STUDY OF AN OUTBREAK OF OFF-FLAVOURS IN BULK-TANK MILK

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Abstract

Off-flavours in milk can be classified using the following general categories: oxidized flavour, feed flavour, rancidity, and other flavours (e.g. unclean, malty, salty, flat or chemical). Off-flavours in milk occur at a low incidence in all milk-producing areas of Canada, with "outbreaks" occurring periodically in certain localities for no clearly defined reason. However, in the late 1990s, the incidence of off-flavours in bulk-tank milk was relatively high in Prince Edward Island (PEI). From the dairy company records it appeared that about 50/330, or 15% of herds, were affected during the winter season of 1999-2000. This presented a major economic problem for producers and dairy industry personnel, and attracted a degree of unwelcome media attention in PEI.

To evaluate the reliability of the method used for identifying off-flavours in milk, a sensory study was carried out with a panel of four milk-tank operators, who constituted the milk flavour quality control personnel. Results showed that the panelists had satisfactory agreement in differentiating off-flavoured milk from milk of good quality. The inter-panelist agreement ranged from substantial (Kappa statistic > 0.61) to almost perfect (Kappa statistic > 0.81), whereas the intra-panelist agreement range was moderate (Kappa statistic > 0.41) to almost perfect, suggesting that in the absence of a more objective diagnostic tool, a panel of trained milk graders was appropriate for the monitoring of the flavour quality of bulk-tank milk.

Results from clustering analyses revealed that this outbreak had not only a seasonal pattern, but also a limited geographical distribution with cases concentrated mostly in intensive dairy farming regions (Queens and Prince counties), and most importantly, a spatial-temporal pattern that usually peaked during fall – early winter months (September to January). Three high-rate space-time clusters (two composed of herds that experienced feed off-flavour and one composed of herds with rancid off-flavour) and two low-rate (areas with low rate of off-flavour occurrence) were identified. It appeared that high-rate clusters tended to receive more precipitation than low-rate clusters during the clustered time frame; temperature data were not as conclusive.

A case-control study was carried out using data collected for a 20-month period, to determine the herd-level factors associated with this sudden outbreak of off-flavours in PEI. It appeared that feed off-flavour was the flavour defect responsible for the outbreak, and that its occurrence was strongly associated with: feeding round-bale silage to lactating cows (OR = 11) or stored forage less that 2 hours before milking (OR = 253) or as a free-choice (OR = 3.2), and poor air quality in the lactating cows’ barn (OR = 41).

Gas chromatographic analyses and a trained sensory panel were used in an experimental study that included nine Holstein Friesian cows from three different PEI dairy herds to (1) develop a model that could reliably reproduce feed off-flavour under commercial dairy farming conditions, and (2) determine the volatile compounds either responsible for or associated with feed off-flavour. Results indicated that feeding lactating cows with freshly opened baled grass silage less than three hours before milking could trigger the development of feed off-flavour. They also showed that the occurrence of this off-flavour...
could be predicted by the concentration difference of either dimethyl sulfide or butane-2-one. On the other hand, results from gas chromatography-olfactometry analysis suggested that off-flavour was probably due to the concentration differences of a certain subset of volatile aromatic compounds, rather than the presence or absence of specific compounds.

In collaboration with Alpha MOS – France, a preliminary step of a feasibility study for the development of an instrument-based diagnostic assay was conducted with 28 milk samples (20 with disclosed off-flavour status, and 8 without) and using a trained sensory panel as a gold standard. Results showed that the developed gas-sensor array (aFOX system), which was successfully calibrated using one subset of the data (10 off-flavoured and 10 good quality milk samples), had a very high discriminative ability and repeatability in assessing the flavour quality of the remaining dataset. The classification of seven of the eight unknown samples by the aFOX system was a perfect match with that of the trained sensory panel. Although these results were satisfactory, additional work is yet to be performed for a full validation protocol.

In summary, the results from this research indicated that feed off-flavour was the driving force behind the sudden outbreak of off-flavours in bulk-tank milk in PEI dairy herds, and that gas-sensor array, commonly referred to as “electronic” or “artificial” nose was a promising technology for the future of the monitoring of flavour quality of not only bulk-tank milk, but also various processed dairy food products.
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This work would not have been possible without the collaboration of the administration of dairy processing companies ("ADL" and "PERFECTION") and the affiliated milk truck operators, and I would like to extend my appreciations to all of them.

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Dedicated To:

To my mother Awa, my sister Amina and Ghislaine
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<tbody>
<tr>
<td>AAC</td>
<td>Aroma-active compound</td>
</tr>
<tr>
<td>ADF</td>
<td>Acid detergent fiber</td>
</tr>
<tr>
<td>Alpha MOS</td>
<td>Alpha Multi Organoleptic System</td>
</tr>
<tr>
<td>ADL</td>
<td>Amalgamated Dairy Limited</td>
</tr>
<tr>
<td>ADSA</td>
<td>American Dairy Science Association</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemist</td>
</tr>
<tr>
<td>BP</td>
<td>Bound protein</td>
</tr>
<tr>
<td>AVC</td>
<td>Atlantic Veterinary College</td>
</tr>
<tr>
<td>BTM</td>
<td>Bulk-tank milk</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>DAFAF</td>
<td>Department of Agriculture, Fisheries, Aquaculture and Forestry</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-O</td>
<td>Gas chromatography-Olfactometry</td>
</tr>
<tr>
<td>HS-SPME</td>
<td>Headspace solid phase micro extraction</td>
</tr>
<tr>
<td>MSD</td>
<td>Mass spectrometry detector</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fiber</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen Radical Absorbance Capacity</td>
</tr>
<tr>
<td>PAF</td>
<td>Population attributable fraction</td>
</tr>
<tr>
<td>PEI</td>
<td>Prince Edward Island</td>
</tr>
<tr>
<td>P_neg</td>
<td>Proportion of negative agreement</td>
</tr>
<tr>
<td>P_pos</td>
<td>Proportion of positive agreement</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>S_e</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>S_p</td>
<td>Specificity</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra high temperature</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile organic compound</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1. Introduction

The Canadian dairy industry is the third largest sector of the national agri-food economy based on net farm cash receipts, and is only exceeded by grains and red meats [1]. In 2002, total net farm cash receipts from the dairy sector generated over $4.0 billion. During the same period, dairy products shipped from approximately 292 federally inspected processing plants were valued at $9.9 billion, representing 13.6% of all processing sales in the Canadian food and beverage sector. Moreover, for the dairy year (August 01st-July 31st) 2001-02, there were approximately 38,000 people working on 18,673 Canadian dairy farms, and roughly 26,000 other workers were employed in the 292 registered Canadian dairy plants [1]. This makes the dairy sector the second largest employer in the Canadian food industry, right behind red meats.

The economic growth and stability experienced by the dairy sector over the above years may be credited to multiple factors, among which are the growth in size of the average dairy farm and dairy processing plant, the diversification of processed dairy products of excellent quality, and the increasing demand of these products for both domestic and international markets. In order to achieve such success, the industry has had to make quality issues a high priority, as high quality milk results in increased yields of manufactured products with greater shelf life and improved organoleptic properties. This results in a product that meets the requirements of consumers who are increasingly demanding high quality foods that are wholesome, nutritious and safe. Also, with international trade in dairy products increasing, some governments demand that the
quality and safety of imported products meet or exceed their internal requirements; such expectations are sometimes used as non-tariff trade barriers. Consequently, milk products should not only be safe products for the public health, but also be both palatable and relatively resistant to bad flavours and losses of nutritive value.

On Prince Edward Island (PEI), just as in other Canadian provinces, quality control of milk and other milk products is subject to the regulations contained in the provincial Dairy Industry Act [2] (1998), and the responsibility for administration of these regulations falls to the jurisdiction of the provincial Department of Agriculture, Fisheries, Aquaculture and Forestry (DAFAF) and the local dairy processing and transportation companies. Because processed milk and milk products can only be as good as the raw materials from which they are made [3], the quality control process should start right at the farm, before the raw milk is transferred from the farm bulk tank to the truck’s tank.

