Sampling whole melons to check for the presence of *Salmonella*

Request from EC DG-Sanco (09-10-2012):
Give advice on the best way to sample whole melons to check for *Salmonella*.

For drafting an advice some experts were consulted as well as (a selection of) literature (see list of references).

Information from experts:
- Ernesto Liebana Criado of EFSA: EFSA has no specific information for the sampling of melons. Ernesto made reference to Mieke Uyttendaele.
- Mieke Uyttendaele, Ghent University, Belgium is coordinator of EU FP7 project Veg-i-Trade. She consulted two colleagues from the Veg-i-Trade project in Spain (Ana Allende, Centre of Edaphology and Applied Biology of the Segura – CEBAS, Spanish National Research council – CSIC, Murcia) and in Norway (Gro Johannessen, Norwegian Veterinary Institute – NVI, Oslo). At her own laboratory the analyses of melons is performed by peeling 25 g of rind, which is used for further analyses.
    - Ana Allende (Spain) indicated that at her laboratory they take approximately 100 g of the rind, combine it with 0.1% peptone water in a ratio of 3:1, mix by stomacher and filter the homogenate. The procedure is based on the procedure of Annous et al., 2004 (see below).
    - Gro Johannessen (Norway) indicated to have no experience with analysing melons, but considered swabbing as 'not a good idea' and suggests that either vigorous rubbing or taking off the rind (at least from the stem scar area) may be better.
- Aarieke de Jong of the Netherlands Food and Consumer Product Safety Authority (NVWA), indicated that some years ago they have analysed melons by peeling of (a part of) the rind and pre-enrich the rind or by rinsing of the rind and pre-enrich the rinse.
- Thomas Hammack of the Food and Drug Administration (FDA) in the USA indicated that the FDA has experiences with analysing melons because of several *Salmonella* outbreaks related to melons (especially cantaloupes). Thomas developed the method for analysing melons (cantaloupes) currently available in the US FDA Bacteriological Analytical Manual (BAM, 2011). This procedure concerns a 'soak' method, where the whole melon is added to pre-enrichment broth and incubated as a whole (see below).

Information from literature:
- Melons have been implicated in several *Salmonella* outbreaks and often this concerned cantaloupes. Some examples are given in Bowen et al. (2006) and Munnoch et al. (2009). Recently, information was published by the Centers for Disease Control and Prevention, USA about a multistate outbreak of *Salmonella* Typhimurium and *Salmonella* Newport linked to cantaloupes in the USA (July-September 2012): http://www.cdc.gov/salmonella/typhimurium-cantaloupe-08-12/
- Cantaloupes may be susceptible to contamination because they grow on the ground. Potential sources of contamination include (Hammack et al., 2004):
    - Runoff from livestock areas;
    - Contaminated irrigation water;
    - Fertilizer;
    - Fecal contamination.
- Lopez-Velasco et al (2012) assessed the root uptake of *Salmonella* Typhimurium by melons. The results of their study show 'that root uptake and systematic transport of *Salmonella* from soil, as a consequence of contaminated irrigation water, is highly unlikely to occur'. They also indicate that 'contamination of the external rind from irrigation sources remain a concern in melon production'. Due to cut processing, pathogens can be transferred from the rind to the flesh of melons.
- In ISO/CEN documents no information was found on (sub) sampling melons for analysing the microbiological quality (which would have been expected in EN ISO 6887-4; Anonymous, 2003).

- In the documents consulted, the analyses of pathogens is mainly performed on the rind of the melons (Annous et al., 2004 and 2005, Beuchat and Scouten, 2004, Castillo et al., 2004, Hammack et al., 2004).

