



Detection and persistence of citrus bark cracking viroid and other viroids in citrus peel oils for agricultural applications

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Abstract

Plant-based agricultural products, like citrus peel oils, are increasingly used as sustainable alternatives to synthetic pesticides. However, in crops such as hop (*Humulus lupulus* L.), where viroid infections can seriously reduce yields, there is concern that products made from infected citrus might transmit viroids, especially citrus bark cracking viroid (CBCVd). This study evaluates the risk of viroid transmission by examining CBCVd, hop stunt viroid (HSVd), and citrus exocortis viroid (CEVd) through orange oil using RNA extraction and RT-qPCR analysis. Two extraction methods were tested, with the chaotropic protocol outperforming the detergent-based approach for isolating RNA from oil matrices. Spiking experiments confirmed consistent detection of CBCVd and the plant RNA marker NAD in mixtures containing 90% RNA and 10% oil, even after seven days, indicating RNA stability in oil-rich environments. In contrast, pure oil samples showed no viroid RNA or NAD detection, suggesting limited RNA persistence in pure oil. Of 32 citrus peel samples tested, CBCVd was detected in one and HSVd in seven, but no viroid RNA or NAD was detected in the corresponding oils. These findings indicate a minimal risk of viroid transmission through orange oil; however, formulations containing surfactants or water may allow RNA to partition into aqueous phases, potentially increasing the risk. This study highlights the need for routine testing of raw materials and final citrus-based products to ensure phytosanitary safety.

Keywords Pospiviroids · Hop stunt viroid · Citrus exocortis viroid · Reverse transcription quantitative PCR · *Humulus lupulus* · Hops

Introduction

Germany is one of the world's leading hop producers, renowned for its high-quality bitter hops, which are predominantly used in beer production (Pavlovič et al. 2012; Šrédli et al. 2020). Germany's high-quality hops result from ideal growing conditions, climate, soil, and a long tradition of hop farming, supporting its global reputation (Ruggeri et al. 2024). However, hop cultivation faces challenges from abiotic stressors like drought, frost, and excessive rainfall, leading to stunted growth and root rot. Biotic stressors, like spider mites (*Tetranychus urticae*) and aphids (*Phorodon humuli*) (Lorenzana et al. 2013), can lead to leaf damage and facilitate fungal growth in case of aphids (Calderwood et al. 2015; EPPO, 1994). Pathogens like powdery mildew (*Podosphaera macularis*) and downy mildew (*Pseudo-peronospora humuli*) are also widespread in hops, causing reduced growth, yield, and cone quality (Gent et al., 2009; Gargani et al. 2017; Weldon et al. 2021). All these stressors can adversely affect the chemical composition of hop cones,

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resulting in altered flavor profiles, reduced bitterness, and diminished aromatic qualities reducing their marketability. Among these biotic stressors are viroids—small, circular, single-stranded RNA pathogens that replicate autonomously within host cells without encoding proteins or a protective coat (Diener 2003; Mishra et al. 2018; Adkar-Purushothama and Perreault 2020). Currently, there are four viroids known to infect hops, *Cocadviroid rimocitri* (citrus bark cracking viroid, CBCVd, synonym citrus viroid IV; CVdIV) (Jakše et al. 2015), *Cocadviroid latenshumuli* (hop latent viroid, HLVD) (Patzak et al. 2021), *Hostuviroid impediuhumuli* (hop stunt viroid, HSVd) (Marquez-Molins et al. 2021; Walker et al. 2021) and apple fruit crinkle viroid (AFCVd) (Sano et al. 2004).

To counter stressors and meet rising hop demand, farmers are adopting alternative crop protection methods. The European Green Deal's pesticide bans (European Commission 2020) have accelerated the shift toward plant-based products for agricultural applications. In Europe, options include seaweed extracts like Algifol® (Neomed Pharma GmbH), neem-based NeemAzal-T/S® (Trifolio-M GmbH) for aphid control, and orange peel oil-based Prev-Am® (Oro Agri International B.V. 2020), which offers insecticidal, fungicidal, and acaricidal effects.

In particular, the orange oils are gaining attention due to their availability as a by-product of the juice industry, along with their straightforward extraction and processing, offering a sustainable, environment friendly solution to pest and disease management (Andrade et al. 2023). They are rich in bioactive compounds, particularly the monoterpene family including limonene, citral, linalool, and geraniol, which provide strong insecticidal and antifungal effects (Moufida and Marzouk 2003). For example they are known to disrupt the nervous systems and cell membranes of pests like aphids (Atanasova and Leather 2018), making them efficient alternatives to synthetic pesticides. Several studies have demonstrated the efficiency of essential oils from *Citrus aurantium* and *Citrus reticulata* in controlling aphid populations in vitro. For example, *Aphis illinoisensis* (grapevine aphid) exhibited 100% mortality with *C. aurantium* oil, while *C. reticulata* oil resulted in 92.8% mortality after 48 h (Alotaibi et al. 2022). This effect is primarily attributed to the high concentration of limonene, a compound known for its aphicidal properties (Sreepian et al. 2022; Alotaibi et al. 2022). Given the abundance of limonene in many citrus species, these plants show great potential as natural alternatives to synthetic insecticides, particularly for alternative pest management strategies. Additionally, their plant-based origin, cost-effectiveness, and rapid biodegradability ensure minimal residues, making them an ideal choice for organic farming systems. Prev-Am®, an example for a orange oil-based product, with insecticidal and antifungal effects, has

