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A predictive microbiology approach for thermal inactivation of Hepatitis A virus in acidified berries

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ABSTRACT

Hepatitis A virus (HAV) is a food-borne enteric virus responsible for outbreaks of hepatitis associated with consumption of raw vegetables. Soft fruits, such as red berries, exposed to faecal contamination are increasingly responsible for collective food-borne illnesses associated with HAV, when eaten raw or used in unprocessed foods. Heat is the most effective measure for the inactivation of HAV. Thermal treatments are used on fruits as a decontamination method, but they have to be adapted to product characteristics; indeed, factors such as sugar or pH may have an impact on the viral sensitivity to thermal treatments. A model was developed for the inactivation of HAV in red berries without supplemented sugar and with different pH values. Nonlinear inactivation curves in acidified raspberries were modelled using an integrated model, with a single equation nesting secondary models of temperature and pH in the primary model. Model predictions were then confronted to experimental results obtained in another laboratory on other berries with different pH values. Excellent predictions were obtained in most cases, while failed predictions provided safe results, with the model predicting higher residual virus titres than what was observed.

1. Introduction

Hepatitis A virus (HAV) is an enteric virus, mainly transmitted via faecal–oral route, either through person-to-person contact or by contaminated water and food. A large percentage of food-borne outbreaks are caused by HAV and numerous epidemiological studies have particularly associated viral hepatitis with the consumption of raw vegetables (Kuritsky et al., 1985; Rosenblum et al., 1990; Warner et al., 1991; Niu et al., 1992; Hernandez et al., 1997; Gaulin et al., 1999; Nygard et al., 2001; Long et al., 2002; Le Guyader et al., 2004). Indeed, soft fruits and vegetables may be exposed to faecal contamination during irrigation with contaminated water, fertilization with inadequately composed manure, or handling by infected persons with poor hygiene (Ward et al., 1982; Niu et al., 1992; Deng and Cliver, 1995; Bidawid et al., 2000a; Calder et al., 2003; Koopmans et al., 2003; Todd et al., 2007). Although raw vegetables are usually consumed after a washing step, pathogenic bacteria, parasites and enteric viruses can survive this minimal treatment (Beuchat et al., 1998; Gulati et al., 2001; Butot et al., 2008). Frozen raspberries, strawberries and blueberries are being recognized as vehicles for hepatitis A virus and responsible for collective food-borne toxi-infections, when used in unprocessed foods (Noah, 1981; Reid and Robinson, 1987; Ramsay and Upton, 1989; Niu et al., 1992; Hutin et al., 1999; Calder et al., 2003). The potential of gamma irradiation and sanitation with low chlorine level to inactivate HAV has been investigated on experimentally contaminated samples of berries (Sattar et al., 2000; Butot et al., 2008). Heating appears as the most effective measure for the inactivation of HAV (Siegl et al., 1984; Murphy et al., 1993). There are many reports that describe the heat tolerance and survival of HAV in various food matrices, including shellfish (Millard et al., 1987; Croci et al., 1999, 2005; Hewitt and Greening, 2006), dairy products (Parry and Mortimer, 1984; Bidawid et al., 2000b) and fruit-based products (Deboosere et al., 2004; Butot et al., 2009). Food constituents and factors can have an impact on the viral sensitivity to thermal treatments: high sugar concentrations can induce a protective effect for enteric viruses (Deboosere et al., 2004), and low pH increased thermal inactivation (Salo and Cliver, 1976). Therefore industrial heat treatment can be used on raw fruits to secure the finished products for consumption in terms of viral risk, but they have to be adapted to product characteristics.

