in the dd-PCR
298 assay showed a good separation between positive droplets (containing celery DNA) and negative
299 droplets (not containing celery DNA), indicating the good discrimination of the method. The dd-
300 PCR assay performances were also conducted. The limit of detection in serially diluted celery
301 extract was 0.20±0.12 Cp/µL, corresponding to ca 5 pg/µL of celery DNA while the limit of
302 quantification was 0.83±0.20 Cp/µL corresponding to 20 pg/µL. Previous investigation conducted
303 on celery extract by real-time PCR method showed a limit of detection of 10 pg/µL (Fuchs, Cichna-
304 Markl, & Hochegger, 2012). The repeatability of the dd-PCR was tested on 3 different celery DNA
305 concentrations which were performed each in ten replicates (intra-assay repeatability) and in two
306 consecutive days (inter-assay repeatability). The method showed a good repeatability with intra-
307 assay and inter-assay coefficient of variation ranging from 13.82% to 20.80%.
308 The evaluation of specificity is essential to determine the applicability of a qualitative and
309 quantitative method. Therefore, cross-reactivity with non-target DNA from laurel, sage, thyme,
310 oregano, black pepper, parsley, onion, cucumber, carrot, pea, potato, mixed spices and peeled
311 tomato was tested. The Cel-*Mtd* primers and probe only amplified target sequences from celery,
312 indicating a good specificity of the dd-PCR assay. To test the effect of the food matrix on the
313 performance of the method, the dd-PCR was conducted on celery-spiked food samples (ground
314 meat, pureed soup and tomato sauce). From an initial celery concentration of 1% (w/w) samples
315 were diluted up to 0.003%. The dd-PCR quantified celery DNA in all spiked samples with a good

316 linearity ($R^2$ >0. 990) indicating no matrix effect on the performance of the method. This result is
317 comparable with the LOD observed in celery spiked sausage by real-time PCR which detected
318 celery at concentration as low as 0.005% (Fuchs, Cichna-Markl, & Hochegger, 2012).

319 After the evaluation of the method performances, the dd-PCR assay was tested on 21
320 commercial samples of various types with celery stated in the ingredient list. In the case of
321 precautionary labelling the dd-PCR assay did not revealed the presence of celery, suggesting that
322 the content was below the detection limit. In one sample, Bolognese meat sauce, where the label
323 declaration indicated the presence of 3% celery, the assay was not able to identify the presence of
324 the allergen. This result could be a consequence of the manufacturing process. While other foods
325 included in the study were either raw or pasteurized, the Bolognese meat sauce was submitted after
326 cooking to a sterilization treatment into an autoclave at 121 °C (Prandi et al., 2019). In highly
327 processed foods (e.g., heated at temperature above 100 °C) the application of PCR techniques for
328 DNA amplification may be negatively affected by DNA degradation (Bansal, Singh, Mangal,
329 Mangal, & Kumar, 2015; Bauer, Weller, Hammes, & Hertel, 2003). Therefore, while analyzing
330 different food matrices including in their composition oils, fats and animal tissues, the potential
331 presence of PCR inhibitors should be accounted in the DNA extraction procedure (Di Pinto et al.,
332 2007).

333

334 **Conclusions**

335 The dd-PCR assay described in the present study represent a novel, accurate and reliable method to
336 determine and quantify the presence of celery in various types of foods (i.e., composite, of animal
337 and plant origin). The advantages of this method are the specificity with no cross-reactivity with
338 other allergens and the little impact of the food matrix. In addition, this DNA-based method is
339 particularly suitable for low protein content allergens such as celery. A limitation of the method is
340 that the obtained results, expressed in copy numbers per microliter, cannot be directly converted in

the content of the allergen in the food (e.g., mg/kg or % w/w). Therefore, more investigation is needed to introduce a constant (conversion factor) to transform the copy numbers into the actual content of allergen in the food. With this aim, specific study should be conducted to compare dd-PCR quantitative results with other analytical techniques such as real-time PCR or liquid chromatography–mass spectrometry (LC–MS). Even with this limitation the dd-PCR could be applied as a routine screening method for quantification of celery when assessing compliance with labeling requirements or to ensure food quality and safety for the presence of accidental contamination.