been shown to effectively control aphids and whiteflies as well as pathogens like powdery and downy mildew (Biofa 2025; Brunelli et al., 2018). It is also recommended for use on hops by the manufacturer for the control of the same diseases, demonstrating the widespread applicability of orange oil-based solutions in sustainable agriculture (Oro Agri International B.V., 2020).

While citrus peel oils offer significant benefits as sustainable pest control agents, they also raise concerns about potential contamination with pathogens, particularly viroids, that are able to infect hop. Hereof, CBCVd is the most relevant viroid due to its severe pathogenicity. It was first detected in hops in Slovenia 2015 and later in Germany in 2019 (Jakše et al. 2015; Julius Kühn-Institut, 2019). CBCVd causes severe stunting and bark cracking in hop plants, often leading to plant death within three to five years (Jakše et al. 2015; Julius Kühn-Institut 2019; Štajner et al. 2019). While CBCVd is the most relevant viroid in hop cultivation, HLVD is the most abundant in global hop production (Puchta et al. 1988). HLVD, while not causing visible symptoms in hops, reduces bitter acid and alters terpene content, thereby diminishing hop quality and altering the favorable aroma (Patzak et al. 2021; Štajner et al. 2019). A third viroid, HSVd, originally identified in hops in Japan, causes a significant reduction in bitter acid content, leading to lower-quality hop cones and substantial economic losses in some cultivars (Hataya et al. 2017; Marquez-Molins et al. 2021). Besides hops, HSVd can also infect citrus plants, causing cachexia, a disease that results in bark scaling, stunted growth, and reduced fruit quality, posing a significant threat to both hop and citrus cultivation worldwide (Levy and Hadidi 1993). Lastly, despite primarily affecting citrus, where it causes bark cracking, stunted growth, and leaf yellowing, *Pospiviroid exocortiscitri* (citrus exocortis viroid; CEVd) has been experimentally shown to infect hops under greenhouse conditions (Hagemann et al. 2023a). Moreover, its ability to contribute to the formation of chimeric viroids, such as CBCVd (Puchta et al. 1991), highlights a potential indirect role in hop infection, particularly given the severe detrimental effects of CBCVd on hops (Jakše et al. 2015).

Hagemann et al. (2023a, b) demonstrated that viroids such as CBCVd, HSVd, and CEVd can be successfully extracted from citrus peels and used to infect hops. These findings suggest that oils derived from citrus peels could also contain viroids and potentially introduce them into clean hop fields when used for agricultural applications. To identify this potential risk is the main objective of this study. However, extracting RNA from oil is challenging due to its lipophilic nature and high levels of secondary metabolites, which interfere with extraction and downstream applications. Even though extraction of nucleic acids from oil have been successfully performed in the past (Busconi et al. 2003;

Raieta et al. 2015), here we analyze the terpene-rich orange oil which is challenging. To further clarify the terminology, the International Organization for Standardization (ISO) defines “essential oils” as products obtained through steam distillation, dry distillation, or by mechanical processing of the citrus flavedo (Salvatore et al. 2022). However, some literature ambiguously applies the term to distilled oils alone. In this study, we used a laboratory-scale mechanical cold-pressing technique on orange flavedo to produce what we refer to as “orange oil”, aligning with industrial practices for cold pressing citrus peels. Up to now the potential for viroid transmission through this process was completely unknown.

To address this, we formulated the hypothesis that viroids are present and can persist in orange oils used for plant protection measures, posing a potential risk of spreading to crops.

To test this, we aimed to develop and validate an RNA extraction protocol tailored for orange oils to enable reliable viroid detection using RT-qPCR, assess viroid stability and detectability in spiking experiments to simulate potential contamination in plant-strengthening products, and determine whether viroids remain detectable in orange oil formulations under different conditions. Initial insights from this study suggest that the risk of viroid transmission via orange oil products may be low. As demand for environment friendly plant-based products grows, ensuring their safety will be essential for protecting sensitive crops and maintaining sustainable practices.