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Likewise, efficiency of treatments depends on the target virus. HAV is an RNA non-enveloped virus, structurally similar to noroviruses, enteroviruses and astroviruses. However numerous studies have addressed the higher stability of HAV under denaturising environmental conditions (McCaustland, 1982; Siegl et al., 1984; Scholz et al., 1989; Hollinger, 1996). A cell-culture-adapted HAV strain (HM175/18f) seemed to be a "relevant" virus in studies aiming at validating the virus inactivation procedures used in agro-food industry (Bidawid et al., 2000b; Deboosere et al., 2004; Butot et al., 2008, 2009), when efforts to cultivate human norovirus have been unsuccessful (Duizer et al., 2004). Indeed, in the absence of a model virus or model system, food safety guidelines need to be based on studies that have been performed with the most resistant enteric RNA viruses. HAV may be thus considered as a good indicator virus or as a surrogate for enteric viruses.

Mathematical models to predict the thermal inactivation of food pathogens during industrial process assist in assessing the risk of contamination for the consumer. A model for the effect of sugar concentration and pH on heat inactivation of HAV in strawberry preparations was published by Deboosere et al. (2004). The results have shown a significant protective effect of sugar contents on the heat resistance of the virus between 80 and 90 °C and a significant effect of pH (range 3.3–4.3) on the D-value at 85 °C. However, high temperatures can not be applied on some fruit matrices (fruit breaks, coulis) for which no sugar is added. In the present paper, we describe a new predictive microbiology approach for thermal inactivation of Hepatitis A virus in acidified red berries without supplemental sugar. This study aims at modelling the behaviour of HAV in acidified berries as a function of thermal treatment and pH. The effects of heat treatments between 65 and 75 °C on survival of HAV were evaluated in experimentally-inoculated fruit purees. This study also aimed at determining if adjustment to more acidic pH values, between 2.5 and 3.5, could promote the thermal inactivation of HAV in these purees.

2. Materials and methods

2.1. Strains and media

HM175/18f strain of HAV (VR-1402) and the foetal rhesus monkey kidney cell line (FRhK-4) were obtained from the American Type Culture Collection. These cells were used throughout the study for the propagation of HAV to prepare inoculums and to measure HAV infectivity. Methods of cultivation, maintenance of cells and preparation of virus pools have been described previously (Flehmig, 1980; Lemon et al., 1985; Cromeans et al., 1987).

2.2. Infectivity assays

Quantitative measurement of the infectivity of the Hepatitis A virus was done by using a titration method by lysis plaque under agar overlayer, described previously (Deboosere et al., 2004). Briefly confluent FRhK-4 monolayers in 6-well cell-culture multiplates were infected with serial ten-fold dilutions of virus samples. Each well was overlaid with a medium containing agar. After 9 to 10 days at 37 °C in humidified 5% CO_2 atmosphere, solid overlayers were removed. The monolayers were fixed and stained with a formalin and crystal violet solution. The average number of plaque-forming units (PFU) was used to determine virus titre of the sampled assayed, expressed in PFU mL^{-1}.

2.3. Fruit products

Raspberries supplied by food industrial partners (Vergers de Boiron; Kerry Ravifruit) were ground to obtain a purée that was used as a reference matrix for the modelling step, and citric acid (anhydrous powder; Arnaud, France) was added to obtain final pH values of 3.3, 3.0, or 2.5. Regarding validation of model predictions, other ground fruits were used with their natural pH: strawberries (pH 3.35), raspberries (pH 3.05) and bilberries (pH 2.87).

2.4. Thermal treatment

Each food matrix was artificially contaminated with HAV to obtain concentrations of 10^6–10^8 PFU mL^{-1} 0.5 g of preparations were then distributed in glass tubes, 100 mm long and 0.5 mm thick (Fisher Bioblock Scientific). Thermal inactivation was performed as described previously (Deboosere et al., 2004), except that samples were left at room temperature for 3 h before heat treatment, as an aggregation step. Heat treatments were performed by simultaneous immersion of the tubes in a glycerol bath set at the desired temperature (65, 70 or 75 °C) for a determined period of time. A thermocouple connected to a data acquisition unit (Agilent Technologies, Actifa, France) was inserted into an uncontaminated aliquot of preparation to monitor the internal temperature throughout the heat treatment. Individual aliquots were removed at periodic time intervals and placed immediately in an ice bath for rapid cooling. The treated media samples were 50-fold diluted in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, France) before virus titration. Each experiment was replicated 3 times.