The regulations stipulate that only persons issued with a milk grader’s certificate and tank truck operator’s license are allowed to grade, collect and transport milk. All milk tank truck operators are required to complete an approved course in milk grading and transportation [2]. Before transferring any milk to a tank truck, the milk tank truck operator must examine the milk in the farm’s tank for temperature, colour, flavour, odours, and foreign matter, and where he finds that the milk examined would not meet the standards outlined in the regulations, he cannot transfer any of the milk from the farm bulk tank to the tank truck. Good quality milk should be kept at a temperature between 1 to 4 °C, and be free of flavours, odours or colours that adversely affect its organoleptic...
characteristics.

The PEI DAFAR analyzes the milk from every dairy farm in the province on a regular basis for bacteria, protein, fat, somatic cell count, inhibitors and drug residues. In addition to monetary penalties and probation on shipping milk for a given period, no milk producer is allowed to sell or offer to sell milk with a somatic cell count in excess of 500,000 cells per milliliter on two successive monthly analyses or milk that has been adulterated with water or milk that has been contaminated with inhibitors or drug residues. The PEI DAFAR also has the mandate to analyze milk for bacterial content as often as deemed necessary, but at least twice a month. Milk containing in excess of 50,000 colonies per milliliter is not allowed for sale. The regulation stipulates that a milk producer whose milk exceeds this standard on two consecutive analyses in an official test period be penalized monetarily in addition to some prohibition of sale for various periods of any milk produced on the producer’s farm [2].

The main aspect of the quality control program for raw milk addressed in the present studies was the organoleptic assessment of milk, which undoubtedly plays a key role in the acceptance of milk and milk products by ordinary consumers. Bradfield [4] observed that taste was a determining factor for increase in milk sales. As mentioned above, milk with objectionable flavour (also known as off-flavoured milk) is not allowed to be used or processed in any way for human consumption. This point in the regulation is more a quality assurance matter than a food safety issue. In the past, off-flavour in milk has been a source of negative media publicity and subsequent economic losses to the dairy
companies and the dairy producers. About a decade ago, the province of New Brunswick (Canada) experienced an outbreak of off-flavour in raw milk [5;6] that attracted nationwide media attention. Coincidentally, reports from the Canadian Dairy Industry Profile [7] indicate that during the same period of time (1982 – 1992), fluid milk products were losing ground in the competition with other beverages such as bottled waters, juices and soft drinks.

Milk of good organoleptic quality is a highly nutritious food that has a pleasing, slightly sweet taste, with very little odor and no unpleasant aftertaste; it also has a smooth and rich feel in the mouth. Because of its relatively bland nature, it is particularly susceptible to aroma defects either in the presence of minute quantities of abnormal constituents or in case of some variations in the concentrations of compounds responsible for its flavour. The phenomenon of off-flavours in milk has been extensively studied through both experimental and observational studies [8-13], and published research reports and reviews [14-18]. Successful control of milk off-flavour outbreaks in most cases depends on correct identification of the flavour defect involved. The committee on Flavour Nomenclature and Reference Standards of the American Dairy Science Association (ADSA) published a comprehensive review on off-flavours in milk and their nomenclature [19]. They developed scorecards for sensory evaluation of dairy products and a practical classification system to aid in training of both research and quality control personnel. The scorecards (Table 1-1) contain a list of flavour criticisms occurring in milk and other products. Table 1-1 gives a summary of the categories of flavour defects in milk using terminologies that reflect the mechanism involved, along with associated
terms generally employed to describe specific characteristics of off-flavours in each category. I will, in the present chapter, review in detail the major flavour defects affecting raw and pasteurized milk ("transmitted", "oxidized", "lipolyzed" and "microbial" off-flavours), followed by a brief overview of two of the flavour defects that are exclusively found in pasteurized milk ("light-induced" and "heated" off-flavours). In recent years, there have been notably fewer publications on off-flavours in milk. Thus, many of the references in the present chapter, which are still as relevant now as then, are twenty or more years old.

1.2. Transmitted off-flavours

The flavour defects in milk represented in this group include any objectionable flavour generated by the transfer of volatile odour-active substances (from the cows' feed and/or environment) into the milk while it is still in the udder of the cow. These off-flavours are generally of less importance economically (not usually occurring as an "outbreak," but rather an individual farm problem). The terminology used for the description of these off-flavours is self-explanatory, as it outlines the suspected source of the defect: "feed," "weedy," "cowy," "and barny" [19]. It is common knowledge that highly volatile anesthetic agents, such as ether or chloroform, can be found in milk collected from animals shortly after anesthesia. Babcock [20] reported that the inhaled odours of wild garlic tops could be detected in the milk, demonstrating the physiological mechanisms of the development of these off-flavours in milk. Additionally, in a series of experiments with cows placed in a specifically designed tent, cows were allowed to inhaled odours or
vapours of various substances (among which were turpentine, benzaldehyde, camphor, vanillin, garlic, onions, manure, corn and alfalfa silages) [21]. Subsequently, the cows were taken to the milking barn after two hours where they were milked and the milk was then submitted to a panel of 3-5 experienced milk graders who established that inhaled odours of manure, crushed garlic, and silages imparted an objectionable feed flavour to the milk. Dougherty and co-workers [10] used ruminal and tracheal fistulas in a series of experiments to determine the depth of penetration of eructated gases into the lungs and the effects of these gases on the flavour of the milk. These studies established that there were two main pathways through which aroma-active substances could gain entrance to the blood (Figure 1-1). Odoriferous substances from some feeds or from the environment surrounding the cows can be breathed into the cow’s lungs, from where they are carried to the udder via the bloodstream (respiratory pathway), or absorbed by the blood from the digestive tract, and then transmitted to the udder (digestive pathway). Petersen and Brereton [21] mentioned a third alternative, which is the passage through the skin from contacted substances. These reports also contend that blood was providing a “two-way” street for transportation of flavoured substances, although this was not substantiated with hard evidence. They suggested that when the source of the off-flavour was removed from the cows’ environment, the volatile materials were transferred from the milk back to the bloodstream and exhausted via the lungs.

1.2.1. Feed off-flavour

Feed off-flavours in milk are undesirable flavour defects usually detected in freshly
collected milk from cows that consume certain types of feed or inhale their aroma. These off-flavours are different from those resulting from the action of potential microbial contaminants, chemical changes occurring during storage, or even those resulting from handling before, during and after processing [22]. The association between feed consumed by the cows and the flavour quality of milk was first reported by Bradley in 1757 (as cited by Dougherty et al. [9]). He indicated that milk produced in an area near London (England) where cows were fed beet and turnip tops and roots had a bitter flavour. A couple of centuries later, works of a number of researchers [9;20;23;24] corroborated the theory that feed and environment are primary factors associated with off-flavours in raw milk.

Feeds that are known to be capable of producing off-flavours in milk include fermented silage (corn, legume, grass or mixed), green forage and hay. Abruptly changing feeding practices were also reported as possible cause of “feed” off-flavour[25], particularly during the spring transition when the cows change from barn feeding with dry winter rations to lush green pasture forage.

A wide variety of organic molecules has been associated with the development of “feed” off-flavours: some researchers [26-28], studying the volatile constituents of grass and corn silage, found mixtures of methyl sulfide, aldehydes, ketones, alcohols, and simple methyl, ethyl, and propyl esters.

General recommendations to avoid the development of “feed” off-flavour in milk have
included: identifying and eliminating feeds likely to result in feed off-flavour, or preventing cows from getting access to these feeds for the few hours prior to milking; providing adequate ventilation in the barn housing these cows; and considering a gradual change in feeding practices during spring transition rather than abrupt changes [25].