Materials and methods

General methods

Plant sample preparation for reference and test material

CBCVd infected hop: Hop plants of the cultivar ‘Herkules’ were grown at the greenhouse at University of Hohenheim, Germany and inoculated using a mixture of CBCVd-positive total RNA extracted using Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, USA) and CBCVd dimer structures at 1 µg/plant (Online Resource 3) introduced directly to plants via scalpel incisions in September 2021. In September 2022, the infection status of the plants was confirmed by RT-qPCR as described in the following paragraphs. Leaf samples of CBCVd-infected plants were sampled in liquid nitrogen and stored until needed at -80°C. Total RNA was extracted from leaves of CBCVd-infected hop plants using the Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, USA) according to the manufacturer’s instructions. After extraction, the RNA was diluted to a concentration of 50 ng/µL using 10 mM Tris

buffer (pH 8.0) and used as reference for spiking experiments and positive control throughout the entire study. Different varieties of orange fruits were collected from various grocery stores and transported to the laboratory for processing (Online Resource 2). Oranges were chosen as source material, since they showed to be a more reliable source of oils (Online Resource 1). Each fruit was sampled separately, by thinly peeling the flavedo using a sterile scalpel blade (Online Resource 1). The individually sampled peels were stored at -80°C for further processing.

Detergent RNA extraction

Samples were prepared by pooling the flavedo from two oranges to create a single sample. The pooled samples were kept frozen and ground with liquid nitrogen to ensure effective cell disruption. A total of 100 mg of the ground peel was used for RNA extraction using the Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, USA) according to the manufacturer’s instructions. The concentration of the extracted RNA was assessed using a NanoDrop™ 1000 spectrophotometer (ThermoFisher, Waltham, USA).

Chaotropic RNA extraction

A 100 µL aliquot of either cold pressed oil, as described below or RNA-oil mixture sample was used for RNA extraction with the NucleoZOL® RNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s guidelines. After extraction, the RNA was purified using NucleoSpin® RNA columns (Macherey-Nagel, Düren, Germany), optimized for high-yield RNA isolation. The RNA quality and concentration were assessed using a NanoDrop™ 1000 spectrophotometer (ThermoFisher, Waltham, USA).

Reverse transcription real-time quantitative PCR

Two reverse transcription-duplex real-time quantitative PCRs (RT-qPCRs) were performed to detect viroids in various RNA or oil samples using the Bioline SensiFAST™ Probe No-ROX One-Step Kit (Bioline, London, UK) on a Rotor-Gene 6000 qPCR cycler (Qiagen, Hilden, Germany), following a modified version of the protocol described by Hagemann et al. (2023b). Each RNA or oil sample was analysed in two volumes, 0.2 µL and 1 µL, respectively. The first duplex RT-qPCR reaction used a FAM-labeled probe for CBCVd, along with the primers CVdIV_qPCR_F and CVdIV_qPCR_R, while the plant mitochondrial NADH dehydrogenase (nad5) gene, associated with nicotinamide adenine dinucleotide (NAD), served as an internal control

for plant RNA, detected using a HEX-labeled probe with the primers nad5_F and nad5_R. In the second duplex RT-qPCR reaction, CEVd was detected using a FAM-labeled probe with the primers CBCV_1 and CEVd_MH_F2, while HSVd was simultaneously detected using a HEX-labeled probe along with the primers HSVd_JK_F2 and HSVd_JK_R2, as detailed in Table 1. The cycling parameters were: reverse transcription at 48 °C for 30 min, followed by an initial step at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 15 s and a combined annealing extension at 60 °C for 30 s. A Ct cut-off of 35 or 35 rounded off was applied to exclude most erratic amplification curves in citrus samples that did not follow a typical sigmoidal pattern, ensuring reliable viroid detection while minimizing false positives.

Experimental procedures

Validation of RNA extraction from orange oil

The extraction and detection of RNA from orange oil were validated using a spiking approach. Compared to the white mesocarp (albedo), the flavedo was identified as a more reliable RNA source (Online Resource 1). To simulate industrial processing, flavedo samples were cut into small pieces and pressed using a cold press (Vevor, Shanghai, China). The resulting pulp was centrifuged at 25,000 rpm for 20 min

Table 1 Primers and probes used in this study

Name	Sequence	Type	Reference
CVdIV_qPCR_F	GGAACAGGAGCTC GTCTC	Forward	Seigner et al. (2020)
CVdIV_qPCR_R	GTCCCGCAGAGAA AT TCC	Reverse	Seigner et al. (2020)
nad5_sense	GATGCTTCTTGGG GCTTCTTGT	Forward	Menzel et al. (2002)
nad5_antisense	CTCCAGTCACCAA CATTGGCATAA	Reverse	Menzel et al. (2002)
CEVd_MH_F2	CTGCAGGCAGGAA AAGAAAAA	Forward	Hagemann et al. (2023b)
CBCV_1	CAAGAGTTGTATC CACCGGG	Reverse	This study
HSVdF2_JK	GACTTACCTGAGA AAGGAGCCC	Forward	Hagemann et al. (2023b)
HSVdR2_JK	ACAAAAAGCAGG TTGGAAGACG	Reverse	Hagemann et al. (2023b)
nad5_probe	AGGATCCGCATAG CCCTCGATTATGT G-BHQ-1	HEX probe	Menzel et al. (2002)
CVdIV-qPCR_P	CATCGCTGGCTCC ACATCCG-BHQ1	FAM probe	Seigner et al. (2020)
HSVd HEX	HEX-AGAGAGGG CCGCGGTGCTC T-BHQ-1	HEX probe	Luigi and Faggioli., (2013)