The desired temperature was reached in approximately 2 min; however, since virus inactivation took place during this step, it was assumed that the target temperature was reached immediately.

2.5. Inactivation model

The experimental data obtained for all heat-inactivation kinetics were processed using the statistical software S-PLUS 2000.

A primary model used to describe viral inactivation kinetics was adapted from a bacterial inactivation model proposed by Albert and Mafart (2005). This model was used to describe bacterial inactivation curves for nonlinear heat treatments and to take into account the residual population observed at the end of heat treatment:

$$\mathrm{Log}_{10}(N) = \mathrm{Log}_{10}[(N_0 - N_{\mathrm{res}})\cdot 10^{-(t/\delta)^p} + N_{\mathrm{res}}] \quad (1)$$

where N is the infectious virus titre, t is time, N_0 represents the initial titre (at time 0), N_{res} is the residual titre at the end of the treatment, δ is the time for first decimal reduction for the population not included in N_{res}, and p is a shape parameter for concavity or convexity of the curve.

The logarithmic reduction, or abatement, in virus titre obtained at the end of the treatment was described using $A = \mathrm{Log}_{10}(N_0) - \mathrm{Log}_{10}(N_{\mathrm{res}})$.

The impacts of temperature and pH on δ and A were described using the following secondary models:

$$\mathrm{Log}_{10}(\delta) = \mathrm{Log}_{10}(\delta^*) - \frac{T - T^*}{Z_T} - \frac{\mathrm{pH} - \mathrm{pH}^*}{Z_{\mathrm{pH}}} \quad (2)$$

$$A = A^* - \frac{T - T^*}{Y_T} - \frac{\mathrm{pH} - \mathrm{pH}^*}{Y_{\mathrm{pH}}} \quad (3)$$

where δ^* (respectively A^*) represents the value of δ (respectively A) in an arbitrary reference condition T^* (65 °C) and pH* (3.3), Z_T (respectively Y_T) is the temperature increase necessary for a 1-unit reduction of $\mathrm{Log}_{10}(\delta)$ (respectively A), and Z_{pH} (respectively Y_{pH}) is

the pH increase necessary for a 1-unit reduction of $\mathrm{Log}_{10}(\delta)$ (respectively A).

It was assumed that the shape parameter p did not depend on temperature and pH, as has been observed previously for bacteria (van Boekel, 2002; Couvert et al., 2005). Likewise, no interaction terms were included in models for bacteria. Based on these results, and for reasons of parsimony, no interaction terms were included here.

A complete model combining the primary and secondary models (Equation (4)) was actually used to evaluate the parameters on the full dataset: the values of δ and N_{res} in Equation (1) were substituted by their expressions from Equations (2) and (3). N_0 values were forced at $N_{(t=0)}$. Titres under the detection threshold were set at the detection threshold (safe assumption). A one-step fitting procedure was thus performed and parameters describing HAV inactivation in raspberries were obtained: p, δ^*, Z_T, Z_{pH}, A^*, Y_T, Y_{pH}.

$$\mathrm{Log}_{10}(N) = \mathrm{Log}_{10}\left[N_0\cdot\left(1-10^{-A^*+\frac{T-T^*}{Y_T}+\frac{\mathrm{pH}-\mathrm{pH}^*}{Y_{pH}}}\right)\cdot 10^{-\left(\frac{t}{10^{\mathrm{Log}(\delta^*)-\frac{T-T^*}{Z_T}-\frac{\mathrm{pH}-\mathrm{pH}^*}{Z_{pH}}}}\right)^p}+N_0\cdot 10^{-A^*+\frac{T-T^*}{Y_T}+\frac{\mathrm{pH}-\mathrm{pH}^*}{Y_{pH}}}\right] \quad (4)$$

