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New developments in safety testing of soft fruits

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DOI: 10.1533/9781782420279.4.292

Abstract: The chapter begins by establishing the definition of soft fruit, then discusses microbial and chemical hazards that might be found in soft fruit. Methods developed for virus detection in soft fruit are reviewed and, finally, the factors that mainly affect virus detection are detailed as these pathogens are currently linked to outbreaks caused by soft fruit consumption.

Key words: berries, foodborne viruses, parasites, sample preparation, molecular detection.

21.1 Introduction

Fruits and derived products are known carriers of foodborne pathogens. In recent years, an increase in their consumption has been observed as fresh fruits have increased in popularity as part of the human diet. As a consequence, an increase in the number of outbreaks related to fresh fruit consumption has been observed. Soft fruits have been frequently linked to outbreaks, especially to emerging pathogens that have been recently identified in this type of fruit. Identification of the causative agents in an outbreak has been facilitated by recent developments in foodborne pathogen detection. New methodologies for identification of pathogens have helped not only to identify emerging pathogens, but also to support improvement of surveillance and agricultural practice systems.

Traditionally, pathogens have been separated from food matrices by homogenization of samples in a diluent. Analysis of the resulting suspension requires selective enrichment and subsequent biochemical identification. Traditional testing of soft fruits has therefore been slow and, in some cases, non-causative agents have been identified as a result of a lack of proper testing methods.

Some standard methods for microbiological testing are still based on conventional microbiological methods. However, the inclusion of molecular methods to microbiology has extensively supported the identification of some non-culturable pathogens such as viruses. As many viruses cannot be propagated *in vitro*, their routine identification could not be included in routine testing.

As part of the laboratory testing for fresh produce safety, this chapter will provide information about new pathogen detection methods developed for soft fruits. Some of considerations for the development of new methodologies include fruit surface factors and pathogen surface factors that allow pathogen survival, extrinsic factors such as where the soft fruit is produced and stored and physical characteristics of the target pathogen. Furthermore, sample preparation is a key step to remove inhibitors present in soft food matrices.

21.2 Soft fruit

Soft fruit is a generic term used for small, fleshy fruit. Although most of the fruits included in this term are berries, botanically, the term berry not only comprises blueberries, gooseberries, blackcurrant and grapes but also avocados, tomatoes and kiwis (Stuppy and Kesseler, 2008). For the purposes of this chapter, the term 'soft fruit' will refer to small edible fruit with multiple seeds. Therefore, berries such as blueberry, strawberry, raspberry, gooseberry, elderberry and currants will be included.

Recent statistics about the major crops of soft fruit worldwide include strawberry, blueberry, raspberry, currants and gooseberry in ascending order of metric tonnes produced (FAOSTAT, 2010). The main producers of soft fruit from 2006 to 2010 were the USA for strawberry and blueberry and the Russian Federation for raspberry and currant production (Fig. 21.1). The Russian Federation was also the major producer of gooseberries during this period of time (FAO, http://faostat.fao.org/site/339/default.aspx). The remaining soft fruit producers include countries such as Turkey, Spain, Poland, Serbia, Canada, Ukraine, Spain and Mexico (Fig. 21.1).

Soft fruits are offered to consumers directly in farms or as U-pick, pre-packed fresh in local or international markets or processed in the form of juice, puree, jams or frozen (Strik, 2007). In general, data observed during 5 years show increased production of soft fruit worldwide. This might be attributable to the popularity that berries have gained because of their content of compounds associated with health benefits. In addition to their high nutritional value because of their vitamin and mineral contents, soft fruits are also highly valued for their antioxidant compounds, flavonoid and phenolic contents (Parish *et al.*, 2012).

Production of soft fruit has also been increased by exportations from new countries. Production of soft fruit in different countries has also expanded their availability in all seasons in most regions (Parish *et al.*, 2012). With the myriad of production and safety systems performed worldwide, it is not surprising that hygienic practices during soft fruit production are quite diverse. The safety of