to separate the oil from the aqueous phase and tissue debris (Fig. 1a).

To assess the efficiency of RNA extraction from oil, orange oil samples were spiked with total RNA extracted from CBCVd-infected hop leaves at a concentration of 50 ng/μL. Two RNA-to-oil ratios were tested: 90% RNA+10% oil (R90/O10) and 10% RNA+90% oil (R10/O90). Additional controls included 100% RNA (room temperature) and −80 °C stored RNA as positive references, as well as 100% oil (O100) as a negative control with no added RNA.

RNA was extracted from all spiked samples using the chaotropic RNA extraction method after incubation in the dark at ambient room temperature for one hour, one day, and one week. The recovered RNA was subsequently analyzed via RT-qPCR to confirm the suitability of this method for detecting viroid RNA in oil-based matrices.

Evaluation of viroid stability in orange oil

To investigate plant RNA and viroid stability and persistence in orange oil, the same sample setup from the validation of the RNA extraction experiment was used. These samples were tested using two methods as shown in Fig. 1b. First, all samples were directly analysed by RT-qPCR for the detection of NAD and CBCVd without specific RNA extraction. Due to the immiscibility of phases, the lipophilic (upper) and aqueous (lower) phases were tested separately, with 0.2 μl and 1.0 μl of each phase used as templates. In parallel, the same set of samples underwent chaotropic RNA extraction, followed by RT-qPCR analysis for the same targets using the same template volumes. The results from both methods were then compared to assess differences in viroid detection. To extend the stability study, three 100% CBCVd RNA samples extracted using the chaotropic method were stored at ambient room temperature for three months and tested for CBCVd and NAD using RT-qPCR.

Assessing viroid transfer from citrus peel to freshly pressed orange oil

To investigate whether viroids detected in orange peel could also be found in the orange oil extracted from the same infected fruits, 32 orange samples were analysed. Initially, RNA was extracted with the detergent method and screened for the presence of NAD and three viroids, CBCVd, CEVd, and HSVd, using RT-qPCR. Fruits testing positive were subjected to oil extraction. The resulting orange oil was used directly as a template in RT-qPCR.

The chaotropic method, though reliable for RNA extraction from oil, could not be applied because it requires a minimum input volume of 100 μL, which was not available for