3. Results

3.1. Parameters estimation

The heat resistance of HAV in a raspberry puree, acidified to pH 2.5, 3 and 3.3 with citric acid, was analyzed by kinetic evaluation of the loss of infectivity in cell culture. The desired internal temperature was reached after approximately 2 min. The profile of the temperature increase showed: firstly, that the target temperatures were almost reached during the first minute of treatment; and secondly, that the second minute was required to obtain the final 2–3° increase to reach the target temperatures. In some cases, mostly for the 65 °C target temperature, but also for 70 °C, no decrease in viral population was observed until 1 to 4 min after the target temperature was reached, thus resulting in a shoulder. On the other hand, for other experiments, viral inactivation ranging from 1.5 to 4 log units was observed during the temperature increase period, especially for fruits preparation at pH 2.5 heated at 75 °C. Therefore, temperature variations during the heating time were neglected for the data analysis: it was assumed that the temperature was constant at its target value from the beginning of the experiment.

Using the Equation (4), the parameters were estimated from HAV inactivation in acidified raspberry data (Table 1). Inactivation kinetics and model curves are shown in Fig. 1.

Since a global primary and secondary model was used, no individual fit was conducted on any given condition. Therefore, the model describes a global behaviour rather than a sum of individual behaviours, which explains why a "perfect fit" is not observed in Fig. 1.

3.2. Model validation

Predictions with the model were confronted to new experimental data, obtained in another laboratory on strawberries, raspberries and bilberries, when heated at 65, 70 and 75 °C. These comparisons are presented in Fig. 2. Apparent δ values were estimated using the model, with kinetics again including the come-up time to reach the target temperature, but not the temperature variations.

Table 1
Parameters estimates from acidified raspberry data.

Parameter	Estimated value	95% confidence interval
p	3.31	2.56; 4.23
$\mathrm{Log}_{10}(\delta^*)$	0.83	0.80; 0.85
Z_T	24.13	22.19; 26.12
Z_{pH}	−4.67	−5.74; −3.86
A^*	2.25	1.78; 2.75
Y_T	−6.67	−9.78; −5.16
Y_{pH}	0.97	0.70; 1.68

In this work, the model used with parameters estimated on acidified raspberries (Table 1) gave excellent predictions of HAV behaviour in other, non acidified berries. Indeed, predicted heat-inactivation kinetics present a close description of the experimental data obtained in the various fruits for most cases. Failed predictions provided safe results, with higher predicted N_{res} values than what was observed.

4. Discussion

pH of various berries are naturally acidic, with values ranging from 2.5 to 3.3. Sugar contents of berries, without addition, were naturally about 5°Brix (corresponding to 5% (wt/wt) of sugar). Sugar concentration and pH have been previously described as important factors in heat resistance studies of bacteria and viruses, in solutions simulating acidic fruit-based products (Silva et al., 1999; Deboosere et al., 2004). HAV is able to survive for a long time in the environment and extremely stable over a large pH range from 1 to 11 at room temperature (Siegl et al., 1984; Scholz et al., 1989). Moreover, low pH was shown to induce aggregation of virus particles (Volkin et al., 1997; Langlet et al., 2007), which may have a stabilizing effect and so increase viral thermoresistance. Since sugar content corresponded only to natural content of red berries in this study, pH was considered as the most likely factor to interfere in viral thermoresistance. In this study, the effects of heat treatments between 65 and 75 °C on survival of HAV were evaluated firstly in experimentally-inoculated and acidified raspberry purees, and secondly, in strawberries, raspberries and bilberries. The experimental protocol used for measuring thermal inactivation has been adapted to work in a system where the state of virus particles was controlled. Indeed, the observation of a reduction in viral titre may be related to the loss of infectivity, to inactivation, aggregation, or adhesion to the substrate. Phenomena of aggregation and adhesion depend both on environmental characteristics and surface properties of the virus (Langlet et al., 2008). The acknowledgement of the state of the viruses and of their electrostatic and hydrophobic properties is essential in studies on the behaviour of viruses in complex matrices. Langlet et al. (2007) showed that aggregated forms of MS2 phage (a model for enteric viruses), with sizes of few micrometres instead of 30 nm at neutral pH, predominated in suspension at acidic pH, especially as the pH was below the isoelectric point of viral particles (pI(MS2) = 3.9), leading to decrease by 3 log in plaque-forming unit counts. The adsorption/aggregation is not always spontaneously reversible upon return to pH higher than pI. Hydrophobic bonds stabilize the interactions between viruses and the matrix. The different properties of viral capsids could also induce different sensitivities to heat treatments. The MS2 phage is widely used as a model to evaluate survival of enteric viruses in water, because its small size