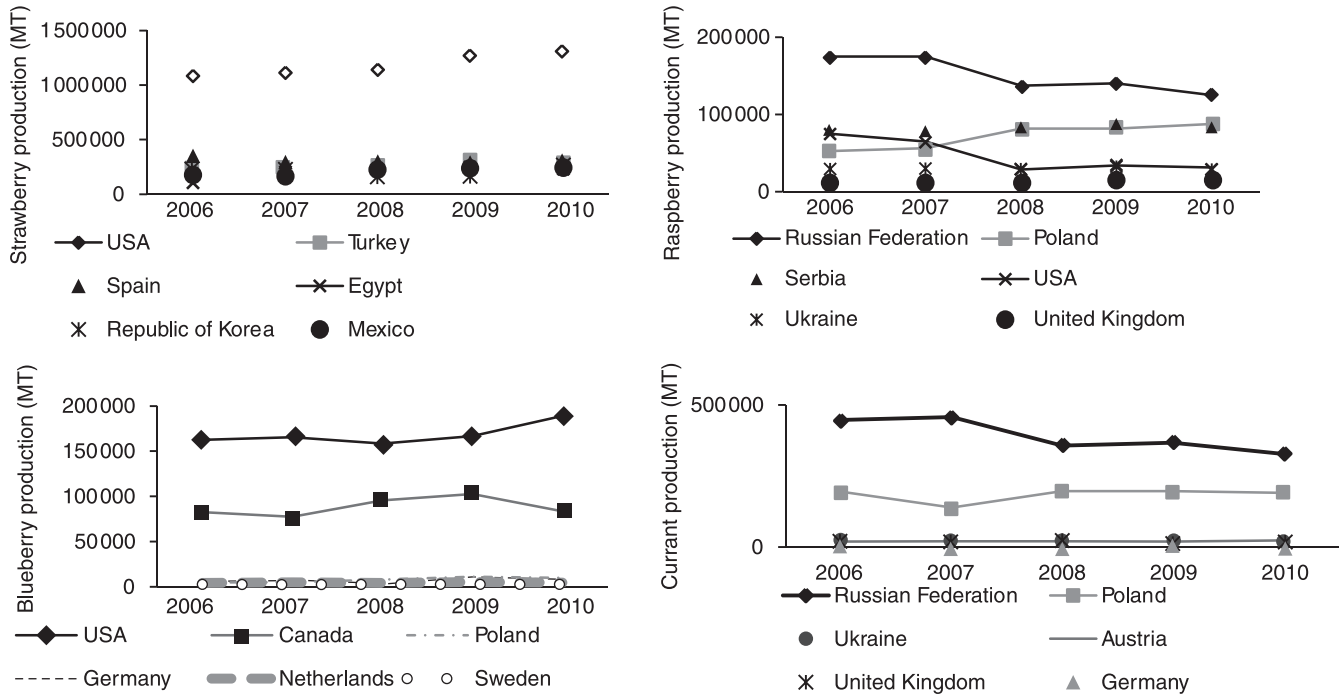

Fig. 21.1 Production of major soft fruit crops by main growers in 2010. Data are presented from 2006 to 2010 but countries included are in order of production in 2010 (derived from United Nations Food and Agriculture Organization, FAO, FAOSTAT, accessed 18 December 2011, at http://faostat.fao.org/site/339/default.aspx).

food, and in this case of soft fruit, involves potential contamination from chemical, physical and microbiological sources (Fig. 21.2). Potential microbiological risks are the main safety issue with fresh fruit. Fresh produce is most often contaminated as a result of poor practice in primary production and/or misuse of natural and environmental resources (Brassard *et al.*, 2012; Newell *et al.*, 2010), for example the irrigation of produce with polluted water (including possible contamination through roots owing to drop irrigation (Urbanucci *et al.*, 2009)), contact with human faeces or faecal soiled materials and poor hygiene practice by food handlers during harvest (León-Félix *et al.*, 2010). Furthermore, contamination may arise by inappropriate practices during processing or at the point of sale/consumption (Boxman *et al.*, 2011; Daniels *et al.*, 2000; Schmid *et al.*, 2007). Finally, there may be cross-contamination from polluted wash water, working instruments or surfaces, which had been previously contaminated by infected food handlers or contaminated food items (D'Souza *et al.*, 2006; Boxman *et al.*, 2009; Dreyfuss, 2009).

Chemical and physical contamination of food may also become a risk to consumers. Physical contamination may not represent a high risk in soft fruit unless the fruits are processed for juices, jam or frozen. Physical contamination of processed soft fruit occurs when any material such as metal fragments, gravel, jewellery and glass particles, which is not normally in processed products, can

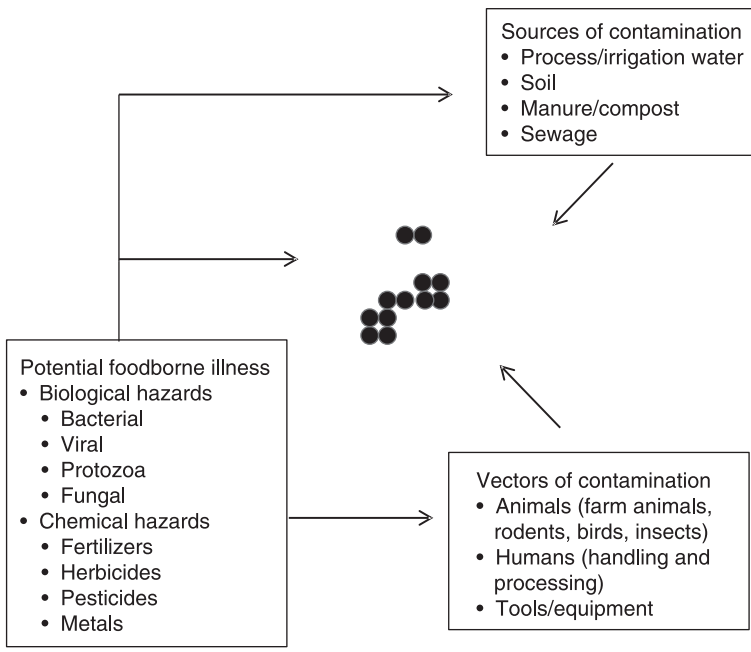

Fig. 21.2 Potential contamination factors for soft fruit during preharvesting, harvesting and processing (adapted from Bower *et al*., 2003).

produce illness or injury to consumers (Daeschel and Pathima, 2007). Chemical contamination might occur during production or processing of soft fruit (Fig. 21.2). Use of pesticides in berry production can be carried to either fresh or processed fruit, representing a high risk to consumers. It is therefore crucial to follow integral agricultural practices in the field to ensure that chemical residues are not passed through the soft fruit processing to reach consumers (Parish *et al.*, 2012). Chemical contaminants can also be introduced during processing when compounds recognized as safe are not used in agreement with regulatory guidelines. Compounds recognized as safe might include antioxidants, preservatives, sulfiting agents and colorants, among others (Daeschel and Pathima, 2007).