  For enumeration of pathogens on the rind of the melon, Annous et al (2004 and 2005) peeled of the whole rind with a mechanical peeler, placed the rind (and the stem scar area and the opposite end of the melon) in a sterile (approx. 1 liter glass) blender jar, together with four equal volumes (wt/vol) of 0.1% peptone water and blended it for 1 min. The resulting blend was filtered through a filter bag, diluted and surface plated. In the publication of 2005, Annous et al. compared this procedure with a procedure where a composite sample of 20 rind plugs were taken at random locations on the surface of the melon. These plugs were taken with a sterile 20 mm diameter stainless steel cork bore and adhering flesh was removed. The disadvantage of the cork bore method is that spot contamination on the rind can easily be missed.

  Castillo et al (2004) used a sponge to sample the rind of a cantaloupe. A surface area of approx. 100 cm^2 of each cantaloupe was sampled using a sterile sponge (3.8 by 7.6 cm), moistened with 25 ml of 0.1% sterile peptone water. The area to be sampled was firmly rubbed with the moistened sponge after which the sponge was placed in a 540 ml plastic bag. Next, 225 ml peptone water was added to the bag and used as a pre-enrichment for *Salmonella* analysis. Disadvantage of this sponge method on a limited area of the rind is again that spot contamination on the rind can be missed. Furthermore, *Salmonella* may form biofilms on the rind, which may be difficult to remove from netted rinds (e.g. of cantaloupes), even when firmly rubbed with a sponge.

  Hammack et al (2004) compared two methods for the recovery of *Salmonella* from whole cantaloupes: the 'soak' method and the 'rinse' method. For this they spot inoculated individual cantaloupes with a suspension of *Salmonella* Anatum, *Salmonella* Poona or *Salmonella* Oranienburg and stored the inoculated cantaloupes at 2-6 °C for 4 days. Cantaloupes were placed in sterile plastic bags with a nonselective pre-enrichment broth at a 1:1.5 cantaloupe weight-to-broth volume ratio. The cantaloupe broths were shaken for 5 min at 100 rpm after which 25-ml aliquots (rinse) were removed from the bags. The 25-ml rinses were pre-enriched in 225-ml portions of the same uninoculated broth type at 35 °C for 24 h (rinse method). The remaining cantaloupe broths were incubated at 35 °C for 24 h (soak method). The pre-enrichment broths used were buffered peptone water (BPW), modified BPW, lactose (LAC) broth, and Universal Pre-enrichment (UP) broth. No differences were seen between the different pre-enrichment broths, while the two sample methods showed significant differences. The soak method detected significantly more *Salmonella*-positive cantaloupes ($P < 0.05$) than did the rinse method: 367/540 *Salmonella*-positive by the soak method and 24/540 *Salmonella*-positive cantaloupes by the rinse method. The soak method showed to be superior to the rinse method for the detection of *Salmonella* on cantaloupes. Hammack et al suggested that the low recovery of *Salmonella* by the rinse method may have been due to the formation of biofilms adhering to the surface of the melons. 'The surface of a cantaloupe is rough on both macroscopic and microscopic scales. One would expect the pathogen to penetrate such a surface to produce pockets of contamination that would not be readily dislodged through shaking. When soaked in a nonselective pre-enrichment medium, such organisms would be expected to grow out of their confinement and into the medium.'

<u>Conclusions</u>

With the information obtained after consulting some experts and literature, the following can be concluded:

- For analysing whole melons for the detection of *Salmonella* it is advisable to analyse the rind of the melon.
- Contamination on the rind of the melon may be heterogeneous. For example only the part which has touched the soil may have become contaminated, or the melon may have become contaminated by 'splashes' due to heavy rainfall, or by contaminated irrigation water. Therefore, if only one or more small parts of the rind (e.g. 25 g samples) are

analysed, there may be a risk for false negative results due to this heterogeneous contamination of the rind. To exclude this risk, it may be considered to analyse the whole rind (including the stem scar area and the opposite end) of the melon.