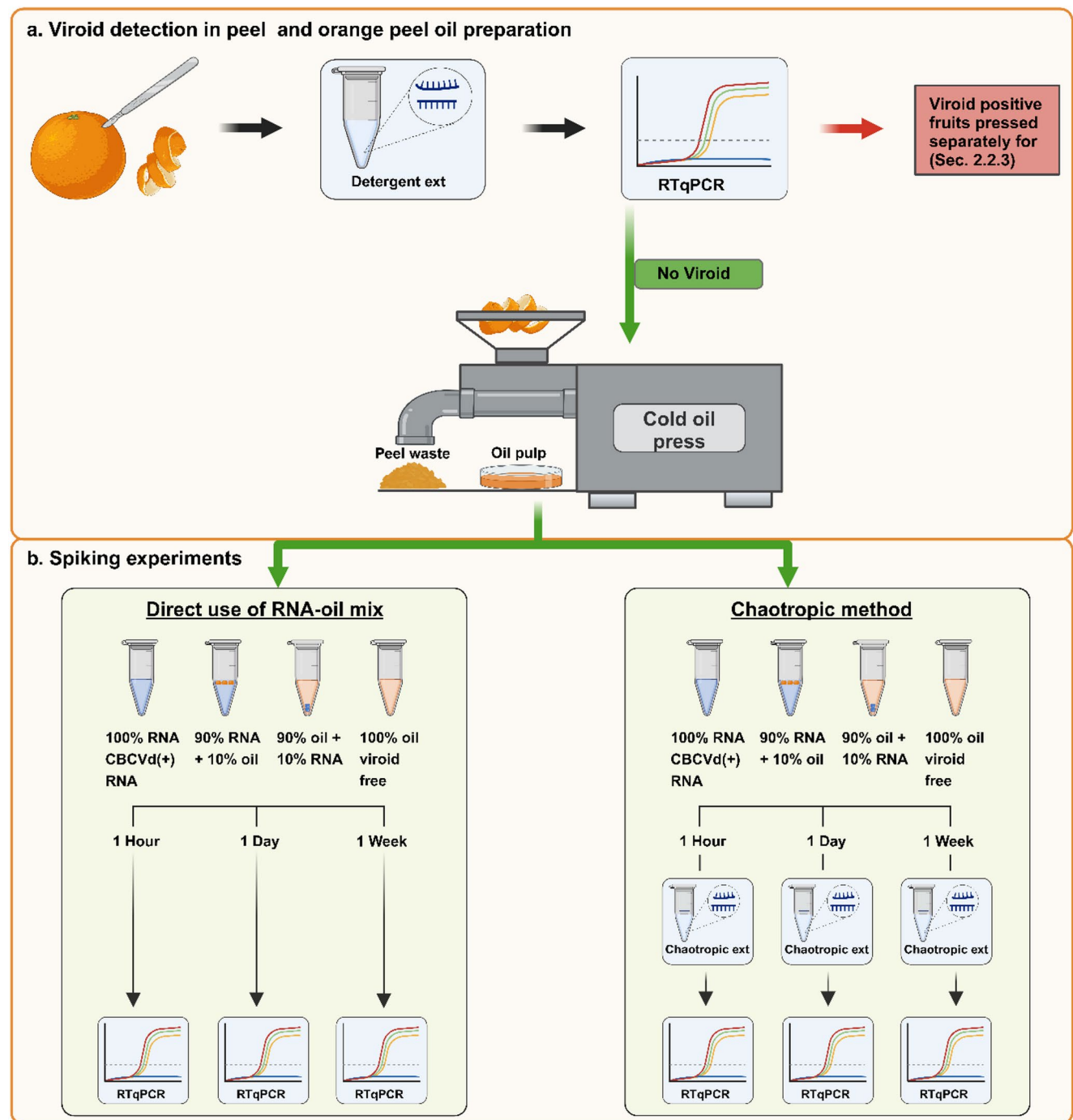


Fig. 1 Schematics of the experimental procedure (Created in BioRender. Hagemann, M. (2023a) <https://BioRender.com/je6xls7>). **(a)** Method establishment, where RNA was extracted from orange peel using a detergent method, followed by viroid detection via RT-qPCR and oil pressing if viroids were absent. **(b)** Spiking experiments, where

orange oil spiked with RNA extract in two ratios (10:90 and 90:10), or pure RNA extract were analysed after one hour, one day, or one week. Samples were either directly subjected to RT-qPCR or processed for RNA extraction using the chaotropic method prior to RT-qPCR

most pressed oils. Additionally, to preserve the oil's composition and avoid alterations such as the evaporation of volatile terpenes and other components (Han et al. 2024), the study prioritized the direct analysis of fresh orange oil.

Results

Validation of viroid extraction and detection in orange oil

Oil extracted from oranges that tested negative in the pre-experiment was subjected to two analytical approaches: direct RT-qPCR analysis using orange oil as a template and chaotropic RNA extraction prior to analysis. The oil buffer mixture was immiscible hence to get an even clearer separation we centrifuged them into two distinct phases. NAD and CBCVd remained detectable for up to seven days in samples containing 100% RNA. In the 90% RNA sample, the upper oil-rich phase showed no detection of NAD or CBCVd within the first 24 h, but both became detectable after one week in both experimental replicates. The lower aqueous phase consistently showed detection of NAD and CBCVd across all time points. In contrast, samples containing 10% RNA were free of NAD and CBCVd in the upper oil-rich phase at all-time points, though sporadic detection was observed in the lower aqueous phase up to one day. The 100% oil control remained free of NAD and CBCVd throughout.

The results from direct use of the samples, while effective, are less practical for routine testing of commercial products due to their varied compositions, which often form multiple phases that complicate analysis. To address this, the chaotropic RNA extraction was applied to the same samples, to validate the findings and explore potential differences in detection outcomes. The results (Table 3) highlight, that all 100% RNA samples tested positive for NAD and CBCVd across all conditions. Whereas samples with 90% RNA showed positive results in both replicates at the one day and one week marks. In contrast, it was observed that NAD and CBCVd were detected only in replicate I at one day, with no detection in replicate II. For samples containing 10% RNA, both targets were detected up to one hour, but by the one day mark, NAD and CBCVd were only observed in replicate I, while replicate II showed no detection. Lastly, all 100% oil control samples were free of NAD and CBCVd, consistent with the direct use of oil results.

For the extended stability study, CBCVd and NAD remained detectable in all three 100% RNA control samples even after three months of storage at ambient temperature (data not shown). The average Ct-values were for CBCVd

were 34.6 for 0.2 μ L and 31.46 for 1 μ L template volume, and 33.1 for 0.2 μ L and 30.5 for 1 μ L for NAD.