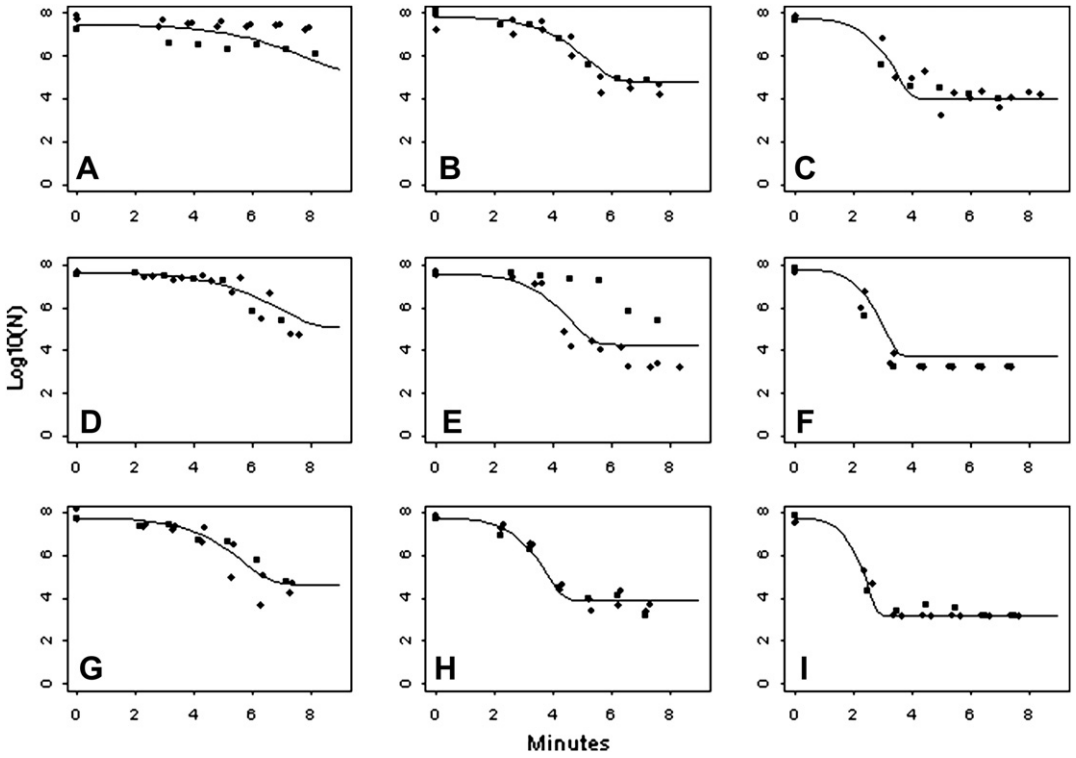

Fig. 1. Inactivation kinetics of HAV in raspberries at 65 °C – pH 3.3 (A), 70 °C – pH 3.3 (B), 75 °C – pH 3.3 (C), 65 °C – pH 3.0 (D), 70 °C – pH 3.0 (E), 75 °C – pH 3.0 (F), 65 °C – pH 2.5 (G), 70 °C – pH 2.5 (H), and 75 °C – pH 2.5 (I); experimental data (symbols, 3 repetitions) and model fit (line).