1.2.2. Weed off-flavour

Weed (or weedy) flavour in milk can arise when cows are allowed to graze weed-infested pasture, or if they are fed hay or silage containing certain weeds. The most common and readily recognized off-flavours in this group have been reported to be those from wild garlic, onion, and related plants; they indicated that off-flavours from some weeds persist for as long as 12 hours after they are consumed [18;19]; consequently, such weeds must be kept out of a cow’s ration.

A number of authors [29-31] have presented summaries of taints found in milk resulting from pasture weeds, and emphasized that benzyl mercaptan, allylisothiocyanate, pulegone and indole were the compounds responsible for off-flavour generated by land cress, penny cress (also known as French weed or stinkweed), penny royal, and pepper grass, respectively. In the case of onion/garlic, di-n-propyl sulfide, isopropyl mercaptan, and poionaldehyde were indicated as the compounds responsible for the weed taint.
1.2.3. **Cowy off-flavour**

Off-flavours resulting from a variety of mechanisms fall in this category. Metabolic disturbances of the cow may result in the production of milk with a flavour defect characterized as "cowy" or "unclean." Josephson and Keeney [32] observed that the odor of the breath of ketosis-affected cows was found in the milk produced by these cows, and that this odour was sometimes so strong (in severe cases of ketosis) that it could be transmitted to the milk of neighboring cows if the barn was not adequately ventilated. They contend that high concentrations of acetone bodies from incomplete metabolism of fat found in the blood of cows with the disease were responsible for the objectionable flavour defect. Later, Potts and Kesler [33] reported that acetone contained in the cows feed, such as silage, could impart a similar flavour defect.

Besides ketosis (acetonemia), mastitis has been reported as the other source of off-flavour organoleptically characterized as "unclean" or "salty" [19]. It was reported that other causes of cowy flavour were improper preparation of the cow’s udder before milking and poorly cleaned milking equipment [25]. Patton and co-workers [34] compared the vapour phase chromatogram of a volatile substances from milk trapped in a 1% aqueous HgCl₂ solution with that of authentic methyl sulfide and concluded that although methyl sulfide may contribute significantly to the flavour characteristics of normal milk, in concentrations greater than threshold values, it can also impart a cowy off-flavour to milk.
1.2.4. Barny off-flavour

Inhalation of odors by lactating cows housed in a poorly ventilated environment results in a flavour defect known as “barny”. The mechanism of odour transmission is similar to that described for feed off-flavour in Figure 1-1 (respiratory pathway). The nature of “barny” off-flavour remains unclear and it may be difficult to distinguish this off-flavour from “cowy-like” flavour [19;35]. However, it is clear that it occurs mostly in farms with cows housed in a contaminated environment, coupled with poor ventilation.

1.2.5. Chemical flavours

This group of flavour defects, which sometimes is classified under transmitted off-flavours, is usually not considered as serious as others described above, because of their isolated occurrence. They are caused by the contamination of milk by a variety of chemical agents contained in the formulation of products frequently used in dairy farms: medications, insect sprays and herbicides [36], cleaners, disinfectants and sanitizers [37;38]. For instance, milk flavour alterations due to the presence of chlorinated and iodized compounds have been the most frequently reported [39-41], as compared to phenol compounds (from disinfectants and pesticides), which are only occasionally incriminated in off-flavour in milk [42].
1.3. Oxidized off-flavour

These flavour defects have been extensively studied [5;43-47] because of their economic importance. They are prevalent in Northern climate areas where cattle are housed for a significant proportion of the year and can occur as an “outbreak” where many herds are affected over a prolonged period. In fluid milk, “oxidized” off-flavour arises from auto-oxidation (by molecular oxygen) of unsaturated fatty acids in the phospholipids of the milk fat globular membrane [5;14;48]. A number of researchers [49;50] have indicated that the concentrations of unsaturated fatty acids in the phospholipids, which are concentrated in the outer layer of the fat globule, were higher than in the fat globule itself, hence more susceptible to oxidation. Additionally, pro-oxidation catalytic agents such as copper, iron, sulphur and cobalt, are concentrated at the surface of the fat globule [50-54]. Iron or copper concentrations in milk as low as 0.3 ppm and 0.01 ppm, respectively, have been reported to be capable of inducing lipid oxidation [55]. Ascorbic acid has been reported to have an anomalous behaviour in relation to oxidized flavour [56]. Riel and Sommer [43] indicated that at concentrations above those in normal milk, ascorbic acid acts as an antioxidant, but at lower concentrations it is a pro-oxidant, while Haase and Dunkley (as cited by Kanner and co-workers [56]) explained the paradoxical anti- and pro-oxidant effects of ascorbic acid by the relative concentration ratios of ascorbate over copper. They noted that ascorbic acid was acting as an anti-oxidant when ascorbate was 10-fold higher than the concentration of copper, whereas at lower ratios, its effect was pro-oxidative.
Oxidized flavours are described by various sensory attributes such as “cardboard,” “metallic,” “fishy,” “tallowy,” “painty,” or “oily,” indicating the great variability of the predominant flavour characteristic and the degree of oxidation [57]. A number of publications describe high variability in the susceptibility of milk to oxidation [48;58;59]. Interestingly, the variability appears to be as much between-cow as between-herd [60-62], perhaps suggesting a genetic predisposition to oxidized milk. Depending on this susceptibility to oxidation, the following classification was proposed by a number of authors[14;63]:

- **Spontaneous milk** – which can develop “oxidized” flavour without any exposure to the potential catalysts (iron or copper);
- **Susceptible milk** - which can develop “oxidized” flavour only if it is contaminated with copper or iron;
- **Non-susceptible milk** - which does not develop “oxidized” flavour at all, even in the presence of iron or copper.

The factors associated with spontaneous oxidation in milk are: type and quality of feed, age of the cows and stage of lactation. Poor quality feed and feeding added unsaturated fats have been linked to oxidized off-flavour [64-67]. Charmley reported that in problem herds in a study of a New Brunswick outbreak [5], 45% of first lactation heifers produced oxidized milk as opposed to 29% of older cows, although other researchers (cited by [18]) did not observe an age prediction. King and co-workers [68;69] attributed a higher susceptibility of milk from first lactation heifers to oxidation to an increased content of copper. They also claimed that copper concentration in milk was highest in early stage of
lactation, followed by a rapid decrease during the first few weeks, then a slow decrease for the remainder of the lactation period.

1.3.1. **Mechanism of development of “oxidized” off-flavour.**

Oxidized off-flavours arise from the oxidation of polyunsaturated fatty acids in the phospholipids at the interface of the milk fat, which leads to hydroperoxide formation and decomposition of the phospholipids, respectively. It is a three-stage chain reaction, which starts with an initiation of the reaction, followed by propagation and termination (Figure 1-2). Lipid hydroperoxides, resulting from the auto-oxidation of unsaturated fatty acids by the free-radical chain reaction, are flavorless and very unstable. In the termination phase, these hydroperoxides break down to produce short chain volatile compounds of the following classification (Figure 1-3): aldehydes, alcohols, ketones, acids, hydrocarbons, lactones, furans and esters [48;70-73]. Because unsaturated aldehydes and ketones have the lowest sensory thresholds, they are most often credited with being responsible for oxidized flavours [57;74-77]. Charmley [5] contended that mixing oxidized milk with good quality milk may result in oxidation of the latter, as the oxidation reaction is self-catalyzing.