Assessing viroid transfer from citrus peel to freshly pressed orange oil

To strengthen the study, we extended the testing with additional fruit and oil samples to assess whether the raw material and oil production processes impact viroid integrity. Among the 32 orange samples analysed, all peel RNA samples tested positive for NAD, one for CBCVd, none for CEVd, and seven for HSVd. However, none of the corresponding oil samples tested positive for NAD or any viroids.

Discussion

In response to increasing restrictions on using synthetic pesticides under the European Green Deal and Farm to Fork strategies (European Commission 2020), farmers and the agricultural sector are adopting plant-based products for agricultural applications such as orange peel oils, which have shown effectiveness as natural alternatives for managing pests and diseases in crops like hops (Brunelli et al., 2018; Oro Agri International B.V., 2020). However, a recent study by Hagemann et al. (2023b) found that peel of tested citrus fruits contained the two most severe hop stunting viroids CBCVd in 5% and HSVd in 30% of tested fruits, respectively. In response to these challenges the current study analysed viroid contamination in terpene-rich matrices, such as orange oil, using different RNA extraction methods and subsequent RT-qPCR. This provides a framework for evaluating viroid-related risks in plant-derived agricultural products and ensuring the integrity of food authentication systems.

Citrus oils, including orange oil, are chemically complex matrices, characterized by high concentrations of monoterpenes like limonene and secondary metabolites such as flavonoids and polyphenols (Njoroge et al. 2005). These compounds are known to interfere with RNA extraction (Sasi et al. 2023). Polyphenolic compounds in orange oil can also bind irreversibly with RNA to form strong, covalent-like interactions, creating inseparable complexes that further hinder RNA recovery and purification in citrus oils (Mattheus et al. 2003). In contrast, nucleic acids have been successfully extracted and analyzed from olive oil, for applications such as food authentication and traceability. Unlike terpene-rich orange oil, olive oil comprises over 98% triglycerides, resulting in a relatively simpler chemical composition (Boskou 2006; Jimenez-Lopez et al. 2020). However, even with this simplicity, the lipid-rich nature of olive oil presents challenges for nucleic acid extraction

Table 2 Direct use of the samples for RT-qPCR for NAD and CBCVd detection using spiked RNA-oil samples, tested in separate upper and lower phases, across three time points. The analysis was performed at two replicates (I, II) at two volumes (a, b), with CBCVd and NAD detection indicated in red and non-detection in green

Incubation		1Hour								1Day								1Week							
Target		NAD				CBCVd				NAD				CBCVd				NAD				CBCVd			
Repetition		Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb
Treatment	Phase																								
RNA (-80°C)	Homogen																								
RNA (21°C)	Homogen																								
R90/O10	Upper																								
R90/O10	Lower																								
R10/O90	Upper																								
R10/O90	Lower																								
O100	Homogen																								

Table 3 Chaotropic RNA extraction followed by RT-qPCR for NAD and CBCVd detection using RNA extracted from spiked oil samples across three time points. The analysis was performed at two replicates (I, II) at two volumes (a, b), with CBCVd and NAD detection indicated in red and non-detection in green

Incubation		1Hour								1Day								1Week							
Target		NAD				CBCVd				NAD				CBCVd				NAD				CBCVd			
Repetition		Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb	Ia	Ib	IIa	IIb
Treatment																									
RNA (-80°C)																									
RNA (21°C)																									
R90/O10																									
R10/O90																									
O100																									

(Birtić and Kranner 2006). Studies employing CTAB-based extraction methods have successfully overcome these challenges, recovering nucleic acids from oils and providing valuable insights (Busconi et al. 2003; Raieta et al. 2015). Building on these findings, we aimed to isolate RNA from orange oil despite its enrichment with challenging secondary metabolites.

Initially, we utilized detergent RNA extraction, which is phenol-free but still highly efficient for isolating RNA from non-fatty matrices like hop leaves and compost residues (Hagemann et al. 2023b, 2024). While effective for orange peel RNA extraction and baseline viroid detection, this method appeared less suitable for oil samples. In contrast, the chaotropic RNA extraction, which is based on the combination of guanidinium thiocyanate and phenol demonstrated superior performance for orange oil formulations, leveraging principles described by Chirgwin et al. (1979). Guanidinium thiocyanate denatures RNases rapidly, protecting RNA from degradation, while phenol enhances the disruption of hydrophobic compounds like terpenes and