21.3 Microbial pathogens of safety concern in soft fruits

In general, fruit and processed fruit products are believed to be safe from pathogenic bacteria because of their low pH (Zhao, 2005). However, contamination of soft fruit can occur during different points at the preharvest, harvest and postharvest (Fig. 21.2) stages. The main factors that contribute to a high microbial load in a soft fruit can be divided into the source of contamination, the vector for contamination and the potential microorganisms that can be found in the different fruits. Outbreaks linked to soft fruit have shown that pathogens are able to survive high acid content products derived from soft fruit, showing that soft fruits are vulnerable to microbial pathogens.

21.3.1 Bacterial and parasitic pathogens in soft fruits

Preliminary analysis of the trend of pathogens present in food has shown a decrease in infections caused by *Campylobacter*, *Listeria*, *E. coli* O157, *Shigella* and *Yersinia* in 2010, compared with 1996–1998 (CDC, 2011). Contrary to infections where *Salmonella* and *Vibrio* were the causative agents, the reduction of illnesses caused by these five pathogens is attributed to improved detection and surveillance systems, regulatory banning of contaminated ground beef with *E. coli* O157 and increased awareness of the risk of consumption of undercooked beef in food service organizations and at home. However, pathogens that are not included in these surveillance systems such as norovirus (NoV), *Clostridium perfringens* and *Toxoplasma* are still the main microorganisms causing human illness. As a result of developments in methods for viral testing in soft fruits and recent strategies to improve the production, quality and safety of soft fruit production, recent data on the presence of bacteria and parasites in soft fruit are scarce. Some reports of outbreaks linked to bacterial and parasitic pathogen presence in soft fruit were reported more than 10 years ago (Table 21.1). From 1973 to 1997, nine outbreaks were linked to berries including strawberry (four), raspberry (four) and blackberry (one). Seven of those outbreaks were associated with *Cyclospora* and one with *Staphylococcus aureus* (Sivapalasingam *et al*., 2004).

Table 21.1 Outbreaks linked to bacterial and parasitic contamination of soft fruits

Pathogen	Type of berry	Year	Location	Notes
Bacteria				
Staphylococcus aureus	Strawberry	1985	New York, USA	14 persons were ill during this outbreak reported by the Foodborne Outbreak Surveillance System
Salmonella	Strawberry	2001	NR	1/143 samples collected for analysis from imported products to USA from five different countries was positive
Parasite				
Cyclospora cayetanensis	Raspberries likely	1995	Florida, USA	Raspberries from Guatemala were involved in 87 cases, but causative agent was not isolated from the berries
C. cayetanensis	Raspberries	1996	20 USA states and two Canadian provinces	Contamination was attributed to contaminated water mixed with insecticides and fungicides that were sprayed onto the fruit
C. cayetanensis	Raspberries	1997	Multiple states in USA and Ontario, Canada	The source of contamination was not identified
C. cayetanensis	Raspberries	1998	Ontario, Canada	The source of contamination was not identified
C. cayetanensis	Blackberries	1999	Ontario, Canada	The source of contamination was not identified
C. cayetanensis	Raspberries	2000*	Philadelphia, USA	The source of contamination was identified after interviewing guests at a wedding reception and DNA identification by PCR

Source: adapted from FDA, *Ho *et al*. (2002).

More recent studies have shown that *Salmonella* and *Cyclospora cayetanensis* are able to survive in juices made from soft fruit (Bower *et al*., 2003). *Listeria monocytogenes* and *Salmonella* have been isolated from frozen blueberries and fresh strawberries respectively (Zhao, 2005). In addition, the attachment of bacteria or parasites to soft fruit surfaces is also another factor that could lead to an increment of the microbial load. Kniel *et al*. (2002) demonstrated that oocysts

of *Toxoplasma gondii* can attach to berry epidermis and persist during 8 weeks. *T. gongii* is a coccidial parasite related to *Cyclospora* and it can be used as a *Cyclospora* surrogate. Raw soft fruit is usually harvested by hand and packed in containers that will eventually reach consumers without being washed. For example, strawberries that will be frozen, are destemmed in the fields, most of the time using thumbnail or metal devices. The extra human handling of the soft fruit therefore represents an opportunity for a pathogen to attach to the food surface and survive. Although no recent data are available about outbreaks linked to contaminated soft fruit with bacterial or parasitic pathogens, the hygienic practices used for their production could eventually lead to high bacterial or parasitic loads.

21.3.2 Viruses in soft fruits

Soft fruits that are eaten raw or lightly heat-treated are frequently causes of outbreaks of viral illnesses. As examples, frozen raspberries have caused NoV outbreaks in Finland, Denmark, Sweden and France (Little and Gillespie, 2008), and, in 2012, Germany experienced their biggest foodborne NoV outbreak ever caused by frozen strawberries implicated in outbreaks that involved 500 institutions with 11 000 cases (Anonymous, 2012). Even though several viruses such as enterovirus, human rotavirus, adenovirus, sapovirus, astrovirus, coronaviruses or aichi virus appear capable of causing foodborne illness or emerging as foodborne pathogens, all recent data show that hepatitis A virus (HAV) and NoV continue to be the most common viral pathogens causing foodborne illness (Yadav *et al*., 2010). Statistical data have shown that HAV is a higher health risk than other pathogens such as *Salmonella* Typhi, *Shigella sonnei* or *Staphylococcus aureus* because of the number of deaths caused by HAV outbreaks (Todd *et al*., 2009). Besides the risk that soft fruits can be contaminated with viruses during growth, the role that food workers play in the spread of illness has been assessed in HAV and NoV outbreaks in which over 50% of the cases were hospitalized. Bidawid *et al.* determined that 9% of HAV and nearly 18% of a NoV surrogate, feline calicivirus (FCV), could be transferred from artificially contaminated finger pads to lettuce by contacts during food handling. When studying the opposite route of transmission, they found that 14% of FCV could be transferred from lettuce to bare hands (Bidawid *et al.*, 2004, 2000a). This ease of reversible viral transfer between workers and produce was later supported in a field study (León-Félix *et al.*, 2010) evaluating the impact of harvesting and packinghouse operations on the contamination of green bell pepper with NoV. When testing the workers' hands for NoV before and after 3 hours of work, they were able to show a change in the rates of positivity of 0% to 14% for pickers, 53% to 33% for classifiers and 45% to 25% for packers. Although these data are for fresh produce, similar viral transmission is expected to occur during soft fruit production because of the similar practices of picking.