Although there is no simple control strategy for the problem of oxidized flavour in milk, while waiting for corrective measures (which consist of reviewing all the potential associated causes one by one until the problem is solved) to take effect, possible short term solutions could include daily shipping of the produced milk for immediate
processing. Because the susceptibility of milk to oxidation depends on the vulnerability of the fat globule membrane, it is essential to apply appropriate management and nutritional measures to ensure strong resistance of this membrane to catalytic or mechanical alterations. Charmley [5] suggested a list of recommendations that include:

- Checking the milking system for excessive air leaks and fat (and other) deposits, milk agitation and foaming.
- Checking the iron, sulphur, and copper levels in the water supply used to wash and sanitize the milking equipment (concentrations above 1 mg per liter are considered high).
- Considering the use of iodine rather than chlorine sanitizer, as iodine is less likely to precipitate metals versus chlorine.
- Checking the cows’ diet for elevated amounts of added (unsaturated) fat and considering substituting with a more saturated fat source as discontinuing the feeding of added fat may have a negative effect on the production.
- Vitamin E/selenium supplementation, followed by subsequent substitution of stored forages with fresh forages if available. Fresh forages contain high levels of vitamin E, which in combination with selenium contribute to the protection of phospholipid membranes from oxidative processes.
- Assessing the body condition and milk of individual cows for evidence of oxidation and identifying “problem” cows that appear to be producing susceptible milk. If cows appear to be losing excessive body
weight in early lactation (i.e. greater than one unit of condition score),
then the ration should be re-evaluated and corrective measures
implemented immediately. Drying off late lactation problem cows or
diverting their milk away from the bulk tank may lead to resolution of
the problem.

Considering selling the "problem" cows if deemed necessary.

Although homogenization and pasteurization have been shown to have an inhibitory
effect on the development of oxidized flavour in milk, there is less agreement regarding
the mechanism by which this occurs [52;78-80], and in many cases milk is already
affected before it can be processed.

1.4. Rancid off-flavour

The term “rancid” in the field of dairy science is used to characterize off-flavour that
results from enzymatic hydrolysis of the milk fat triglycerides rather than fat oxidation
[19]. This flavour defect is also sometimes termed “butyric,” “soapy,” “goaty,” or
“bitter.” The enzymes responsible either originate from the milk itself [endogenous
lipoprotein lipase (LPL)], or are produced by psychrotrophic bacteria [(with
* pseudomonas species (particularly *P. fluorescens* and *P. fragi*)] constituting the largest
proportion in raw milk][81]. Deeth and Fitz-Gerald [81] speculated that unlike the natural
milk lipase (LPL), most lipases of microbial origin are capable of hydrolyzing the
triglycerides in non-disrupted milk fat globule membranes. They indicated that it was not
known whether this was due to the penetration of the membrane by the lipases or whether the membrane was first disrupted by other enzymes such as the phospholipases.

In normal conditions, this enzymatic reaction is not possible, as the milk fat globule membrane protects the triglycerides. However, if the integrity of this membrane is disrupted, the natural lipases come into contact with the triglyceride substrate and induce the hydrolysis of esterified fatty acids [82]. Consequently, a “rancid” flavour defect can be induced anywhere from milk harvesting until the time of pasteurization. Willey and Duthie [83] indicated that depending on the mechanism of flavour development, there were two types of “rancid” flavour defect: “unclean” flavour, resulting from foaming or spontaneous lipolysis, and “sickening” flavour, which can result from mixing raw milk with homogenized milk and also from intense agitation during milking, or temperature fluctuations such as cooling milk, warming it to about 86 °F (30 °C) and then promptly cooling it again [63;81]. Any process that alters the membrane, such as homogenization, agitation, foaming, and cooling will accelerate rancidity.

In the 1950’s there was a marked increase in the incidence of rancid off-flavour that Thomas (as cited in [15]) attributed to the widespread installation of the bulk tanks and pipeline milking systems. Since then, improvement of the technology and changes in the design and operation of this equipment has significantly contributed to the control of rancid flavour in milk of this origin. However, milk from some cows has been reported to be highly susceptible to so-called “spontaneous” lipolysis [16;81]. Factors such as late lactation and/or low milk yield [16;84-86], estrus [16], and changes in the mammary
glands due to subclinical mastitis [87;88] have been reported to be potentially associated with spontaneous lipolysis. It has been speculated that as stage of lactation advances, the fat globule membrane weakens progressively due to the changes in the make-up of phospholipid available for the formation of the membrane [5;86;89;90], hence increasing the susceptibility of milk fat globules to enzymatic degradation. However, Weaver and Bachmann (as cited in [81]) have suggested with no proposed mechanism that stage of pregnancy was the critical factor leading to susceptibility to spontaneous lipolysis rather than advanced lactation. Numerous other authors [85;91;92] pointed out that increased susceptibility of milk from late lactation cows due to enzymatic degradation is not necessarily due to the stage of lactation or pregnancy, but most likely to poor quality feed that is generally fed to these cows. Jellema [85] and Jellema and Schipper [93] substantiated this argument by stating that in many countries where rancid flavour defect in milk is often a problem, a relatively high percentage of cows usually get to their late stage of lactation during the period of the year when there is no (or inadequate) pasture feeding. Also, excessive dietary energy intake has been [94-97] linked with softening and structural changes of the milk fat, whereas insufficient energy intake was reported to be associated with weakening of the fat globule membrane, respectively, hence increasing the vulnerability to lipolytic mechanical alterations.

Dairy rations containing increased amounts of raw protein have been shown to encourage the incorporation of long-chain unsaturated fatty acids in the milk fat (as cited by Kirst [94]). Lactating cows with energy-deficient diet mobilize long-chain unsaturated fatty acids from the body fat, and the rate of metabolism of these fatty acids is slower than that
of the short-chain saturated ones (for energy supply), thus a considerable amount of long-chain fatty acids enters the udder. Fat supplements containing fatty acids such as palmitic \((\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H})\) and/or myristic \((\text{CH}_3(\text{CH}_2)_{12}\text{CO}_2\text{H})\) acids have been reported to promote spontaneous lipolysis as well; whereas stearic acid \((\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H})\) and fatty acids with chain length shorter than myristic acid were found to not promote lipolysis [98]. Detailed review of the effect of feed and nutrition on susceptibility to lipolysis is provided by Jellema [85].

Lipase-producing microbial contaminants (psychrotrophic bacteria: \textit{Pseudomonas} and \textit{Acinetobacter} species) in milk are capable of producing an off-flavour with a "bitter" characteristic, which is in part a result of elaboration of proteolytic enzymes that subsequently induce protein degradation [99-103]. Therefore, the term "bitter," when used to describe rancid milk, may reflect proteolysis which is occurring in concert with lipolysis.

The compounds responsible for "rancid" flavour in milk are believed to be the milk fat triglycerides derived from the \(\text{C}_4\) through \(\text{C}_{12}\) even-numbered fatty acids, specifically: butyric, caproic, caprilic, capric, and lauric acids [75;104;105]. A comprehensive review of the "rancid" flavour defect in milk is provided elsewhere [3;71;90].

Control measures for hydrolytic rancidity include those listed for oxidized off-flavour, because any physical or oxidative damage to the fat globule membrane may result in the
liberation of the fat globule itself, which becomes exposed to the lipases. In addition to these measures, it is recommended [5]:

- to ensure appropriate installation, use and maintenance of the milking equipment (agitator, compressor, and milk line),
- to dry off cows relatively early, in order to avoid milk from cows in advanced lactation that have been reported to be more susceptible to rancidity, and
- to balance the cows’ ration according to their production level.