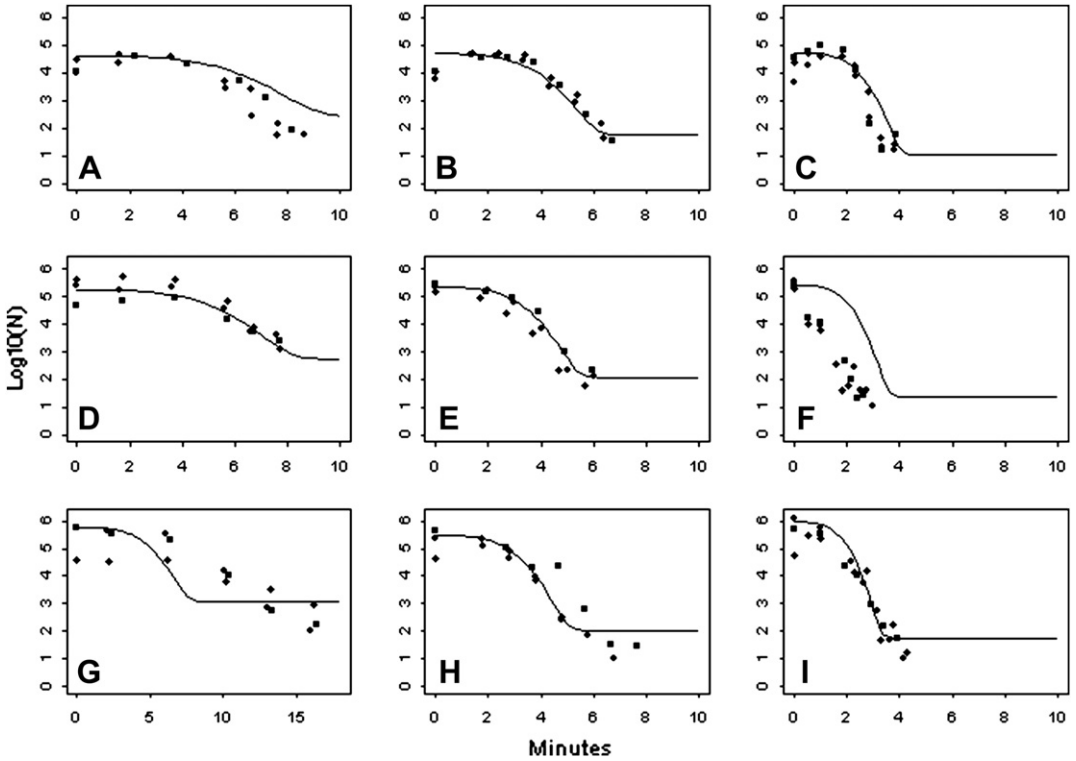

Fig. 2. Inactivation kinetics of HAV in strawberries (pH 3.35) at 65 °C (A), 70 °C (B) and 75 °C (C), in raspberries (pH 3.05) at 65 °C (D), 70 °C (E) and 75 °C (F), and in bilberries (pH 2.87) at 65 °C (G), 70 °C (H) and 75 °C (I); experimental data (symbols, 3 repetitions) and model prediction (line).

and its structure are comparable to that of human pathogenic viruses. Therefore, these results were used to define the conditions of experimental studies carried out for thermo-inactivation, suggesting a preliminary incubation for 3 h at room temperature to aggregate virus particles and subsequently measure only the reduction of infectivity associated with treatment technology.

From these experiments, it appeared that a reduced pH led to a faster thermal inactivation in the tested range. The final reduction in viral titre (parameter A) is greater for higher temperatures and lower pH. Despite the narrow pH range (from 2.5 to 3.3) that was studied, a pH increase has an effect in increasing heat resistance of HAV. Moreover, the acidic pH conditions under which HAV was incubated showed that influence of pH was more pronounced at higher processing temperatures and increase of acidity favoured viral inactivation at higher temperature, as previously observed (Salo and Cliver, 1976; Deboosere et al., 2004).