The recent year's significant progress in the method development for virus detection in foods reflects the growing acknowledgement of the risk of food acting as a vector for virus transmission. With the introduction of TaqMan real-time

RT-PCR (RT-qPCR), it has been possible to simultaneously confirm the amplified product (Mattison *et al.*, 2009b; Lees and CEN/WG6/TAG4, 2010) as well as to estimate the amount of detectable virus genomes (Le Guyader *et al.*, 2009). However, the lack of reliable and harmonized sampling plans and standardization in the detection methods limits their suitability for routine analysis of viruses in soft fruit. Therefore, data on the prevalence and titres of viruses in these matrices are rare. In a collection of data on outbreaks that occurred in USA during 1990–2005 with known aetiology, fresh produce accounted for 13% (713/5416) of all foodborne related outbreaks (DeWaal and Bhuiya, 2007; Doyle and Erickson, 2008). Of these, NoV was the most common pathogen accounting for 40% of outbreaks associated with fresh produce (i.e. fruits and vegetables). Specifically, NoV in lettuce and salad greens alone accounted for nearly 25% of the produce-associated outbreaks (Doyle and Erickson, 2008). Data from the European Rapid Alert System for Food and Feed (RASFF), outbreak investigations, and a few monitoring studies verify this importance of fresh produce as a high-risk food product for NoV. The application of recently developed methods in outbreak investigations has begun to provide an insight into the magnitude of contamination of soft fruits that has resulted in disease after consumption. Several outbreak reports of NoV-contaminated raspberries describe large numbers (up to 11 000) of people being infected (Falkenhorst *et al.*, 2005; Sarvikivi *et al.*, 2011a; Anonymous, 2012). Table 21.2 illustrates published outbreaks where NoV contaminated soft fruits were epidemiologically linked to the patients and available for viral testing. Only a few screening studies have been conducted of viruses in soft fruits. In a French study of soft red fruits, 6.7% of 150 samples tested positive for NoV genogroup I (GI), GII or both (Baert *et al.*, 2011). In a Canadian study of irrigated, field-grown strawberries, 26% (16/60) of samples were positive for NoV GI, and a few samples also tested positive for swine hepatitis E virus genogroup 3 and human rotavirus (Brassard *et al.*, 2012). In a minor screening study in Belgium, five samples from each of two lots of raspberries and 10 samples from each of two lots of strawberries were tested for NoV. Infrequently, samples from all four lots were found to be positive for NoV GI, GII or both (Stals *et al.*, 2011). The quantified levels of NoV found in these studies are summarized in Table 21.3. No illnesses could be connected to these batches of soft fruit, despite some being contaminated with NoV in levels similar to or exceeding the estimated infectivity dose of 18–1000 viral particles (Teunis, 2008) and what is commonly found for oysters associated with disease outbreaks (10^2–10^4 NoV genomic copies/g digestive tissue) (Dore *et al.*, 2010; Le Guyader *et al.*, 2008). According to the authors, suggested reasons for this could be under-reporting of sporadic gastroenteritis and that the amount of NoV genomic copies detected by RT-qPCR (which does not differentiate between infective and non-infective viruses) may overestimate the amount of infectious viruses (Baert *et al.*, 2011).

Finally, although rather similar analytical principles are used among the laboratories when analyzing soft red fruits, there are important factors which differ such as variation in the number of samples (29 to 150) and most likely also batches tested, which may affect the prevalence of positive findings. Moreover,

Table 21.2 Outbreaks caused by NoV and HAV contaminated soft fruits where foods were available for analysis

Country	Year	Product	Origin	Outbreaks	Cases	Patient sample	Food sample	Reference
Canada	1997	Raspberries	Bosnia	2	100	GI*	GI	Sarvikivi *et al*. (2011b)
Finland	1998	Raspberries	East Europe	1	108	GII	Neg.	Ponka *et al*. (1999)
Sweden	2001	Raspberries	Slovakia	1	30	GI	GI, GIIb	Le Guyader *et al*. (2004)
New Zealand	2002	Blueberries	New Zealand	1	39	HAV	HAV	Calder *et al.* (2003)
Denmark	2005	Raspberries	Poland	6	1100	GII.4, II.b, II.7	GII.4	Falkenhorst *et al*. (2005)
Germany	2005	Blackberries	Unknown	1	241	GI	Neg.	Fell *et al*. (2007)
France	2005	Strawberries	Serbia?	1	75	GI.5	Neg.	Cotterelle *et al*. (2005)
Sweden	2006	Raspberries	China	4	43	NoV*	Neg.	Hjertqvist *et al*. (2006)
Finland	2009	Raspberries	Poland/Serbia	13	900	GI.4, GII, II.b, II.4, II.7	GII	Sarvikivi *et al*. (2011a)
Denmark	2009	Raspberries	Serbia	1	6	GII.8	GI, GII	Anonymous (2011)
Finland	2009	Raspberries	Poland	3	200	GI.4	GI.4	Maunula *et al*. (2009)
Denmark	2010	Raspberries	China	1	10	NoV*	GII	Anonymous (2011)
Denmark	2010–11	Raspberries†	Serbia	7	224	GI.b / I.6	GI, GI.6 GII	Anonymous (2011)
Denmark	2011	Raspberries	China	1	8	GI.4	GII	Anonymous (2011)

Source: Schultz *et al*. (2012) PhD thesis.