1.5. Microbial (bacterial) off-flavours

Milk is a highly favourable culture medium for microbial life because of its chemical composition, which is rich in various nutrients (protein, carbohydrate, fat, minerals, vitamins), and because of its water content [106]. Thus it is highly vulnerable to contamination that can occur at any stage of production, processing or marketing. Many undesirable changes in the sensory quality of milk (raw or pasteurized) are possible when environmental conditions are conducive to microbial proliferation and enzyme activity [107;108]. Bacterial growth, contamination with bacterial metabolic products, and enzyme secretion are the most common means by which microorganisms produce off-flavour in milk. Microbial enzymes are known to be stable to heat treatment, hence surviving pasteurization and sterilization processes [31;109]. Three different off-flavours are generally described as microbial off-flavours: Acid, malty and fruity flavours.
1.5.1. Acid flavour

Lactic flora (*Streptococcus lactis*, and *Streptococcus cremoris*), being predominantly mesophilic in character, are one of the main natural contaminants of milk [110;111]. The defect generated by these microorganisms is described as sour, and it has been reported by Sommer, Van Slyke and Baker (as cited by Bassette and others [18]) to be not due to the formation of lactic acid (which is barely volatile because of its low vapour pressure), but rather to the presence of volatile organic compounds such as acetic acid, propionic acid, formic acid, acetaldehyde, acetone, diacetyl, and acetyl methyl carbinol. This off-flavour can develop in milk if it is not cooled immediately to 4.4° C or below after collection [111;112].

1.5.2. Malty flavour

Malty flavour, also known as “caramel,” “cooked,” “burnt,” or “grapenut-like,” occurs occasionally in milk contaminated with *Streptococcus lactis* subsp. *maltigenes* [113;114] or *Lactobacillus maltaromicus* [18;108;115;116], followed by a period of storage of the milk at 10° C or above. The volatile compounds produced by *Streptococcus lactis maltigenes* include a variety of aldehydes and alcohols deriving from amino acids. However, the characteristic “malty” aroma and flavour have been reported to be due to 3-methylbutanal derived from leucine [108;117-119] (Figure 1-4).
1.5.3. Fruity flavour

This flavour defect in milk, also described as strawberry-like or ester-like, has been traced back to the presence in milk of *Pseudomonas fragi*, a psychrotrophic gram-positive bacteria that produces a lipase that initially hydrolyzes short chain fatty acids from the milk lipids and then esterifies them to their corresponding ethyl esters. Ethyl butyrate and ethyl caproate, which are the products of the lipolytic esterification of butyric and caproic acids, have been reported to be responsible for “fruity” off-flavour in milk [19;102;104]. Hosono and collaborators [120;121] elucidated the presence of an esterase in crude cell extracts from *Pseudomonas* cells in milk capable of esterifying butyric and caproic acids with ethanol. Figure 1-5 suggested by Morgan [115] summarizes the chemical reactions involved in the formation of ethyl ester by *Pseudomonas fragi*.

Using sensory analysis alone for milk quality control would identify only the flavour defects described as “acid”, “malty” and “fruity” as off-flavours of microbial origin; whereas other defects such as “stale,” “barny,” “bitter,” “rancid,” “unclean,” or even “feed” may well be of microbial origin, but because of the similarities with off-flavours of other sources, the determination of actual causes is possible only if additional bacteriological analyses are performed [18;107]. Rapid cooling and holding raw milk at 4.4 °C or below would prevent the proliferation of such contaminants, thus maintaining the flavour quality of raw milk until pasteurization. Since pasteurization ensures the destruction of all vegetative pathogens, and a considerable percentage of non-pathogenic
microorganisms present in milk, the development of acid flavour following pasteurization is unlikely; therefore, if any flavour defects of microbial origin are detected in pasteurized milk, it is likely to have occurred either at the earlier stage of production or during post-pasteurization period through contamination with psychrotrophic bacteria [120-124], which are capable of growing at 7°C and below [125]. Although the microorganisms responsible for these flavour defects are destroyed by adequate pasteurization, the volatile products of their metabolism are not affected by this process [18;19], thus making the development of microbial off-flavours irreversible. Generally, off-flavours caused by the growth of bacteria in milk are not detectable until large numbers of bacteria (10⁶ per millimeter and above) are present [124]; such milk would not meet legal standards for bacterial quality in Canada and other developed dairy nations.

1.6. Unclean, bitter and putrid flavours

These categories of flavour defects are often detected in pasteurized milk only, and certain gram-positive, heat-resistant psychrotrophic bacteria are responsible for their development. The spoilage of milk may occur during their growth or indirectly through the involvement of specific enzymes that these microorganisms produced [126]. Schroder [127] reported that two groups of extra-cellular enzymes were of major importance in this process: proteinases and lipases that could act directly on micellar casein or on the fat globules in milk, thus producing the characteristic unclean, bitter, or putrid flavour. Increases in the free fatty acid concentrations in stored raw milk in which psychrotrophic
bacteria multiplied have been reported [128], which suggests that the enzymatic processes of casein and milk lipid degradation may well also take place in raw milk while it is being stored, and then continue after heat-treatment.

1.7. Light-induced off-flavour

Given the current conditions of raw milk production and transportation (stainless steel pipelines and holding tanks), light-induced off-flavour is unlikely to be a problem for raw fresh milk. It is more of a concern for pasteurized milk and fluid milk products. This problem used to be very common when milk was bottled and distributed in clear glass containers (often because it was usually left on consumers’ doorsteps, exposed to the sunlight). Changes of packaging material (plastic-coated paper) and distribution limited through supermarkets nearly eliminated this problem for many years. However, dairy products in translucent packages (e.g. Plastic bags) in the supermarkets are exposed to fluorescent lighting which can trigger a series of chemical reactions leading to the development of light-induced off-flavour during retail storage and display [129-132].

These flavour defects consist of two distinct components, each leading to the development of a particular off-flavour: the primary and predominant off-flavour, also known as a sunlight or activated flavour, has been attributed to protein degradation and is usually described as burnt, burnt feather or protein, cabbage or cooked cabbage, and mushroom; whereas the second off-flavour, which becomes pronounced only 2-3 days later [19] has been reported to be due to the so-called photo-induced lipid oxidation [133-
136]. This off-flavour is usually described as cardboard-like or metallic. Further details on these flavour defects are available elsewhere [137-143].

1.8. Deficiencies in the traditional approaches to milk off-flavour problems

Due to the differences in flavour quality control policies in different milk-producing regions, it is difficult to obtain actual data on the incidence of off-flavour problems worldwide. While it is possible to have relatively accurate estimates in countries where milk flavour quality control is a mandatory routine process, this is nearly impossible for countries or dairy regions where it is absent or practiced on a random basis. The nationwide awareness raised by the off-flavour outbreak in New Brunswick in the mid-eighties [5] led to the introduction of systematic flavour quality control in New Brunswick and Prince Edward Island. Currently, only a few Canadian provinces (PEI, Alberta and Manitoba) rigorously follow this program at every pick-up; all the other provinces (including New Brunswick) randomly check the flavour quality of a given proportion of farms on a monthly basis (Personal communications with the New Brunswick and Nova Scotia Milk Marketing Boards). It is important to mention that, regardless of the mode of application, industry-based flavour quality control programs of raw milk are usually carried out as a screening test where milk is classified as good or tainted. Conversely, government-based institution’s such as the PEI Milk Quality Laboratory, which performs parallel testing, also classify the type of off-flavour defect involved.
To date, no off-flavour outbreak has been investigated using controlled epidemiological procedures, even though this would seem to be an ideal approach in outbreaks. The traditional approach used in outbreak situations has been to attempt to classify the off-flavour based on subjective assessment (i.e. to rely on anecdotal field work and past experiences, with little standardization in the response to an off-flavour problem), followed by the application of remedial actions using “trial and error” (such as the removal of feed, vitamin E supplementation, checking milking equipment) until the problem resolved [5]. With regard to the PEI outbreak described herein, it was believed by both the dairy producers and the dairy industry that feeding cows forages made from grass harvested from fields that have been previously used for potato farming for relatively a long time (at least three consecutive years) was the main cause of the off-flavour in milk. Copper contamination was also incriminated, although copper piping has been substantially eliminated for sometime from water supply or milking systems in PEI dairy farms.

The general lack of an established systematic approach to solving milk off-flavour outbreaks provided the impetus to embark on the studies described in this thesis.