- A rinse procedure or a 'light' swab method may not be sufficient to remove *Salmonella* from the biofilm on the rind of the melons, resulting in possible false negative results.
- Insufficient information was found on the effectiveness of firmly rubbing the (whole) rind with a sponge. In any case, firmly rubbing whole melons may be very labor intensive.
- For analysing the whole rind, two methods may be of use: the 'soak' method as described by the BAM or by peeling off the whole rind (including the stem scar area and the opposite end) and pre-enrich either the whole melon or the whole rind. No information is available on possible differences in recovery of *Salmonella* between these two suggested methods. A disadvantage of peeling of the whole rind is the fact that it is more labor intensive and that it may give a higher risk for cross contamination (due to more intensive handling of the melon) than the soak method.
- It may therefore be considered to follow the BAM procedure for the sample treatment of whole melons (especially cantaloupes), followed by the procedure for detection of *Salmonella* as described in EN ISO 6579 (see below)

<u>Analysing whole melons (combination of BAM, 2011 and EN ISO 6579:2002)</u>

- For whole melons (**cantaloupes**), do not rinse even if there is visible dirt. Examine the cantaloupes 'as it is'.
- Place the cantaloupe into a sterile plastic bag. Add enough Buffered Peptone Water (BPW) to allow the cantaloupe to float. Normally this volume of BPW is 1.5 times the weight of the cantaloupe. For instance, a cantaloupe weighing 1200g may need a volume of approx. 1800 ml BPW. Add more BPW if necessary. Mix gently to make sure that the whole melon is moistened with BPW.
- Place the plastic bag, with cantaloupe and BPW, into a 5 liter beaker, or other appropriate container, for support during incubation. Make sure that the stem scar is immersed in the BPW during pre-enrichment. Allow the open-end flap of the plastic bag to 'fold over' so as to form a secure, but not air-tight, closure during incubation. Incubate the bag with its content at 37 °C ± 1 °C for 18 h ± 2h.
- After incubation, manually mix the contents of the bag containing the cantaloupe and the pre-enrichment broth.
- Perform selective enrichment, plating out and confirmation as described in EN ISO 6579: 2002. In short:
    - Transfer 0.1 ml of the pre-enriched culture to 10 ml RVS, incubate at 41.5 °C ± 1 °C for 24 h ± 3 h and transfer 1 ml of the pre-enriched culture to 10 ml MKTTn, incubate at 37 °C ± 1 °C for 24 h ± 3 h;
    - Inoculate XLD and a second selective plating-out agar medium from each selective enriched culture of RVS and MKTTn and incubate at 37 °C ± 1 °C for 24 h ± 3 h;
    - Confirm typical/suspect colonies from XLD and the second isolation medium.

Additional request from DG-Sanco (12-11-2012)
To which extent are the conclusions applicable to **watermelons** in particular?
Further specify the sampling protocol taking into account the rather huge size of such melons. It is also requested how to end up with a 25g sample from an import consignment of several tons.
Preferably the link to our food-safety-criterion of "Absence of Salmonella spp. in 25g" (5x) would be applicable. It would then be possible to proceed further with the protocol laid down in R 2073/2003.

How many samples to be analysed from a large batch of melons?
Regulation 2073/2003 does not give answers to this type of question for none of the products specified. The problem is most likely not different from other products delivered in large quantities (fruits, animal feed, vegetables,….). The probability of tracing a pathogen on a subsample from a very large batch of samples very much depends on factors like: Is the contamination homogeneously distributed over all products in the batch? Is the contamination homogeneously distributed per product? Is the contamination level sufficient high to detect it? Assuming a full homogeneous distribution of the contamination over all products and on each product of a large batch of products, the following can be calculated on statistical basis (ICMSF, 1986; Evers, 2001).
In case 300 samples are analysed and all are found negative, the probability of the presence of the pathogen in the whole batch of products is approximately <1%.
In case 100 samples are analysed and all are found negative, the probability of the presence of the pathogen in the whole batch of products is approximately <3%.
In case 10 samples are analysed and all are found negative, the probability of the presence of the pathogen in the whole batch of products is approximately <30%.

