Detection of Norovirus Genogroups I and II and Hepatitis A Virus in Raspberries by RT-PCR

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Abstract: As a leading country in raspberry production in the region, Serbia plays an important role in the global raspberry market. Since raspberries are frequently consumed fresh or frozen and often undergo minimal processing before consumption, their microbiological safety is therefore of major public-health and economic importance. Among foodborne viral hazards, noroviruses and hepatitis A virus are particularly relevant because they can persist in fresh and frozen fruit matrices, including raspberries. Although several analytical approaches are available, molecular diagnostics currently represent the dominant strategy for virus detection in food. Real-time RT-PCR is increasingly applied as a standardized ISO-based method, particularly within the framework of ISO 15216:2019, enabling rapid and sensitive detection of potential viral contamination in tested samples.

In this study, 40 raspberry samples collected between January and December 2023 were analyzed. Viral RNA was isolated from samples originating from 10 raspberry producers from the Arilje and Požega regions. The analytical procedure was performed in accordance with the ISO method and simultaneously targeted norovirus GI, norovirus GII, and hepatitis A virus. Overall, 80% of the samples were negative for all tested viruses, 10% were positive for norovirus GI, and 5% each were positive for hepatitis A virus and norovirus GII.

The obtained results indicated that the presence of these viruses was not limited to a specific season, since norovirus GI, norovirus GII, and hepatitis A virus were detected in both fresh and frozen raspberries. These findings suggest that raspberries may serve as a potential vehicle for the transmission of enteric viruses along the food chain. Routine monitoring of norovirus and hepatitis A virus in raspberries is therefore important for food safety assurance and consumer protection, particularly when testing is performed in accordance with harmonized international standards.

Keywords: raspberries; foodborne viruses; norovirus; hepatitis A virus; food safety.

1. Introduction

Raspberries represent one of the most economically important berry crops and are among the principal export products of the Serbian fruit production and processing sector. Owing to the high quality of the fruit, favorable agroecological conditions, and strong demand in both domestic and international markets, raspberry production holds a prominent position in Serbian agriculture and food industry (Nedeljković *et al.* 2024; Kljajić *et al.* 2025). According to the Statistical Office of the Republic of Serbia, raspberry production in Serbia reached approximately 94,051 t in 2024, while the preliminary value for 2025 was 82,577 t. Compared with the latest clearly available FAOSTAT-based estimate of world raspberry production (940,979 t in 2023), these values correspond approximately to 10.00% and 8.78%, respectively. The biological characteristics of raspberry cultivation, its high economic profitability, favorable climatic conditions, and the increasing demand for fresh and frozen berries have all contributed to the continued growth of this sector (Kljajić *et al.* 2025). However, the expansion of raspberry production must be accompanied by continuous attention to food safety. Raspberries are commonly consumed fresh or frozen, often without any virus-inactivating treatment before consumption, which makes the control of microbiological hazards particularly important (Nasheri *et al.* 2019). In such products, food safety concerns are not limited to bacterial contamination, since foodborne viruses may remain undetected in the absence of visible spoilage while retaining infectivity during storage and distribution.

Among the viral hazards associated with soft fruits, human norovirus and hepatitis A virus (HAV) are of particular concern. Human noroviruses are recognized as the leading cause of acute gastroenteritis worldwide, affecting all age groups and producing especially severe consequences in vulnerable populations such as young children, the elderly, and immunocompromised individuals (Carlson *et al.* 2024). Within the human norovirus group, genogroups I and II (GI and GII) are particularly relevant in food virology, since they are responsible for the great majority of human infections and represent the principal analytical targets in foodborne outbreak investigations and surveillance systems (Carlson *et al.* 2024). In outbreak settings, GII strains are generally more frequently reported than GI strains (Verhoef *et al.* 2015). Hepatitis A virus remains another important foodborne pathogen and is generally considered more clinically severe than norovirus because of its hepatic involvement (Olaimat *et al.* 2024). Both viruses are transmitted primarily by the fecal–oral route and may enter the food chain through contaminated irrigation or wash water, contaminated harvesting equipment, infected food handlers, or inadequate hygiene during postharvest handling (Koopmans and Duizer, 2004).