1.9. Overall objective and scope of the thesis.

The present research project was established with the objectives of investigating the reliability of organoleptic testing for off-flavours, the major reported off-flavours that
were involved in the increase in milk rejection from dairy farms in PEI, and the
associated risk factors for those off-flavours. At the time of project initiation, the industry
was unable to identify the cause(s) or the factors associated with the sudden outbreak of
reported off-flavours in raw milk in PEI. It was intended to approach the problem in a
methodical fashion applying appropriate epidemiological techniques to identify farm-
level factors for off-flavours and to propose further investigations to specifically identify
the cause and suggest remedial actions. Seven issues were addressed within the
framework of this study:

- Evaluation of inter- and intra-rater reliability of the sensory panel used
  for the assessment of organoleptic quality of raw fresh milk (Chapter 2).
- Investigation of geographical and temporal associations for this
  outbreak over a 20-month period in PEI (Chapter 3).
- Description of reported off-flavours of major importance in the
  observed outbreak (Chapter 4),
- Identification of the associated risk factors of reported off-flavours
  (Chapter 4).
- Determination of the volatile compounds associated with these major
  off-flavour(s) (Chapter 5).
- Development of a model to predictably reproduce the major off-
  flavours involved in this outbreak (chapter 5).
- Development of a diagnostic assay, either as an alternative to sensory
  panels or a complementary tool, for the detection of off-flavours in milk
  (Chapter 6).
1.10. Reference List


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Table 1-1. Flavour criticisms from the ASDA scorecard and associated sensory characteristics.

<table>
<thead>
<tr>
<th>Causes</th>
<th>Descriptive or associative terms</th>
<th>Sensory characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRANSMITTED</td>
<td>Feed</td>
<td>Readily detected by odor; taste of silage, alfafa and brewers grains disappear quickly.</td>
</tr>
<tr>
<td></td>
<td>Cowy (acetone)</td>
<td>Detected by smell and taste; cow’s breath-like odor and medicinal aftertaste.</td>
</tr>
<tr>
<td></td>
<td>Barny</td>
<td>Detected by smell and taste; leaves unclean aftertaste after expectorating; associated with a foul smelling environment.</td>
</tr>
<tr>
<td></td>
<td>Garlic/Onion (weedy)</td>
<td>Pungent odor and persistent aftertaste; bitter, characteristic of the weed</td>
</tr>
<tr>
<td>OXIDIZED (metal-induced)</td>
<td>Papery, cardboard</td>
<td>Detected by taste when intense by odor; cardboard-like, metallic, painty, fishy, puckery, copper penny on tongue.</td>
</tr>
<tr>
<td>LIPOLYZED</td>
<td>Rancid (lipolyzed)</td>
<td>Detected by smell and taste; fatty acid odor, soapy, bitter, sour, unclean aftertaste, puckery in mouth; persistent</td>
</tr>
<tr>
<td>MICROBIAL</td>
<td>Acid</td>
<td>Detected by smell and taste; imparts tingling effect to tip of tongue.</td>
</tr>
<tr>
<td></td>
<td>Malty</td>
<td>Detected by smell and taste; malt or grapenut-like flavour</td>
</tr>
<tr>
<td></td>
<td>Fermented /fruity</td>
<td>Readily detected by odor; characterized by a vinegar or fruity taste and odor.</td>
</tr>
<tr>
<td></td>
<td>Bitter*</td>
<td>Detected by smell and taste; develops slowly on back of tongue; persists.</td>
</tr>
<tr>
<td></td>
<td>Unclean (psychrotrophic)</td>
<td>Detected by smell and taste; extreme staleness, mustiness, putrid odor, closed barn odor; objectionable aftertaste.</td>
</tr>
<tr>
<td></td>
<td>Oxidized</td>
<td>Detected by smell and taste; burnt feathers, cabbagey, chemical-like odor and taste</td>
</tr>
</tbody>
</table>
**Heat-induced**

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooked, caramelized, scorched</td>
<td>Detected by both smell and taste; includes sulfurous, heated or rich, caramelized and scorched flavours.</td>
</tr>
</tbody>
</table>

**Miscellaneous**

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat</td>
<td>Detected by smell and taste; mouthfeel, watery, lacks characteristic sweetness.</td>
</tr>
<tr>
<td>Astringent</td>
<td>Normally detected after milk is expectorated; the tongue and lining of the mouth tend to feel shriveled, almost puckered.</td>
</tr>
<tr>
<td>Foreign</td>
<td>Detected by smell and taste; characteristics differ with the causative agent; i.e., farm chemicals, fumes, insecticides, and medication.</td>
</tr>
<tr>
<td>Lacks freshness (stale)</td>
<td>Lacks identifiable sensory characteristics; precursor to other, more objectionable off-flavours.</td>
</tr>
<tr>
<td>Salty</td>
<td>Detected by taste; salty, cleansing feeling to mouth.</td>
</tr>
</tbody>
</table>

* Bitter flavour may arise from a number of different causes (lipolyzed, microbial or other). If the specific cause is not known, it should be classified under miscellaneous.
Figure 1-1. Mechanism of transmission of odoriferous substances from feeds or environment to the milk in the udder

(A) Respiratory pathway:

| Feed/air | Cow’s nose/mouth | Lungs | Blood | Udder | Milk |

(B) Digestive pathway

| Feed | Cow’s digestive tract | Blood | Udder | Milk |
Figure 1-2. Mechanism of Auto-oxidation of lipids in milk (Deman, 1980).

<table>
<thead>
<tr>
<th>(I) Initiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( RH + {^1}O_2 \rightarrow R^* + H^* )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(II) Propagation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R^* + O_2 \rightarrow ROO^* )</td>
</tr>
<tr>
<td>( ROO^* + R'H \rightarrow ROOH + R^* )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(III) Termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R^* + R^* \rightarrow R - R )</td>
</tr>
<tr>
<td>( R^* + ROO^* \rightarrow ROOR )</td>
</tr>
<tr>
<td>( ROO^* + ROO^* \rightarrow (ROO)_2 )</td>
</tr>
</tbody>
</table>

Where:  
\( H^* = \) Hydrogen  
\( {^1}O_2 = \) Singlet oxygen (= Active species involved in initiating the reaction)  
\( RH (R'H) = \) Unsaturated fatty acid;  
\( R^* (or R''^*) = \) Lipid radical;  
\( RO^* = \) Alkoxyl radical  
\( HO^* = \) Hydroxyl radical  
\( ROO^* = \) Lipid peroxy radical

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Figure 1-3. Formation of aldehydes, alcohols and ketones from the decomposition of hydroperoxides (Deman, 1980).

(A) Decomposition of hydroperoxide

\[
R - \text{CH (OOH)} - R \rightarrow R - \text{CH} - R + \cdot \text{OH}
\]

(B) Aldehyde formation

\[
R - \text{CH} - R \rightarrow R^* + R - \text{CH} - H
\]

(C) Alcohol formation

\[
R - \text{CH} - R + R'H \rightarrow R - \text{CH} - R + R'^*
\]

(D) Ketone formation

\[
R - \text{CH} - R + R'^* \rightarrow R - \text{C} - R + R'H
\]
Figure 1-4. Formation of 3-Methylbutanal from the action of *S. lactis* *maltigenes* on leucine (development of malty flavour defect in milk)
Figure 1-5. Summarized mechanism involved in the formation of ethyl ester by *Peudomonas fragi* (Morgan, 1976).