*NoV genogroup or type is not described.
†Multiple batches of different package date from same producer.

Table 21.3 Amount of detected NoV in monitoring studies of soft fruits

Sample source	NoV GC/g min–max*		Reference
	GI	GII	
Raspberries/ strawberries (BE)	2.0×10^{1}–1.3×10^{3}†	1.0×10^{2}–6.3×10^{2}†	Baert *et al*. (2011); Stals *et al*. (2011)
Raspberries (FR)	2.5×10^{2}–1.0×10^{5}†	1.0×10^{2}–6.3×10^{5}†	Baert *et al*. (2011)
Strawberries (CA)	5.0×10^{1}–3.0×10^{3}	Neg.	Brassard *et al.* (2012)

* Samples were considered valid for analysis if the recoveries of the process control were higher than 0.1% or if NoV was detected in the sample.
† The GC/g-values (detectable genomic copies/g) are reverted from the log values originally given in the references.
Abbreviations: BE, Belgium; FR, France; CA, Canada.

the difference in the initial and analyzed sample sizes (10–25 g or 0.025–1.25 respectively) is likely to affect the detection limit of the target virus as well as the risk of co-extracted RT-PCR inhibitors. Thus, interpretation of the NoV prevalence and quantitative findings in relation to general occurrence and comparison of results between laboratories should be handled with care. To assist source attribution studies, a recent EFSA (European Food Safety Authority) report (EFSA BIOHAZ, 2011) recommended systematic strain typing in combination with routine harmonized surveillance of viral outbreaks, and of virus occurrence in food commodities. Successful typing of the NoV findings was achieved in only a few of the outbreak studies listed in Table 21.2 (Maunula *et al.*, 2009; Ponka *et al.*, 1999; Le Guyader *et al.*, 2004). The failure to obtain NoV sequences from these samples could be because the strains initially detected by RT-qPCR were not recognized by the applied primers used for conventional RT-PCR or below the assay detection limit (Baert *et al.*, 2011). Other common issues that can challenge successful sequencing of virus strains detected in soft fruits could be insufficiently pure RNA extract or simultaneous RT-PCR amplification of multiple strains (Sarvikivi *et al.*, 2011a).

21.4 Methods for evaluation of microbial safety in soft fruit

Procedures to evaluate microbial load in soft fruit aim to detect and quantify pathogens that represent a risk to human health. The final objective of the evaluation is, therefore, to determine the number of microorganisms for which regulatory systems have adopted a zero tolerance level. For emerging pathogens for which no safety regulations are well established, such as NoV or sapovirus, the objective is set to detect the infectious dose causing infection in consumers. Consequently, the detection limit of methods for evaluation of microbial load in soft fruit requires the amount of colony forming units (cfu), plaque forming units (pfu) or oocysts (infectious stage of some parasites). In order to quantify any microbial pathogen, the objective of the methodology is to concentrate and

separate the target from the food matrix. The particular case of soft fruit represents a challenge as fruits are fleshy and easily create big particulates, which could entrap the microbial target for detection. Furthermore, some soft fruit surfaces contain crevices that could obstruct the separation of the microbial target from the soft fruit surface (Fig. 21.3). In addition to physical entrapment in a rough surface of a berry, smooth surfaces can interact with microbial particles by electrostatic interactions. Methods developed for quantification and detection of foodborne pathogens must take into account the rupture of these interactions in order to improve the microbial recovery, and, hence, promote a sensitive assessment of soft fruits.

Conventional methods for detection of pathogens in food are based on cell resuscitation in the case of bacteria or on identification by indirect methods. In the case of pathogens for which there are no *in vitro* propagation methods, the use of cultivation-independent methods represents the only alternative for evaluation of soft fruit safety. Therefore, new developments in testing of soft fruit have been based on the molecular identification of pathogen. As has been stated in previous sections, recent outbreaks linked to soft fruit consumption are related to the presence of viruses. Therefore, most of the methods reviewed in this section will be focused mainly on these microorganisms.

21.4.1 Sampling

The major challenges for conventional detection of pathogens in soft fruits as well as for molecular identification are: the uneven distribution of presumably low

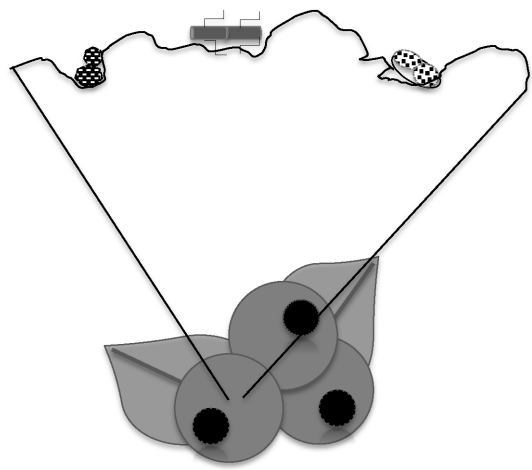

Fig. 21.3 Representation of the interaction between soft fruit surfaces and bacterial, viral and parasitic pathogens. Interaction on a smoother surface, such as blueberry, might be similar because of neutral, hydrophilic and hydrophobic forces (adapted from Wang *et al*., 2009 and Morales-Rayas and Jean, 2011).

viral loads, the limited reproducibility of certain pathogens or nucleic acid extraction efficiencies, the presence of inhibiting substances that jeopardize molecular detection and the genetic variability of pathogens such as NoV (Le Guyader *et al.*, 2006).