However, it is not very likely that *Salmonella* contamination is homogeneously distributed over all melons in one large batch, nor is it expected that each melon is homogeneously contaminated. Hence, analysing a few melons (e.g. 5-10) out of a large batch can result in a hypocrisy of the batch when the few tested melons are found negative for *Salmonella*. Therefore, testing of the end product (here the melons) only will give little to no information on the status of the batch of products. Information on the culture conditions of the products and its quality controls (e.g. quality of the soil, irrigation water, risk on contamination by wild life, etc) may be more informative (Park et al., 2012).

Analysing whole watermelons for Salmonella
As indicated earlier, when whole melons are contaminated with *Salmonella*, it is not likely that *Salmonella* is inside the melon as long as it is intact. Any contamination is most likely to be at the rind of the melon. Therefore, in case of analysing whole melons it is advisable to analyse the rind.
Also for watermelons it may be expected that the contamination is not homogeneously distributed over the rind and it would therefore be preferred to analyse the whole rind. However, analysing the whole rind by applying the ‘soak’ method, as described for cantaloupes, is more complicated for watermelons due to the size of the watermelons. But also analysing the whole rind by peeling it from the melon has some practical limitations as a whole rind of a watermelon (without adhering flesh) may still weigh 500 g or more. Preparing a 10 fold dilution in Buffered Peptone Water (as needed for the pre-enrichment step in the analyses of *Salmonella*) results in practical limitations with this size of sample (e.g. 500 g peeled rind in 4500 ml BPW).
It is therefore suggested to take several sub-samples of the rind of one melon, by using a cork borer, and to pool these sub-samples for further analyses. The sub-samples should be taken from these parts of the rinds where contamination is most likely (at stem scar, opposite end, place of the rind likely to have been in contact with the soil, places containing visible dirt, cracks, etc.)
Below a procedure for analysing a melon by taking (cork bore) sub-samples is suggested (derived from Annous et al., 2005 and Sapers et al., 2001).

Analysing pooled sub-samples of the rind of one melon

Mind: Take precautions during sampling to avoid cross contamination between different melons, e.g. use disposable gloves during treatment of a melon and change gloves between different melons. Also use sterile cork borers, knifes, cutting boards, etc for treatment of each melon and sanitize the materials between treatment of different melons.

- Take at least 10 sub-samples from the rind of a melon so that in total 100 cm^2 surface area of the rind is obtained. For this purpose a sterile cork borer with e.g. a diameter of 2,5 cm (surface area of 5 cm^2) can be used. With such a cork borer, 20 sub-samples need to be taken. If a cork borer is not available, use a sharp sterile knife to take the sub-samples from the rind, together with templates of 5 or 10 cm^2 to indicate the size of the cut per sub-sample.
- Take cork bore samples (or take sub-samples by using a knife at determined template surfaces) from different places of the rind (up to a total of 100 cm^2). Take at least samples at the stem scar and the opposite end of the melon. Take other sub-samples at other places of the rind most likely to be contaminated, e.g. place of the rind likely to have been in contact with the soil, places containing visible dirt, cracks or visible affected sites of the rind.
- By using a sterile knife, remove adhering flesh from the sub-samples. The flesh is not used for further analyses.
- Bring all rind sub-samples in one sterile container/bag.
- Measure the total weight of the rind sub-samples and add a volume of Buffered Peptone Water (BPW) equivalent to 9 times the combined rind weight;
- Mix the samples with BPW for 1 min e.g. by using a stomacher;
- Incubate the mixed sample at 37 °C ± 1 °C for 18 h ± 2h;
- After incubation, perform selective enrichment, plating out and confirmation as described in EN ISO 6579 (also see earlier).

Note:
When using a cork borer with a diameter of 2,5 cm (surface area of 5 cm^2) , one plug of rind (without adhering flesh) may weigh approximately 2,5-3 g (see picture in Annex 1).

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Annex 1 Pictures

Picture 1.1 Watermelon (total weight 3250 g)

Picture 1.2 Cork bore samples of the rind of a watermelon, including the stem scar and the opposite end (without adhering flesh)