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**Table 1.** Limit of detection (LOD) and limit of quantification (LOQ) of celery ddPCR assay (n = 20)

| | | | | Limit of quantification[b] (LOQ) | | | | Limit of detection[c] (LOD) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Celery DNA dilution** | **Celery DNA concentration (ng/µL)** | **Expected celery DNA (Cp/µL)** | **Average celery DNA by ddPCR (Cp/µL) ± SD** | **CV[a] (%)** | **Average number of positive droplets** | **Average number of negative droplets** | **Average number of total droplets** | **Celery positive** | **Celery negative** | **Detection rate (%)** |
| I | 5.0 | 200 | 178.2±30.6 | 17.17 | 2,196 | 13,647 | 15,843 | 20 | 0 | 100 |
| $S_1$ | 2.5 | 100 | 87.1±11.7 | 13.42 | 1,114 | 14,696 | 15,810 | 20 | 0 | 100 |
| $S_2$ | 1.2 | 50 | 48.4±9.05 | 18.72 | 649 | 15,516 | 16,165 | 20 | 0 | 100 |
| $S_3$ | 0.6 | 25 | 24.0±3.2 | 13.23 | 345 | 16,765 | 17,110 | 20 | 0 | 100 |
| $S_4$ | 0.3 | 12.5 | 12.7±1.7 | 13.60 | 178 | 16,421 | 16,599 | 20 | 0 | 100 |
| $S_5$ | 0.15 | 6.25 | 6.2±0.9 | 14.36 | 90 | 16,993 | 17,083 | 20 | 0 | 100 |
| $S_6$ | 0.08 | 3.13 | 3.3±0.5 | 16.25 | 46 | 16,482 | 16,528 | 20 | 0 | 100 |
| $S_7$ | 0.04 | 1.56 | 1.6±0.4 | 23.49 | 24 | 17,371 | 17,395 | 20 | 0 | 100 |
| $S_8$ | 0.02 | 0.78 | 0.8±0.2 | 23.51 | 12 | 16,684 | 16,696 | 20 | 0 | 100 |
| $S_9$ | 0.01 | 0.39 | 0.5±0.2 | 40.93 | 7 | 16,855 | 16,862 | 20 | 0 | 100 |
| $S_{10}$ | 0.005 | 0.20 | 0.2±0.10 | 61.42 | 3 | 17,086 | 17,089 | 20 | 0 | 100 |
| $S_{11}$ | 0.002 | 0.10 | 0.10±0.09 | 85.14 | 2 | 17,289 | 17,291 | 15 | 5 | 75 |
| $S_{12}$ | 0.001 | 0.05 | 0.06±0.06 | 105.76 | 1 | 16,761 | 16,762 | 11 | 9 | 55 |
| - | NTC | NTC | ND | NC | 0 | 17,995 | 17,995 | 0 | 20 | 0 |

I is the initial DNA dilution corresponding to 1:20 while $S_1$-$S_{12}$ are successive serial two-fold dilution of I. NTC = no template control. ND = not detected. NC = not calculated. [a]CV = Coefficient of variation. [b]Limit of quantification (LOQ) = lowest DNA concentration showing CV% < 25%. [c]Limit of Detection (LOD) = last DNA dilution detected in 95% of replicates.

**Table 2**. Repeatability of dd-PCR celery assay for celery DNA.

| DNA concentration (ng/µL) | Day | N of replicates | ddPCR assay Mean values (Cps /µ±SD) | CV (%) |
|---|---|---|---|---|
| 0.0691 | 1 | 10 | 3.6±0.5 | 13.8 |
| | 2 | 10 | 3.05±0.5 | 14.9 |
| 0.0346 | 1 | 10 | 1.9±0.3 | 16.8 |
| | 2 | 10 | 1.3±0.3 | 20.2 |
| 0.0173 | 1 | 10 | 0.9±0.2 | 20.8 |
| | 2 | 10 | 0.7±0.2 | 20.6 |

The dd-PCR assay was performed on 3 celery DNA concentrations, each concentration was tested in two different days (inter-assay repeatability) and on 10 replicates (intra-assay repeatability).