As for other types of foods, an appropriate sampling strategy is important to undertake effective pathogen monitoring. This is particularly important for methods applied within a legislative context and used for risk management and control (Bosch *et al.*, 2011). A plan for sampling should be established on a risk-based approach and the sample or subsamples must represent the original matrix. In addition, the sampling process must not alter the condition of the sample and thus not affect the subsequent analysis (Bosch *et al.*, 2011). To develop a sampling plan for pathogen analyses, it is important to consider the characteristics of the matrix to be analyzed (nature, food category, composition and amount) as the choice of sample size may rely on the capability of the available method to remove inhibitors along the sample processing (Rodriguez-Lazaro *et al.*, 2011). Currently, there is no official standard for sampling for virus detection in foods (Rodriguez-Lazaro *et al.*, 2011) and the limited amount of available harmonized data makes it difficult to enable effective application of virological methods for monitoring virus contamination in food. The low viral infectious dose requires sampling and testing of relatively large volumes of food. In general, published protocols for viral analyses of soft fruits use an initial sample size of 10–100 g (Table 21.4). Although viruses have been recovered from relatively small amounts of soft fruit (Stals *et al.*, 2011), the concentration of virus particles often varies within a consignment, between sub-batches, and even between single pieces of food because of uneven distribution of contamination (Falkenhorst *et al.*, 2005).

21.4.2 Parasitic and bacterial testing in soft fruit

Conventional methods for bacteria in food are generally used for the analysis of pathogens in soft fruit. Standard methods in different countries are based on the methods recommended by the International Organization of Standardization (ISO). Methods for bacteria such as *Salmonella*, *Listeria*, *Campylobacter* or *E. coli* have been published basing the detection on enrichment of cells in selective media. Some of the new methods for detection of bacteria in soft fruit base the pathogen identification on combining culture methods followed by a molecular identification of the target. Although the advantage of this strategy is the speed in assessment of microbial safety, the downside is the identification of the total microbial load. This is because selective media and culture conditions are used for a specific target, making undetectable other infectious pathogens that could be present in low numbers in the soft fruit analyzed. The identification of parasites is based on indirect quantification of the number of oocysts present in a food matrix. Immunoassays are most commonly used for the separation of oocysts; however, the bottleneck in the identification of pathogens is still the sample preparation (Ortega and Sterling, 2006). A recent study compared the use of different wash solutions for identification of *C. cayetanensis* in raspberries (Shields, 2012). A

Table 21.4 Selected methods used for virus detection in soft fruits

Food sample (s)	Sample mass (g)	Viral elution	Concentration and clarification	RNA extraction	References
Blueberries	5	Distilled water, glycine buffer	Receptor-binding capture and magnetic sequestration	TIANamp viral RNA extraction	Pan *et al.* (2012)
Blueberries Raspberries Strawberries	15	TGBE buffer (pectinase), alkaline TGBE buffer, pectinase	Ultra-filtration, PEG, chloroform-butanol	QIAamp (or NucliSens miniMAG), RNeasy Mini kit	Butot *et al.* (2007)
Raspberries	10	Glycine, chloroform-butanol, CatFloc	PEG	Prot. K, phenol-chloroform CTAB	Le Guyader *et al.* (2004)
	60	Alkaline	Ultracentrifugation	QIAamp	Rzezutka *et al.* (2005)
	25	Alkaline TGBE buffer, pectinase, alkaline glycine buffer	PEG NaCl, chloroform-butanol, PEG, recirculating magnetic capture with cationic beads	QIAamp, viral RNA Mini Kit, NucliSens miniMAG, TrizolTM	Dubois *et al.* (2007)
	50	Alkaline TGBE buffer, pectinase	PEG, chloroform-butanol	RNEasy kit	Baert *et al.* (2008)
Raspberries Strawberries	60–90	Alkaline sodium bicarbonate+soya protein	Ultracentrifugation	QIAamp	Rzezutka *et al.* (2006)
Strawberry	One piece of food	PBS	Immunomagnetic separation, positively charged filter filtration	95 °C for 5 min	Bidawid *et al.* (2000b)
Strawberry rinses	25	PBS, immunomagnetic separation, monoclonal antibody	PBS	TRIzol reagent	Shan *et al.* (2005)
Strawberries	20	Beef extract, immunomagnetic separation, polyclonal antibody	Chloroform, PEG, sodium phosphate buffer	95 °C for 5 min	Park *et al.* (2008)
	25	Glycine buffer, cationic beads	Pathatrix™	QIAamp, viral RNA Mini Kit	Mattison *et al.* (2010, 2009a)

Source: modified from Schultz *et al*. (2009).