Experiments on acidified raspberries were performed in triplicate to account for the variability of heat-inactivation kinetics. We obtained nonlinear inactivation curves in acidified raspberries, whereas linear curves had been observed with high sugar concentrations (more than 28%) at 85 °C, which justified the use of linear regression (Deboosere et al., 2004). In this study, three-phase curves with delayed initial decrease and a residual titre at the end of the treatment were observed, mainly at 70 and 75 °C. The chosen model thus took into account the residual population observed at the end of heat treatment, which seemed to be well adapted in order to describe and model experimental inactivation kinetics. The initial shoulder observed in many inactivation curves in this study may be influenced by the fact that experiments were started at room temperature, and that a significant come-up time (around 2 min) was required to reach the target temperature. However, for a number of experiments with target temperatures of 65 °C or even 70 °C, no viral inactivation was observed during this temperature increase period, or for 1 to 4 min after the target temperature was reached, thus indicating that this shoulder could not be considered entirely as an experimental artefact. Yet, the inclusion of the come-up time in inactivation kinetics obviously led to biased estimates of the parameters. Assuming a constant target temperature during the whole kinetics, including the come-up time, is a very simplifying assumption, which will be addressed in future studies.

An integrated model with a single equation nesting secondary models of temperature and pH in the previous model was built to predict the inactivation of HAV in red berries, taking into account heating treatment ranging from 65 to 75 °C and pH range from 2.5 to 3.3. Using this combined approach, the estimated values are expected to be more objective and robust, and the variability in kinetic data is taken into account (Pouillot et al., 2003). This methodology approach is widely used in predictive microbiology studies in the agro-food area. However, no model predictions can be used in confidence unless they were validated on independent data on foods (Delignette-Muller, 1997). Model predictions were then confronted to experimental results obtained in another laboratory on other berries with different pH values. Considering Fig. 2, few differences were observed between predicted and measured infectious virus titre values ($\log_{10}(N)$) in acidic red berries. In some cases, the predicted viral inactivation appeared to be generally safer than the inactivation kinetics experimentally observed for various berry purees (Fig. 2A and F). When predictions failed (Fig. 2G and H), the model predicted higher residual virus titres than what was experimentally observed at the end of experiment and so provided safe results. Consequently the model could be used to predict relatively reliable heat inactivation in soft fruits, acidified or not, in regard to pH variations. While a high sugar content has been shown to increase the heat stability of HAV in fruit-based product (Deboosere et al., 2004), in the present study, pH variation seemed sufficient to explain different heat-inactivation kinetics. Indeed, parameters obtained on raspberries could be applied successfully on strawberries and bilberries, using the measured pH of these fruits: no matrix effect was evidenced in this study. Results confirmed that pH exerted a significant effect on HAV thermoresistance in fruit-based products and that adjustment to acidic pH values, i.e. less than pH 3.3, could promote the thermal inactivation of HAV in red berries-based products without supplemented sugar. In strawberry mashes, although more than 5 min were required at 80 °C with a sucrose concentration above 28% and pH 3.8 to obtain a HAV reduction of 4 log (Deboosere et al., 2004), less than 4 min were required at 75 °C with no added sugar and pH below 3.3 in the present study.

Modelling the thermal inactivation of HAV on berries is very informative to secure the finished berries-based products for consumption. The new modelling approach introduced in this paper is for the second time applied to food virology. A model for thermal inactivation of HAV in red berries, without supplemented sugar, taking into account either heating treatment and acidic pH was successfully developed and validated. In accordance with the objective of predictive microbiology, the use of an equation seems to be suitable to study the virus behaviour of an enteric virus model, i.e. HAV, in a complex food matrix. However, additional studies are clearly needed to take into account heat-inactivation kinetics during the phase of temperature increase to reach the target temperature.

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