**Table 3**. Identification of celery DNA by dd-PCR in commercial food samples.

| Lane[1] | Type of Sample | QD* | Mean celery DNA Cp/µL±SD | CV (%) |
|---|---|---|---|---|
| A | Bolognese meat sauce | (3.0%) | ND | - |
| B | Delicate Minestrone | *% not declared* | 39.8±4.1 | 10.3% |
| C | Chopped vegetables | (3.0%) | 36.0±1.9 | 5.3% |
| D | Vegetable Bouillon cube | *% not declared* | 9.1±0.6 | 7.0% |
| E | Meatball | (1.0%) | 22.8±0.1 | 0.6% |
| F | Hamburger | (0.10%) | 1.5±0.0 | 0.9% |
| G | Tomato sauce A | (1.0%) | 44.3±0.6 | 1.3% |
| H | Minestrone | (3.9%) | 413.0±26.9 | 6.5% |
| I | Pureed vegetable soup A | (1.0%) | 120.1±14.4 | 12.0% |
| L | Beef bouillon cube | *% not declared* | 519±25.5 | 4.9% |
| M | Vegetable soup | *% not declared* | 136.7±0.1 | 0.10% |
| N | Tomato sauce B | *% not declared* | 29.0±0.8 | 2.7% |
| O | Meat sauce | *% not declared* | 243.5±3.5 | 1.5% |
| P | Zucchini and potato soup | *% not declared* | 75.8±5.4 | 7.1% |
| Q | Hake in green sauce | *% not declared* | 352.0±9.9 | 2.8% |
| R | Fish stock | *trace* | ND | - |
| S | Meat loaf | (0.3%) | 31.4±1.8 | 5.9% |
| T | Tomato sauce C | (0.10%) | 20.3±1.3 | 6.2% |
| U | Pureed vegetable soup B | (0.10%) | 22.7±0.6 | 2.5% |
| V | Chicken soup | (0.03%) | 2.6±0.4 | 16.3% |
| Z | Pureed vegetable soup C | *trace* | ND | - |

[1]see correspondence with figure 4; *Quantitative declaration; Within brackets the % celery content when declared on the label. ND = not detected.