R-CH-COOH + HOOC-C\textsubscript{2}H\textsubscript{4}C-COOH \xrightarrow{Pyridosial phosphate} R-C-COOH + HOOC-CH\textsubscript{2}-CH-COOH

\[ \text{NH}_2 \quad \text{O} \quad \text{O} \quad \text{NH}_2 \]

R-C-COOH \xrightarrow{Thiamine pyrophosphate} R-CH + CO\textsubscript{2}

\[ \text{O} \quad \text{O} \]

Aldehydes

R-C-COOH \xrightarrow{NADH} R-CH\textsubscript{2}OH

\[ \text{O} \]

Alcohols

Where: \( R = (\text{CH}_3)_2-\text{CH}^{-} \) \( \quad (\text{C}_6\text{H}_5)-\text{CH}^{-} \)

\( (\text{CH}_3)_2-\text{CH}_2-\text{CH}^{-}-(\text{CH}_3)^- \) \( \quad (\text{CH}_3)_2-\text{CH}-\text{CH}_2^- \)

\( (\text{CH}_3)_2-\text{CH}-\text{CH}_2^- \)

\( (\text{CH}_3)_2-\text{CH}_2-\text{CH}^{-}-(\text{CH}_3)^- \) \( \quad (\text{CH}_3)_2-\text{CH}-\text{CH}_2^- \)

\( (\text{CH}_3)_2-\text{CH}-\text{CH}_2^- \)
CHAPTER 2

EVALUATION OF SENSORY ASSESSMENT OF MILK QUALITY AS A DIAGNOSTIC TEST FOR ABNORMAL FLAVOURS IN RAW MILK

Submitted to: International Dairy Journal
Abstract

A 2-phase experimental study was conducted to evaluate the inter- and intra-rater reliability of organoleptic testing of bulk tank milk, the method of choice for quality control of raw milk. In Phase 1, twenty-one bulk tank milk samples (10 off-flavoured and 11 controls) were submitted to a panel of four experienced milk graders for discriminative (off-flavoured versus non off-flavoured) and descriptive (off-flavour categorization and intensity scoring) assessments. In order to further investigate the reliability of discriminative organoleptic assessment, which is the underlying sensory attribute for pre-processing flavour quality control of raw milk, in phase 2, twenty-seven samples from nine Holstein Friesian cows were collaboratively analyzed by two trained panels of five milk graders each, for discriminative assessment only. Results suggested that the agreement beyond what was expected due to chance alone among the panelists/panels in both phases of the study ranged from substantial ($kappa=0.71-0.80$) to almost perfect ($kappa>0.81$) when assessing the presence or absence of off-flavour in milk. Alternative statistics such as ACI-statistic and indices of agreement in both positive and negative responses yielded similar results. Such high agreements were not obtained in the categorization or the intensity scoring of off-flavours ($kappa = 0.33-0.68$). However, because pre-processing (on-farm) flavour quality control programs for raw milk are essentially based on discriminative assessment, the findings of the present investigation strongly support its continued field application.

Key words: Off-flavour; organoleptic; inter- & intra-rater reliability; agreement; panelist.
Abbreviation keys: PEI = Prince Edward Island, BTM = Bulk-tank milk.
2.1. Introduction

Organoleptic sensory evaluation of milk (milk grading) has long been recognized as a timely, practical and cheap way to collect information on sensory attributes of samples. Its importance in bulk-tank milk (BTM) quality control lies in ensuring consumer acceptance of processed milk and other dairy products, which are only as good as the raw materials from which they are made. Consequently milk flavour is highly regarded in milk quality control programs. Sensory evaluation by trained milk graders traditionally has been the major method for detecting flavour defects in milk [1;2]. In Prince Edward Island (PEI) and elsewhere, milk truck drivers are required to be certified through a specifically designed milk-grading course.

There have been substantial developments in chemical instrumentation and improved methods for extracting volatile and semi-volatile organic compounds from complex food systems to assist in the identification of off-flavours. Although gas chromatography systems (gas chromatography mass spectrometry, gas chromatography-olfactometry detector) or electronic nose systems have been proven to be more reliable and sensitive in determining the concentration, identity and odor characteristics of each component extracted from a milk sample, these tools are still not practical, timely or inexpensive enough for routine use on every farm. Therefore, sensory assessment is still accepted worldwide as the method of choice for the evaluation of various food and dairy products [3-6], including conventionally pasteurized milk [7-9], ice cream [10], and cheese [11]. This study was proposed following an outbreak of off-flavoured milk occurring on PEI
during the late 1990s [12], based on on-farm discriminative sensory assessment (taste and smell) by trained and experienced dairy quality-control personnel. However, to our knowledge the reliability of this form of milk grading has never been formally evaluated.

There was concern within the dairy industry at the time that the testing process was too subjective and therefore subject to misclassification, potentially costing dairy farmers or dairy processing companies substantial amounts of money due to rejected bulk-tank milk (without good cause) or rejected truck loads (found off-flavoured at the processing plant).

This investigation was a two-phase study: the first phase addressed the inter- and intra-rater reliability of both discriminative and descriptive (qualitative and semi-quantitative) assessments, and then phase 2 further investigated the full discriminative assessment process as it would be completed in the field. Emphasis was placed on the discriminative testing (accept/reject), because it is the ultimate attribute that determines the fate of raw milk during routine on-farm flavour quality control.

### 2.2. Materials and methods

The discriminative testing protocols used in both phases were designed to mirror, as much as possible, those used by the dairy quality-control personnel for on-farm routine milk flavour grading, i.e. a sample was of good quality if it was graded “accept,” and off-flavoured if it was graded “reject.” For descriptive tests, on the other hand, categorical scales (nominal for flavour type and ordinal for flavour intensity scale) were provided [13], as described in more detail below.
2.2.1. Common sample handling and preparation between phases

Once retrieved, all milk samples (bulk-tank milk and individual cow samples) were stored at +4°C and always transported in a cooler packed with ice to maintain refrigeration. Any milk sample that was more than 72 hours old was automatically eliminated from the batch submitted for the test.

In order to enhance organoleptic characteristics, the milk samples were held at room temperature for 2-3 hours before the scheduled organoleptic test session to allow the serving temperature to rise (approximately 14-16°C) to a level above that which is generally considered as normal drinking temperature for milk [13].

2.2.2. Phase 1

2.2.2.1. Bulk-tank milk samples

A total of 10 off-flavoured case samples of bulk-tank milk (BTM) samples were collected at the time of rejection of bulk-tank milk for this phase of the study. A bulk-tank was considered rejected when the milk-tank driver (certified grader) detected an objectionable flavour, with the result confirmed by a second milk grader (the milk receiver) at the processing plant of the dairy company. Each of these off-flavoured samples was matched with a control sample from a non-case farm collected within an hour of the rejected sample, with one extra control sample taken to ensure that each of 7 assessment sessions contained 3 tested samples. The number of samples to be tested during each test session
was limited to three original samples (with a maximum of two and a minimum of one being off-flavoured), plus their respective replicates, because of the 72 hour limitation of requiring fresh, field-based, milk samples for testing.

The samples were collected in one-liter sterile plastic containers and cooled to +4°C within 30 min of collection. Samples were decanted into identical 30 ml semi-transparent odorless and sterile plastic containers and labeled with five-digit random codes from a list of a computer-generated random numbers.

2.2.2.2. Panel

The milk graders (all male) constituting the sensory panels involved in both phases of this study were selected from the milk transport personnel of the PEI dairy processing industry. For Phase 1 (BTM samples), a panel of four milk graders was constituted. The panelists were selected by consulting the work schedule of all the drivers affiliated with the dairy company; this helped identify those that could be available on all chosen test days. Selected panelists all had experience (> 2 years) in detecting and categorizing off-flavours in milk. In order to avoid interference with normal functions of taste and smell, graders were not allowed to participate at a test session if they had any health problems.

No pre-test training was organized, but results from an unpublished pilot study carried out a year earlier showed that the panelists were comfortable with a four-point intensity scale ("none," "moderate," "strong," and "very strong") and the four-level category scale

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(“feed,” “oxidized,” “rancid” and “malty”) for type of off-flavours. These were the scales they used routinely.