CatFloc, polydiallyldimethylammonium chloride; CTAB, cethyl trimethyl ammonium bromide; GuSCN, guanidinium thiocyanate; HAV, hepatitis A virus; NoV, norovirus; PBS, phosphate buffered saline; PCRU, RT-PCR units; PEG, polyethylene glycol; pfu, plaque forming units; Prot. K, proteinase K; $TCID_{50}$, 50% tissue culture infective dose.

high recovery of oocysts was obtained (80.2 ± 11.3%) using 0.1% of a solution containing a synthetic detergent and surfactant and a food additive with emulsifying and dispersing properties, sodium dodecylbenzenesulfonate ($C_{12}H_{25}C_6H_4SO_3Na$) and tetrasodium pyrophosphate ($Na_4P_2O_7$) respectively. The recovery using this solution represented about twofold of the recovery obtained from raspberries using 1 M glycine pH 5.5 in a standard method reported by Cook *et al*. (2006a). Although the recovery was improved, the detection method for oocysts was microscopy in both cases, which is the conventional detection method for oocysts in water (Ortega and Sterling, 2006; Cook *et al*., 2006a, b).

21.4.3 Viral testing in soft fruit

Evaluation of the safety of soft fruits has recently focused on detection of viruses as a result of the frequent soft-fruit-borne outbreaks of NoV and HAV (Table 21.2). As viruses do not replicate in food and the most important foodborne viruses (such as NoV) cannot be propagated *in vitro*, conventional identification for clinical samples based on microscopy or immunoassays, which requires a large amount of viral particles, cannot be used. Therefore, the development of molecular methods for identification of viruses in different food matrices has greatly helped in identification of pathogens that were not conventionally identified some years ago. However, compared with bacterial pathogens that can be cultured, the demands of high assay sensitivity and the lack of ability to differentiate between PCR-detected infectious and non-infectious particles are still major drawbacks in the detection methods for viruses in foods. New developments in the safety testing of soft fruit therefore aim to improve the sample preparation methods to overcome the low number challenge. Whichever method is used, it is crucial to ensure that issues associated with low virus numbers and the complexity of the matrix do not result in false negative or false positive interpretations of results. Therefore, effective quality controls must be included as part of the detection method. Such controls were included to a varying degree in the methods used for the screening and outbreak studies listed in Tables 21.2 and 21.3 and are also described by D'Agostino *et al*. (2011). After the initial sampling, most methods for the detection of viruses in soft-fruits include the following three steps: elution and concentration of virus from the food matrix; extraction of nucleic acid; and detection, confirmation and quantification of target viral RNA. The most critical part of sample processing before detection of viral RNA is the elution and concentration of virus particles from the food matrix. Each step must be designed to achieve reduction of sample volume while simultaneously recovering the viruses and eliminating matrix-associated inhibitors. Various strategies for each step have been described, and an overview of selected methods used for soft fruit is summarized in Table 21.4.

In Fig. 21.4, a flow diagram of a procedure for virus detection in raspberries is shown as an example of virus detection in soft fruit. Promising new developments for the concentration of virus from eluates focus on virus capture using cationic charged beads or filters, or anion exchange filtration (Morales-Rayas *et al.*, 2010;

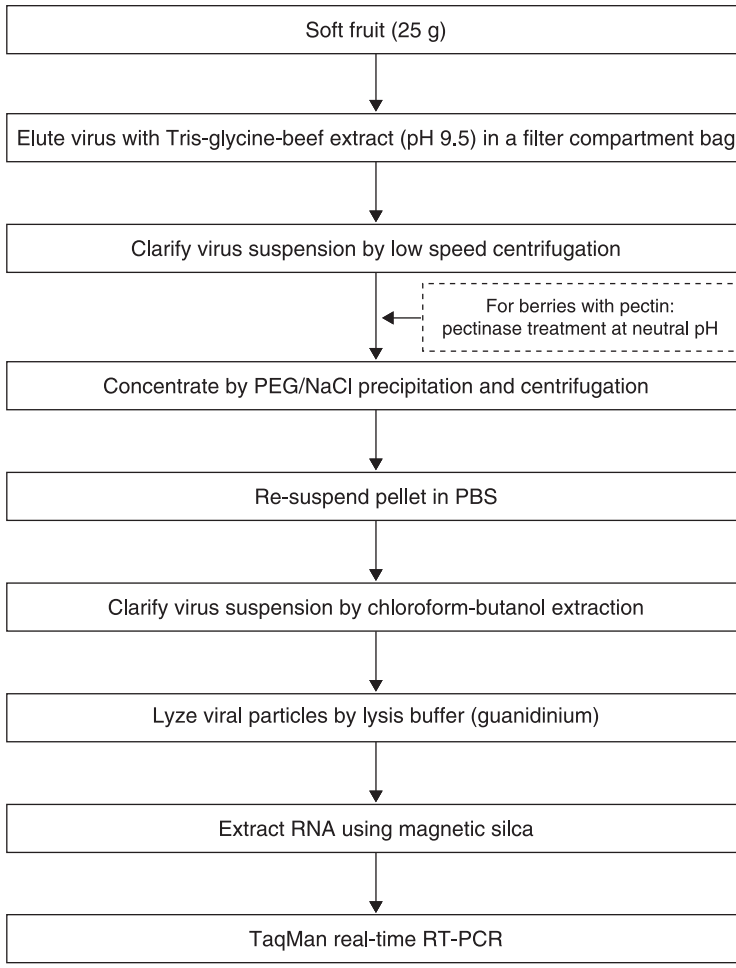

Fig. 21.4 Procedure for norovirus detection in raspberries (Blaise-Boisseau *et al.*, 2010).