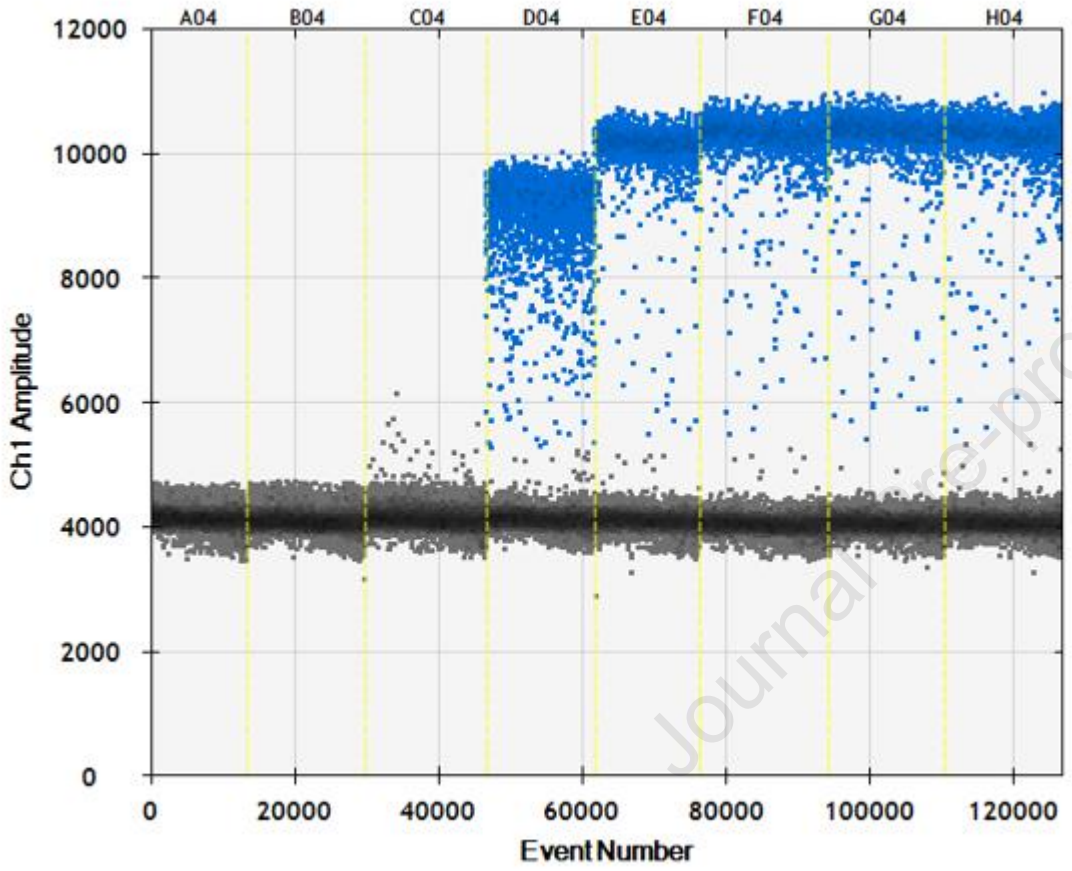




**Figure 1.** Optimization of dd-PCR annealing temperature. The horizontal axis indicates the thermal gradient tested: 65 °C (lane A04), 64.3°C (lane B04), 63°C (lane C04), 61.3°C (lane D04), 58.8°C (lane E04), 56.9°C (lane F04), 55.7°C (lane G04) and 55°C (H04). The vertical axis indicates the amplitude of samples. The graphs were plotted between fluorescence intensity versus droplet numbers. Blue dots represent positive droplets for celery DNA, while black dots represent negative droplets. DNA dilution 1:20, primer F 0.9 µM, primer R 0.9 µM, probe 0.250 µM.