Berry fruits, particularly frozen raspberries and strawberries, have repeatedly been implicated in foodborne outbreaks caused mainly by norovirus and hepatitis A virus, including multiple norovirus outbreaks in Denmark and Finland and large hepatitis A outbreaks in Europe linked to frozen berry products (Le Guyader *et al.* 2004; Falkenhorst *et al.* 2005; Sarvikivi *et al.* 2012; Severi *et al.* 2015; Scavia *et al.* 2017; Enkirch *et al.* 2018). Such epidemiological findings have reinforced the importance of harmonized molecular methods for the detection of foodborne viruses in berry matrices. In Serbian food testing laboratories, molecular methods for the detection of norovirus and hepatitis A virus are routinely applied and are generally harmonized with the international standard ISO 15216:2019, *Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR — Part 2: Method for qualitative detection*. This standard specifically targets norovirus genogroups I and II together with HAV, making these viral agents highly relevant for the virological examination of raspberries. At the same time, currently available RNA virus extraction procedures may be time-consuming, require specialized laboratory infrastructure, or involve steps that can compromise viral recovery during sample preparation (Velebit *et al.* 2025). Therefore, there is a continuing need for rapid, efficient, and reliable analytical workflows suitable for routine virological monitoring.

Accordingly, the aim of this study was to detect norovirus genogroup I (NoV GI), norovirus genogroup II (NoV GII), and hepatitis A virus (HAV) in raspberry samples by real-time RT-PCR performed in accordance with ISO 15216. Raspberry samples were collected from ten different producers in the municipalities of Arilje and Požega (Serbia) between January and December 2023. The application of a standardized analytical workflow was intended to generate data relevant to risk assessment, routine food safety surveillance, and the improvement of preventive measures in raspberry production and handling.

2. Materials and methods

2.1. Sample collection, handling and preparation

Raspberry samples were collected from ten producers in the municipalities of Arilje and Požega (Serbia), a region known for its favorable climatic conditions and long tradition of raspberry cultivation. Both fresh and frozen raspberries, intended for the domestic market and export, were analyzed for the presence of norovirus (NoV) and HAV during 2023 (January–December). After harvesting, raspberries were pre-cooled at −5 °C for up to 12 hours, followed by individual quick freezing (IQF) in a tunnel system and storage at −20 °C for up to 30 days before sorting and packaging. Composite samples consisted of approximately 300 g of fruit (one commercial package), from which 25 g subsamples (8–10 fruits) were used for analysis. Samples were properly packaged, labelled, and transported to the Laboratory for Molecular Methods in Food Analysis. In the laboratory, raspberries were weighed without prior washing, homogenization, or cutting to preserve fruit integrity. Only samples of adequate quality were analyzed, while those showing visible mould or condensation were excluded. Each analytical sample (25 ± 0.3 g) was transferred into a sterile 400 mL filter bag for further processing.

2.2. Virus Concentration from Soft Fruit

Virus concentration was performed according to ISO 15216-2:2019, using the polyethylene glycol (PEG) precipitation method for soft fruit matrices.

Prior to extraction, 10 ± 0.5 µL of process control virus (MS2 bacteriophage) was added directly to the raspberry surface. Then, 40 ± 1 mL of TGBE buffer and ≥30 units of pectinase (from *Aspergillus niger*) were added. Samples were incubated at room temperature with continuous mixing (~60 oscillations/min) for 20 minutes.