Papafragkou *et al.*, 2008) and carbohydrate-conjugated magnetic beads in a recirculating affinity magnetic separation system (Papafragkou *et al.*, 2008; Tian *et al.*, 2011). For detection of NoV and HAV RNA, a large variety of published RT-qPCR assays are available in the scientific literature. For NoV, some of these can be found in the references in Tables 21.2–4, and for HAV, assays developed by, for example, Costafreda *et al*. (2006) and Jothikumar *et al*. (2005) have been successfully used for analysis of foods. Examples of the use of quality controls can also be found in some of these studies (Baert *et al.*, 2011; Costafreda *et al.*, 2006). To quantify NoV detected by RT-qPCR, the value of a positive signal must be converted into RNA copies by interpolation to a standard curve. Based on the primers and probe

used in the detection assay, a standard curve can be generated from different types of nucleic acids: purified synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon, plasmid DNA constructs or *in vitro* transcribed RNA (Costafreda *et al.*, 2006; Le Guyader *et al.*, 2009). Because of lack of validation and harmonization of the type and use of standard curves, exact comparison between viral loads detected in different studies may not be possible.

A qualitative and quantitative standard (including a suite of proper controls) for NoV and HAV detection in foods has recently been developed by the technical working group within the European Committee on Standardisation, CEN/TC275/WG6/TAG4 and accepted by EU member states. This standard is expected as a technical specification in 2013, with the potential of being incorporated into EU legislation as a reference method for virus control (Lees, 2010). However, as the method does not distinguish between detection of infective and non-infective viruses, better understanding on how to interpret a positive result in terms of health risk must be further discussed (Lees and CEN/WG6/TAG4, 2010; Le Guyader *et al.*, 2009).

21.5 Conclusion and future trends

Despite the lack of evidence and limited knowledge of the exact hygiene and growing conditions for soft fruit products, it is realistic to believe that irrigation water is among the main routes of virus transmission (Newell *et al.*, 2010; Kurdziel *et al.*, 2001). It is therefore necessary to develop ways to control and monitor for pathogen introduction during production (EFSA BIOHAZ, 2011). Clear legislation on the use, handling and treatment of water applied in industrial fresh produce production should be efficient to reduce the risk of pathogens from faecal contamination (EFSA BIOHAZ, 2011). Moreover, it has been shown that handling of fresh produce by workers during harvesting and packing is an important risk factor for not least NoV contamination of fresh produce (León-Félix *et al.*, 2010). Implementation of improved hygienic measures is therefore needed in agricultural operations to avoid risk of viral contamination (Rodriguez-Lazaro *et al.*, 2011). Farm and restaurant owners are responsible for the safety of the food they harvest or prepare, and for providing sufficient training to their employees who handle foods (Hicks *et al.*, 1996). Decontamination processes are also important, as they affect pathogen viability, survival, persistence and cross-contamination between food items (Hirneisen *et al.*, 2010). However, for fragile foods, for example raspberries, which lose their visual and sensory qualities when exposed to washing, heating or high pressure processing, there may not be many options for decontamination. To implement control measures such as detection of viruses in the food production, the EU requires the inclusion of a standardized and quality controlled reference method. To meet this requirement, a protocol for viral RNA extraction and detection has been developed within the CEN/TC275/WG6/TAG4 (virus detection in foods). Still, it is essential to gain more information about the practical application (e.g. sampling plans and result interpretation) of these methods for the assessment of product safety (Lees and CEN/WG6/TAG4, 2010). Current

molecular assays to detect viruses can supplement prevention efforts as part of the HACCP program of producers of soft fruit (Baert *et al.*, 2011; Brassard *et al.*, 2012) and provide knowledge of the extent of viral distribution (Mattison *et al.*, 2010). However, the available molecular detection methods must be accommodated by harmonized sampling plans and procedures to interpret results before they can be used for routine monitoring and control (Rodriguez-Lazaro *et al.*, 2011).

As a result of the development of molecular detection methods, the presence of human pathogens not quantified by conventional methods is now evidenced in specific food matrices. In this sense, soft fruits thought to be a conventionally 'safe' food, have turned out to be one of the main channels for introduction of viral pathogens to the food supply. Therefore, new developments on testing of soft fruit are focused towards sample preparation methods—fast, easy to use and able to remove inhibitors to molecular identification. The viral load in soft fruits, as with other complex matrices, still represents a challenge because of inhibitors that may be introduced downstream to molecular identification. It is therefore evident that until the sample preparation bottleneck is passed, availability of molecular methods such as real-time PCR, microarrays, immunoassays or next generation sequencing to identify/quantify a pathogen is still restrained. Furthermore, the lack of differentiation between infectious and non-infectious viral particles, and possible bacterial and parasitic pathogens, remains a major issue in standardization of soft fruit testing. As future remarks, the detection step in pathogen identification should evolve towards identification of bacterial, viral and parasitic targets in one step. In addition, a continuous record of the presence of each pathogen in soft fruit as well as development of in-field applications for identification should be expanded to satisfy the demands of the dynamic food chain.

21.6 Questions for discussion

1. What are the potential contamination factors in soft fruit during preharvest?
2. What are the potential contamination factors in soft fruit during postharvest?
3. What are the potential contamination factors for soft fruit during processing?
4. What microbial contaminants in soft fruits are of main concern for human risk?
5. What viruses are more commonly found in soft fruits?
6. What methods can be applied for detection of bacteria in soft fruits?
7. What methods can be applied for detection of parasites in soft fruits?
8. What detection methods can be applied for screening of viruses in soft fruit?
9. Why are soft fruits and other foods currently not routinely tested for viruses?

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