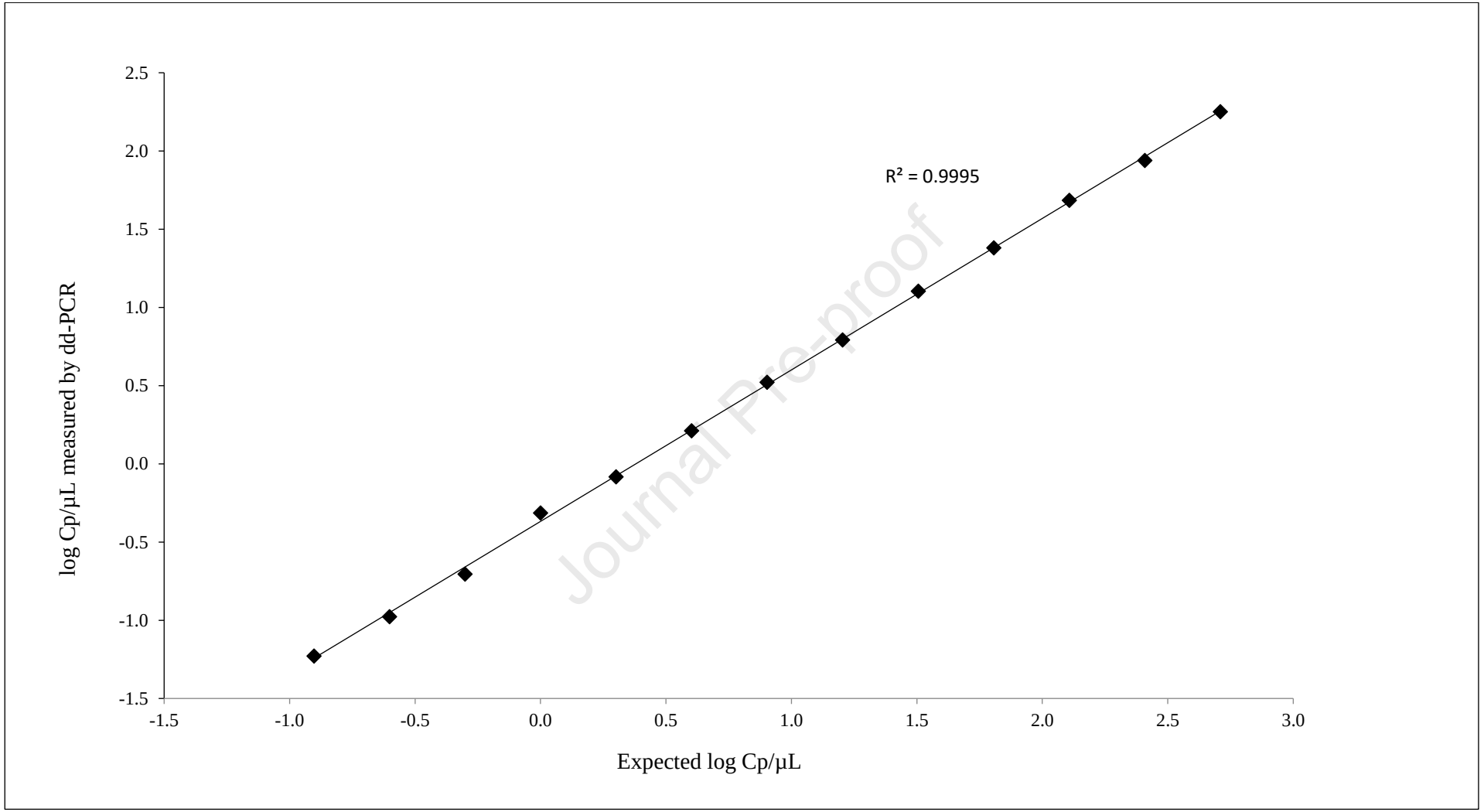


**Figure 2.** Linearity of the dd-PCR assay for celery DNA. The vertical axis represents the log values of measured Cp/µL by dd-PCR. The horizontal axis shows the log values of expected Cp/µL. Each datapoint is the average of 20 replicates.