During incubation, the pH of the eluate was monitored and, if necessary, adjusted to pH 9.5 ± 0.5 using

Table 1. Primer sequences of HAV

HAV68 (FW):	TCA CCG CCG TTT GCC TAG
HAV240 (REV):	GGA GAG CCC TGG AAG AAA G
HAV150(-) (PROBE):	CCT GAA CCT GCA GGA ATT AA

Table 2. Primer sequences of GI

QNIF4 (FW):	CGC TGG ATG CGN TTC CAT
NV1LCR (REV):	CCT TAG ACG CCA TCA TCA TTT AC
NVGG1p (PROBE):	TGG ACA GGA GAY CGC RAT CT

Table 3. Primer sequences of GII

QNIF2 (FW):	ATG TTC AGR TGG ATG AGR TTC TCW GA
COG2R (REV):	TCG ACG CCA TCT TCA TTC ACA
QNIFs (PROBE):	AGC ACG TGG GAG GGC GAT CG

NaOH. After incubation, the eluate was transferred into a 50 mL centrifuge tube and centrifuged at 10,000 × g for 30 minutes at 5 °C.

The supernatant was transferred to a new tube, and the pH was adjusted to 7.0 ± 0.5 using HCl. Virus precipitation was performed by adding 0.25 volumes of 5× PEG/NaCl solution, followed by incubation with mixing and centrifugation at 10,000 × g for 30 minutes at 5 °C.

The resulting pellet was resuspended in 500 µL phosphate-buffered saline (PBS). An additional purification step was performed by adding a chloroform/butanol mixture, followed by vortexing, incubation, and centrifugation. The aqueous phase containing viral RNA was then transferred to a new tube for further processing.

Extracts were either analysed immediately or stored at 5 °C (≤24 hours), −15 °C (≤6 months), or −70 °C for long-term storage.

2.3. Viral RNA Extraction

Viral RNA extraction was performed using the *foodproof® Sample Preparation Kit IV* (HYGIENA Diagnostic, Germany) according to the manufacturer's instructions.

Viral particles were lysed by incubation with lysis buffer, releasing viral RNA. The addition of carrier RNA (tRNA) enhanced RNA binding to the silica membrane, thereby improving extraction efficiency and assay sensitivity.

After addition of absolute ethanol, RNA selectively bound to silica membrane fibers in filter tubes. Bound RNA was purified through two washing steps followed by centrifugation to remove potential PCR inhibitors. Finally, RNA was eluted using an elution buffer and collected for downstream analysis.

2.4. Detection of Viral RNA by Real-Time RT-PCR

Detection of norovirus genogroups I (GI) and II (GII) and hepatitis A virus (HAV) was performed using one-step real-time reverse transcription PCR (RT-PCR).

Amplification was carried out using the *foodproof® Norovirus (GI, GII) plus Hepatitis A Virus Detection Kit®* (HYGIENA Diagnostic, Germany). The assay contains specific primers and hydrolysis TaqMan probes targeting viral genomic regions, as well as a process control for result validation.

Primer and probe sequences used in the assay comply with the ISO 15216:2019 standard (Table 1-3).

2.5. Process Control

To prevent false-negative results caused by potential PCR inhibition from complex food matrices, a process control (MS2 bacteriophage) was added to each sample before virus concentration.

During reverse transcription, viral RNA was converted into complementary DNA (cDNA), which served as the template for amplification.

Table 3. Primer sequences of GII

Cy5	process control (MS2 bacteriophage)
FAM	hepatitis A virus (HAV)
HEX	norovirus genogroup I (NoV GI)
ROX	norovirus genogroup II (NoV GII)