solubilizes secondary metabolites. Its one-phase system simplifies the process, reducing RNA loss and ensuring high-quality RNA recovery from lipid-rich matrices, making it a promising choice for commercial testing. Using this optimized protocol, we successfully extracted RNA from RNA-oil mixtures. RNA extraction from spiked orange oil consistently detected both viroid RNA and NAD as an internal control, confirming our first hypothesis that a specialized RNA extraction method enables reliable viroid detection in orange oil via RT-qPCR. The results also set the foundation for our second hypothesis that viroids remain detectable in orange oil or orange oil formulations when subjected to spiking experiments. Using RNA-oil mixtures directly as a template for RT-qPCR and comparing them to RNA-extracted samples (Fig. 1b), we confirmed that viroid RNA remains detectable in both cases, thereby validating the hypothesis (Tables 2 and 3). The direct use of RNA-oil formulations showed that the detection of CBCVd and NAD was consistent in both RNA 100% (-80 °C and room temperature) controls (Table 2), also after incubation at room temperature

for over three months (Data not shown). However, samples with varying RNA-oil concentrations, 90% RNA and 10% oil samples showed intriguing results; CBCVd was undetectable at one hour and one day but became detectable after one week in the lipophilic phase containing the orange oil. This may be attributed to the gradual diffusion of viroid RNA at the water-oil interface over time, eventually reaching detectable levels in the oil phase. Alternatively, the evaporation of volatile compounds in orange oil, as noted by Han et al. (2024), may alter oil's overall chemical composition, facilitating RNA dispersal or stabilization within the oil-rich phase. In contrast, 10% RNA samples exhibited sporadic CBCVd detection, likely due to the lower RNA concentration, which may result in levels near or below the RT-qPCR detection threshold. These findings highlight the complexity of viroid detection in lipophilic heterogeneous matrices but also demonstrate the capability of our methods to uncover viroid dynamics in such chemically complex systems.

In 100% oil samples, NAD and viroids were undetectable irrespectively if used directly or after chaotropic RNA extraction. We hypothesize that RNA, due to its hydrophilic nature, partitions into the aqueous and debris layers during centrifugation, which removes non-lipid components from the hydrophobic oil matrix. This observation highlights the physical and chemical limitations of RNA retention in oil, a key aspect explored in subsequent hypotheses regarding RNA persistence through oil extraction processes. The absence of RNA in pure oil suggests that nucleic acids are unlikely to integrate into the oil matrix during production. However, the presence of CBCVd and NAD following the chaotropic RNA extraction of 100% RNA highlights the potential for viroid and plant RNA to maintain its integrity over extended periods (Table 3). NAD, despite its linear structure, remained detectable for up to seven days in samples with low oil concentrations, suggesting RNA stability under these conditions. Viroids, with their robust circular RNA conformation, are expected to exhibit even greater stability (Riesner et al. 1983). Supporting this, Hagemann et al. (2024) observed HLVD detectability in compost residues for over 15 days under controlled conditions (50 °C, pH 5–7), further underscoring viroid resilience in challenging environments.

A study by Hagemann et al. (2023b) highlighted the frequent detection of viroids in fruits imported into grocery stores from various global locations but did not investigate their persistence in oils. The potential reintroduction of viroids into agricultural systems through byproducts like orange peels remains a significant concern. These byproducts are often repurposed for oil manufacturing, which is commonly used in agricultural applications (Teigiserova et al. 2021). This observation led to the formulation of

Table 4 Fresh oil pressing experiment where RNA was first extracted from 32 citrus peels using the detergent-based RNA extraction method and analysed by RT-qPCR for NAD, CBCVd, HSVd, and CEVd. For each sample, the corresponding orange oil was directly tested in the RT-qPCR setup for viroid detection

	Peel RNA	Oil
Samples	32	32
NAD	32	0
CBCVd	1	0
CEVd	0	0
HSVd	7	0

our third hypothesis, which is that viroid RNA can persist through industrial orange oil extraction processes, posing a potential risk of viroid transmission to sensitive crops like hops. However, our findings suggest otherwise, because despite all peel samples being NAD-positive and eight being viroid-positive, neither viroid RNA nor NAD was detected in the corresponding oils (Table 4). These results reinforce the conclusion that RNA, including viroid RNA, is unlikely to integrate into the oil matrix under the processing conditions tested, thereby mitigating the potential risk of RNA transmission through orange oil products. Global orange production exceeds 71 million tons annually (FAO), with a substantial 50–60% of the fruit, primarily the peels, discarded as waste (Manthey and Grohmann 2001). These peels are often repurposed for the extraction of orange oils, primarily through methods like cold pressing or steam distillation. Cold pressing uses mechanical force to extract oil from the peel at low temperatures, preserving the natural components of the oil (Aydeniz-Guneser 2020) whereas methods like steam distillation, are more heat-intensive processes, which involve passing steam through the peels to vaporize the volatile oil components, which are then condensed and collected (Sikdar et al. 2016). Hydrodistillation, although less commonly used for orange oil, involves boiling a water-peel mixture to release the oil (Tran et al. 2023). These different extraction methods, particularly the temperatures and pressures applied, impose distinct physical and chemical conditions that could influence the stability and detectability of viroids, should they be present. For instance, an optimized steam distillation method demonstrated optimal oil recovery from *C. sinensis* and *C. reticulata* peels when heated for 30 min at 50 °C (Mercy et al. 2015). Similarly, a study by Tran et al. (2023) demonstrated that essential oil from *C. sinensis* was effectively extracted using a 3:1 water-to-raw material ratio by heating the mixture at 130 °C for 60 min. Such high-temperature methods are particularly relevant in studies on viroid degradation. Hagemann et al. (2021) showed that treating fermentation residues at 70 °C reduced HLVD concentrations by four decimal powers within a single day, while Matousek et al. (1995) observed a 70–90% decrease in HLVD concentrations in hop meristems