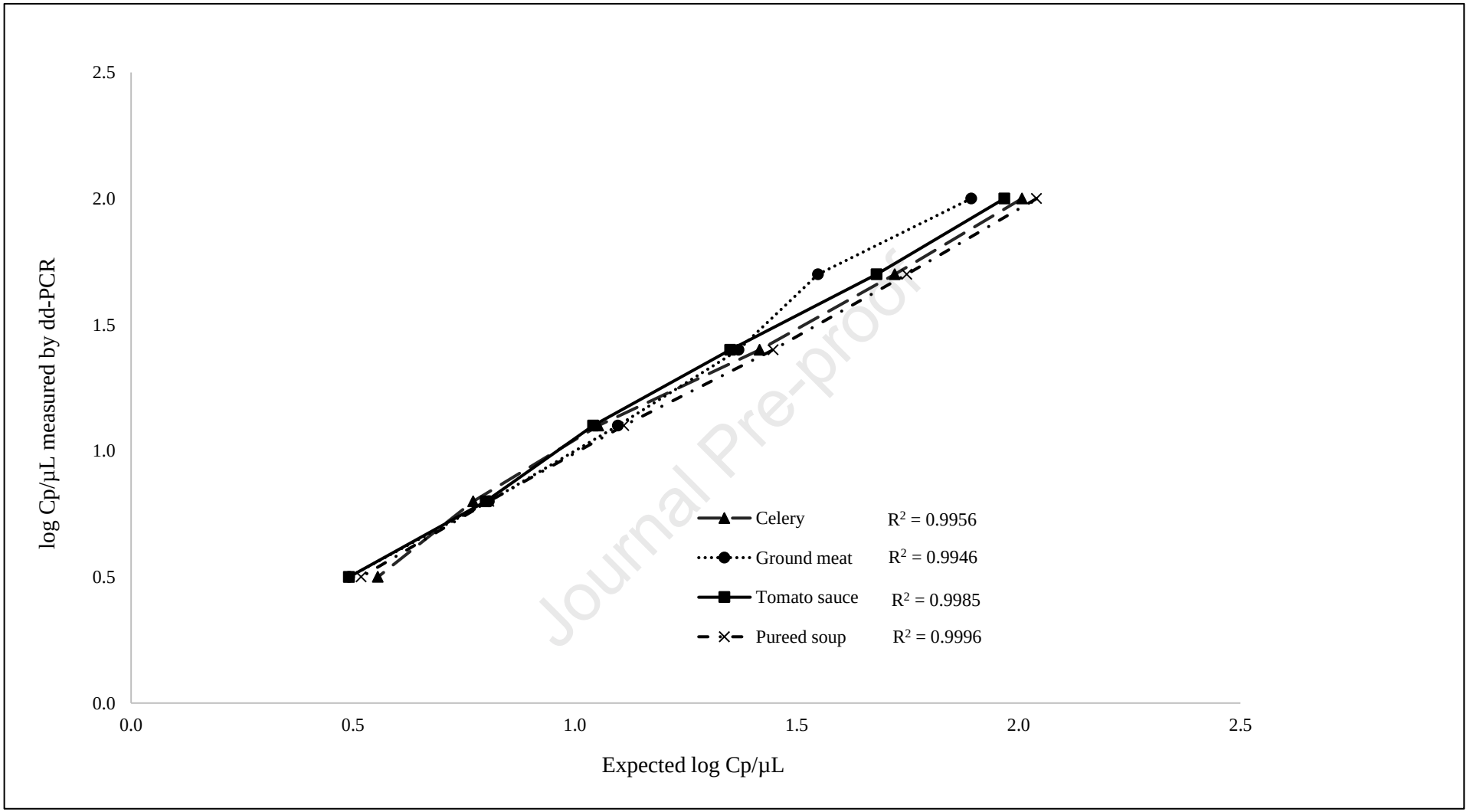


**Figure 3.** Matrix effect**.** Linearity of the dd-PCR assay for celery DNA. The vertical axis represents the log values of measured Cp/µL by dd-PCR on celery powder and spiked food samples. The horizontal axis shows the log values of expected Cp/µL. Each datapoint is the average of 2 replicates.