Table 5. Overview of RT-PCR reaction results

Sample	Hepatitis A FAM	Norovirus GI HEX	Norovirus GII ROX	Process control Cy5
1	Positive	Negative	Negative	Positive
2	Positive	Negative	Negative	Positive
3	Negative	Negative	Positive	Positive
4	Negative	Negative	Positive	Positive
5	Negative	Positive	Negative	Positive
6	Negative	Positive	Negative	Positive
7	Negative	Positive	Negative	Positive
8	Negative	Positive	Negative	Positive
9	Negative	Negative	Negative	Positive
10	Negative	Negative	Negative	Positive
11	Negative	Negative	Negative	Positive
12	Negative	Negative	Negative	Positive
13	Negative	Negative	Negative	Positive
14	Negative	Negative	Negative	Positive
15	Negative	Negative	Negative	Positive
16	Negative	Negative	Negative	Positive
17	Negative	Negative	Negative	Positive
18	Negative	Negative	Negative	Positive
19	Negative	Negative	Negative	Positive
20	Negative	Negative	Negative	Positive
21	Negative	Negative	Negative	Positive
22	Negative	Negative	Negative	Positive
23	Negative	Negative	Negative	Positive
24	Negative	Negative	Negative	Positive
25	Negative	Negative	Negative	Positive
26	Negative	Negative	Negative	Positive
27	Negative	Negative	Negative	Positive
28	Negative	Negative	Negative	Positive
29	Negative	Negative	Negative	Positive
30	Negative	Negative	Negative	Positive
31	Negative	Negative	Negative	Positive
32	Negative	Negative	Negative	Positive
33	Negative	Negative	Negative	Positive
34	Negative	Negative	Negative	Positive
35	Negative	Negative	Negative	Positive
36	Negative	Negative	Negative	Positive
37	Negative	Negative	Negative	Positive
38	Negative	Negative	Negative	Positive
39	Negative	Negative	Negative	Positive
40	Negative	Negative	Negative	Positive

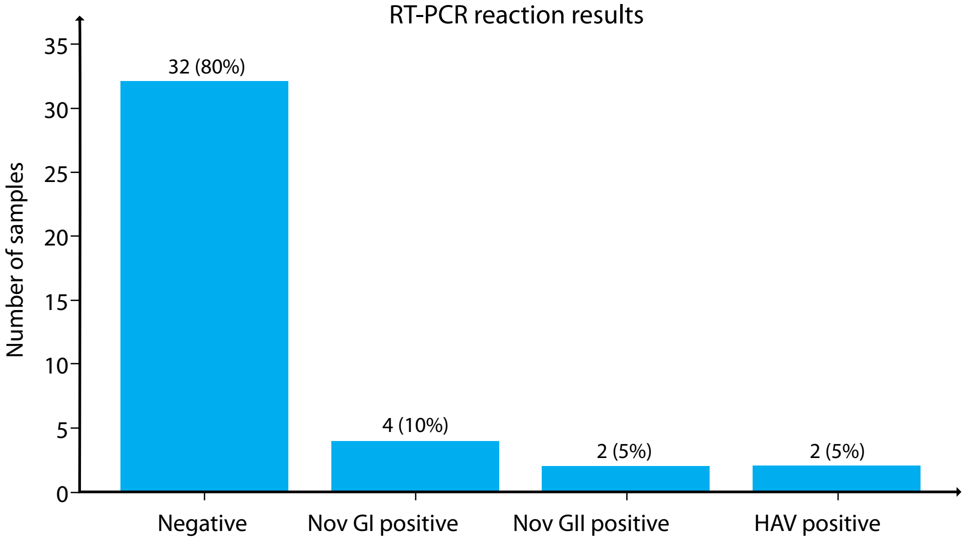

Figure 1. Presentation of RT-PCR reaction results

The multiplex assay enabled simultaneous detection of all three viral targets and the process control in a single reaction. The analytical sensitivity of the assay was 3–10 copies per reaction.

Amplification reactions were performed on an Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific, USA).

3. Results

It was determined that, out of a total of 40 samples, 32 samples were negative for the presence of NoV GI, NoV GII, and HAV, 4 samples were positive for NoV GI (negative for NoV GII and HAV), 2 samples were positive for NoV GII (negative for NoV GI and HAV), and 2 samples were positive for HAV (negative for NoV GI and NoV GII).