after two weeks of heat treatment at 35 °C. These studies underscore that the high-temperature conditions inherent in commonly used oil production methods may effectively minimize the risk of viroid persistence in oil-based products. Cold pressing, the most common method for essential oil extraction, preserves natural oil components by operating at low temperatures (Aydeniz-Guneser 2020; Baser & Buchbauer 2020). Industrial processes typically involve crushing fruit peels with water and using centrifugal force to separate oil at 40–50 °C (JBT Operating Manual). In our study, a laboratory-scale cold-pressing method was used to simulate commercial conditions. The process was conducted at ambient temperature, which means that the conditions are even more in favour of viroid stability compared to commercial conditions making our approach rather conservative regarding risk assessment. The resulting orange oil was free of detectable RNA and viroid contamination, addressing a critical gap in viroid detection within complex fatty matrices.

Citrus-based products, including orange oil, are not mentioned in the PRA of CBCVd by Radišek and Benko-Beloglavec (2016). Together with Wilstermann et al. (2020) and EPPO (2021), they assessed CBCVd's risk of entry, establishment, spread, and impact in Slovenia, Germany, and the EPPO region. When combined with the findings from this study, these PRAs help evaluate the overall risk of viroid transmission through plant-based products, especially orange oil used in hop cultivation. Based on the results, this risk appears low but cannot be ruled out.

In this study, neither viroid RNA nor NAD was detected in freshly pressed orange oil under tested conditions (Table 4). A separate preliminary analysis of three commercial citrus oil-based products also showed no traces of viroids (Jagani et al. 2023). Furthermore, seven commercial products tested in triplicate using the same method showed no RT-qPCR signals (data under disclosure). Similar findings were reported by Pasha and Ziebell (2025), who investigated the potential presence and infectivity of CBCVd and HLVd in commercially available citrus essential oils. No viroid RNA was detected in the oils, and infection assays using tomato and eggplant as hosts showed no evidence of transmission, even when the oils were spiked with infected plant material. Together, these results combined suggest that the presence of viroids in pure oil matrices is very low. Still, CBCVd is highly stable and damaging to hops, causing significant economic losses (Jakše et al. 2015; Radišek and Benko-Beloglavec 2016; Wilstermann et al. 2020; EPPO 2021). Its mechanical transmissibility via tools and machinery (Barbosa et al. 2005; Seigner et al. 2020) underscores the need for strict biosecurity and surveillance. Pure citrus oil, which showed phytotoxicity in our greenhouse trials (data not shown), is not applied directly in agriculture. Instead, commercial formulations (e.g., PREV-AM: 2 L/ha

in 500–1,000 L water/ha; Biofa, Germany) are formulations based on water and surfactants. Mitigating viroid transmission requires that each component of citrus oil-based products, especially those in new or reformulated batches are to be tested separately, and that sourcing raw materials from viroid-free suppliers is prioritized. These precautions not only safeguard product integrity but also help protect agricultural supply chains and food security.

In conclusion, this research successfully validated an RNA extraction protocol suitable for terpene-rich matrices, confirming its effectiveness for viroid detection. Beyond viroid research, this protocol has broad applications, such as detecting various pathogens in essential oil-rich crops, profiling secondary metabolites in medicinal plants like cannabis, and verifying the authenticity and safety of food and agricultural products. Notably, the absence of viroids in fresh-pressed oils from viroid-positive fruit peels under the tested conditions suggests a low, but not negligible, risk of transmission through citrus-based oils. These findings underscore the importance of stringent testing and verified sourcing, offering a solid foundation for risk assessment and supporting the development of safe, sustainable agricultural practices to protect vulnerable crops from emerging pathogens.

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Data availability All data generated or analyzed during this study can be requested from the corresponding author.

Declarations

Ethical approval This work is not dealing with humans or animals.

Informed consent All authors declare that they have no conflict of interest.

Conflict of interest The authors declare that they have no financial interests or personal relationships that could have influenced the work presented in this article. The work was self-funded.

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