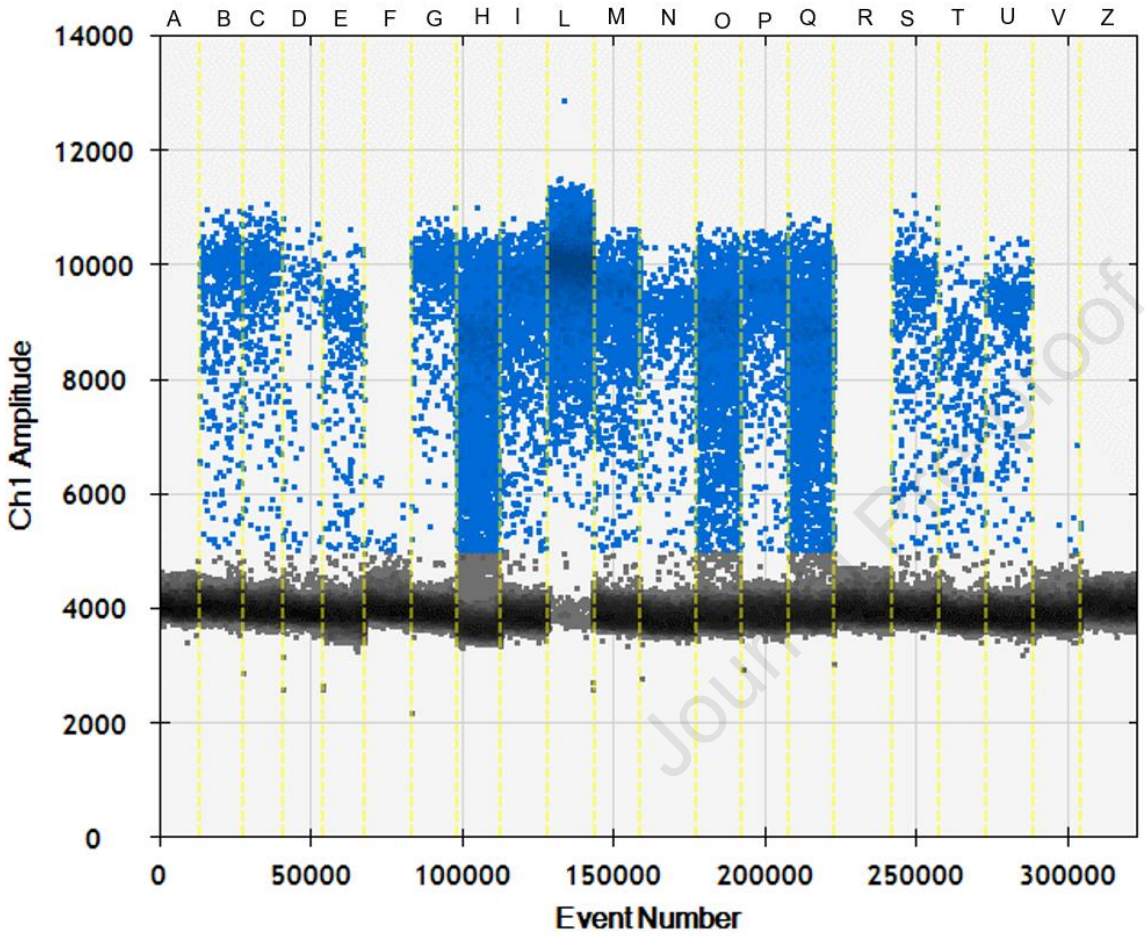


**Figure 4.** dd-PCR conducted on commercial samples**.** The horizontal axis indicates the event number of 21 food samples: bolognese meat sauce (lane A), delicate minestrone (lane B), chopped vegetables (lane C), vegetable bouillon cube (lane D), meatball (lane E), hamburger (lane F), tomato sauce A (lane G), minestrone (lane H), pureed vegetable soup A (lane I), beef bouillon cube (lane L), vegetable soup (lane M), tomato sauce B (lane N), meat sauce (O), zucchini and potato soup (lane P), hake in green sauce (lane Q), fish stock (lane R), meat loaf (lane S), tomato sauce C (lane T), pureed vegetable soup B (lane U), chicken soup (lane V), pureed vegetable soup C (lane Z). The vertical axis indicates the amplitude of samples. The

graphs were plotted between fluorescence intensity versus droplet numbers. Blue dots represent positive droplets for celery DNA, while black dots represent negative droplets.

**Highlights**

- A dd-PCR assay for detection of celery allergen in foods was evaluated;
- Primers and probe were designed to target the mannitol dehydrogenase (*Mtd*) gene;
- The limit of detection was 0.20±0.12 Cp/μL of celery DNA;
- The limit of quantification was 0.83±0.20 Cp/μL of celery DNA;
- The dd-PCR assay showed no cross-reactivity with non-target DNA;

**Declaration of interest to FOOD CONTROL**

Declarations of interest: none

To the attention of the editor-in-chief of the scientific journal Food Control, G. Campbell-Platt
Dear editor
On behalf of my co-authors

I attest to the fact that no financial and personal relationships with other people or organizations inappropriately influenced the work.

Date: May 13th, 2021 Dott. Carlo Spanu