The table below presents an overview of the RT-PCR reaction results for 40 samples, obtained using a multiplex kit for the detection of Norovirus group I (GI) and II (GII), hepatitis A virus (genotypes I, II, and III), as well as the process/internal control.

The graphical presentation provides an overview of the positive and negative RT-PCR reaction results.

Eighty percent of the samples were negative for all three targets, 5% were positive for HAV, 5% were positive for NoV GII, and 10% were positive for NoV GI (Figure 1).

4. Discusssion

Previous studies have clearly shown that NoV and HAV can persist in fruit matrices, including both fresh and frozen berries, which represents an important challenge for food safety surveillance and public health (Rodríguez-Lázaro *et al.* 2012). The persistence of enteric viruses in berries is influenced by several factors, including temperature, pH, matrix composition, and water activity, while freezing itself is generally regarded as a preservation process rather than an effective viral inactivation step (Bozkurt *et al.*,2021). Experimental and review data indicate that HAV can remain infectious on frozen berries for prolonged periods, and that freezing and frozen storage often result in only limited reductions in viral infectivity, thereby allowing contaminated products to remain a potential source of infection during distribution and consumption (Rzezutka & Cook, 2004).

The present results support the relevance of molecular surveillance of berry fruits for norovirus genogroups I and II and HAV. The detection of these viral targets in both fresh and frozen raspberry samples indicates that contamination may occur under different production, handling, storage, and distribution conditions, and confirms that both product categories require virological attention. These findings are consistent with earlier reports demonstrating the occurrence of enteric viruses in berry supply chains and highlighting multiple contamination routes, including contaminated irrigation water, infected food handlers, and postharvest handling environments (Maunula *et al.* 2013; Mäde *et al.* 2013; Bozkurt *et al.* 2021).

The use of real-time RT-PCR in this study proved appropriate for routine virological screening, particularly because molecular methods remain the basis of internationally approved detection protocols for norovirus and HAV in berries and other soft fruit. At the same time, the broader literature emphasizes that molecular detection, although highly sensitive and indispensable for surveillance, does not by itself resolve all analytical challenges associated with heterogeneous contamination, low viral loads, and the inability of RT-PCR alone to directly determine infectivity. For this reason, routine monitoring should be interpreted as one component of a broader preventive strategy that includes strict hygienic control during primary production, harvesting, processing, and distribution (Maunula *et al.* 2013).

An additional point of importance is that positive findings in raspberries are not only relevant from a public health perspective, but may also have substantial economic implications. Berry-associated outbreaks of NoV and HAV have repeatedly triggered regulatory concern, product recalls, and intensified control measures in international trade (Severi *et al.* 2015; Scavia *et al.* 2017; Tavoschi *et al.* 2015). Because Serbia is an important raspberry-producing country, the implementation of reliable and standardized virological monitoring may contribute both to consumer protection and to maintaining confidence in export chains.

Looking ahead, further improvement of analytical workflows for virus detection in berries remains necessary. Recent literature continues to emphasize the need for faster, more robust, and more informative detection strategies, including improved recovery methods, better characterization of low-level contamination, and the integration of advanced molecular tools for strain-level investigation where appropriate (Velebit *et al.* 2025). Such developments could strengthen outbreak tracing, improve source attribution, and support more effective risk management across the berry production chain.

5. Conclusion

In conclusion, the results of this study confirm the presence of NoV GI, NoV GII, and HAV in raspberry samples, demonstrating that raspberries may represent a relevant vehicle for foodborne viral contamination. The detection of these viruses in samples collected from different producers and during different periods of the year underlines the importance of continuous virological surveillance of both fresh and frozen berries. The application of a standardized real-time RT-PCR approach in accordance with ISO-based principles provides a suitable basis for routine food safety monitoring. Nevertheless, sustained improvement of preventive measures, sampling strategies, and analytical workflows remains essential for reducing viral risk, protecting public health, and supporting the safety and marketability of raspberry products.

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