2.2.2.3. Test session

The test sessions were conducted in a well-ventilated and sanitized room at one of the facilities of the local dairy processing industry. The analyses were performed under artificial fluorescent lighting. To obtain independent responses, the panelists were invited into the testing room sequentially. Communication between the panelists was prohibited during the session in order to ensure independence in the evaluations. The panelists rated each sample as “accept” or “reject,” identified the type of off-flavour (if present) and rated the intensity. The time-interval between consecutive evaluations was about 2-3 min. The samples were tested in a random order and panelists were provided with unsalted crackers and filtered water (at room temperature) to rinse their mouth and to aid in removing any residual flavours from the palate [13].

The frequency of test sessions depended on the frequency with which off-flavoured cases arose, but taking into consideration that any sample to be submitted to the sensory panel had to be less than 72 hours old. Overall, seven test sessions were organized, using 3 samples per session and their replicates, with an interval of at least one week between consecutive sessions. Therefore, a total of 42 samples were assessed for discriminative and descriptive assessment in this phase of the study.
2.2.2.4. Data analyses

Kappa statistic [14], as well as other alternative statistics (indices of positive and negative agreements [15] and $A_{CI}$-statistic [16]) were used to assess the intra- and inter-panelist reliabilities of discriminative organoleptic assessment of the milk samples. For intrapanelist reliability, the evaluations of original samples were compared to those of their respective replicates for each panelist, whereas for the inter-panelist reliability, the panelists’ evaluations of original samples only, were compared to each other [17].

The validity of organoleptic testing in Phase 1 was also assessed by calculating the sensitivity ($S_e$) and specificity ($S_p$) [18] for each panelist, with the assumption that the original collaborative assessment performed by the milk drivers and receivers was the “gold standard.” Therefore sensitivity would be interpreted as the ability of each panelist to detect off-flavours for the 10 off-flavour samples, and specificity would be interpreted as the ability of each panelist to detect normal milk, for the 11 control milk samples.

The assessment of the agreement among all of the panelists on the categorization of off-flavours was addressed using unweighted kappa [19]. The amount of agreement that 2 randomly selected panelists from the studied panel would have in assigning a given category to any tested sample was evaluated as well [20]. To determine the reliability of the semi-quantitative (intensity scoring) assessments, Cohen weighted kappa [21] was computed using the weight matrix shown in Table 4-1.
For all of the interpretations of kappa values, we followed the categorization proposed by Landis & Koch [22], wherein kappa values of less than 0.20 represent "slight" agreement, values from 0.21 to 0.40 represent "fair" agreement, values from 0.41 to 0.60 represent "moderate" agreement, values from 0.61 to 0.80 represent "substantial" agreement, and "almost perfect" agreement represents values greater than 0.81.

The Kruskal-Wallis one-way analysis-of-variance test was also used to test whether there were significant differences in the panelists' flavour categorizing and scoring cards. We used the statistical software STATA 8.0 [23] for this test and for the computation of kappa statistics.

2.2.3. Phase 2

2.2.3.1. Individual milk samples

A total of 27 milk samples were collected from 3 Holstein Friesian cows from each of 3 selected commercial dairy herds on PEI. A milk sample was collected from each of these 9 cows before feeding freshly opened baled silage (early morning during the producers' scheduled milking) and then 30 minutes and 3 hours after silage feeding. Detailed description of the study design is reported elsewhere [24].

2.2.3.2. Panel and test sessions

Two panels of 5 milk graders each were formed and once again selected under similar
conditions as in Phase 1, from the milk transport personnel of the local dairy processing industry. Unlike Phase 1 where individual panelists assessed the samples independently, panelists evaluated the 27 individual cow samples collaboratively. They were asked to discuss their opinions with other graders on their panel in order to harmonize their overall assessment (that was entered in the evaluation questionnaire) into a final team-decision as to whether the milk was acceptable or rejected. This team decision would mimic the full discriminative assessment process, as it would be completed back at the processing plant.

Given the high number of samples to be tested in this phase (27 originals), replications were not included for evaluation during the same test session (as in Phase 1) in order to avoid both sensory and mental fatigue of the panelists [25]. Consequently, replicated samples were stored over night at +4°C for testing the next day by the same panels and under similar conditions.

2.2.3.3. Data analyses

Inter-panel reliability on the original samples on day 1 were carried out using not only the kappa statistics [14;21], but also the indices of observed agreement in positive and negative directions proposed by Cicchetti & Feinstein [15] and the $AC_T$-statistic [16]. Unlike the Cohen kappa statistic, the $AC_T$-statistic has been reported [16] as the more consistent omnibus index with the level of agreement between two graders (regardless of the trait prevalence in the subject population or the differences in the graders marginal probabilities from a 2X2 contingency table). Cicchetti and Feinstein [15] recommend that
the kappa statistic always be reported along with separate individual values of the indices of agreement in both positive and negative responses. They argued that in doing so, not only is the problem of the dependency of kappa or other omnibus indices to the trait prevalence and to the differences in the marginal probabilities is avoided, but also, emphases are put on the consistency of the two graders when tests are in the opposite directions of positive and negative evaluations, which is totally overshadowed when a single omnibus index is used. Intra-panel reliability for each panel, comparing assessments between day 1 and 2, was also determined using the same statistics as the inter-panel reliability determination. These alternative statistics to Cohen’s kappa (AC)-statistic, $P_{pos}$ and $P_{neg}$) were computed manually as described by their authors.

2.3. Results

2.3.1. Phase 1

2.3.1.1. Discriminative organoleptic analysis (Accept/Reject assessment)

The discriminative assessment of the milk samples by the panelists resulted in a very good level of agreement between all four panelists. The overall index of consistency between the panelists (Cohen kappa statistic for inter-grader reliability) was 0.84 ($p<0.01$). Similar results were obtained when pair-wise comparisons of individual panelist evaluations were made, producing both kappa and $AC$-statistic ranging from 0.71 ($p<0.01$) to 0.91 ($p<0.01$). The corresponding indices of agreement ($P_{obs}$ – proportion of overall observed agreement, $P_{pos}$ – proportion (index) of agreement on off-flavour positive samples, and $P_{neg}$ – proportion of agreement on off-flavour negative
samples) were over 85.0% (Table 4-2).

Intra-panelist reliability determinations yielded satisfactory results as well: Cohen’s kappa and AC1 statistics ranged from 0.52 ($p<0.01$) to 0.82 ($p<0.01$), with indices of agreement ranging from 76.2% to 92.3% (Table 4-2). Results also suggested high sensitivity (81.8% to 100%) and specificity (90% to 100%) for each panelist (Table 4-3).

2.3.1.2. Qualitative (categorization) and semi-quantitative (intensity scoring) descriptive analyses

Table 4-4 gives summaries of statistics for each of the five categories ("good," "feed," "oxidized," "rancid" and "malty") of off-flavour used by the panelists when describing the milk samples. The overall kappa value was 0.57 ($p<0.01$), suggesting only a moderate agreement between panelists beyond what was expected due to chance alone, which was less than that for the discriminative assessments. However, detailed analyses revealed that the panelists had "almost perfect" agreement in assigning the organoleptic category "good" to assessed samples (kappa = 0.84; $p<0.05$) and moderate agreement (kappa = 0.57; $p<0.05$) for category "feed." As for the other remaining categories ("oxidized," "rancid," and "malty"), it appeared that there was no agreement beyond that expected due to chance alone among the panelists in assigning these categories to suspected off-flavoured samples. Also, the results suggested that the amount of agreement ($T_j$) that two randomly selected panelists of the studied panel would have in assigning a given category to any tested sample [19] would be about 74.0% (Table 4-4).

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This index of agreement was about 92.0%, 73.0% and 13.0% respectively for “good,” “feed” and “oxidized” samples, and zero for “malty” and “rancid.”

Table 4-5 shows the resulting observed and expected agreement, and kappa statistics generated from the reliability testing of the scoring of the intensity of off-flavours. The kappa statistics for the intra-panelist reliability testing ranged from fair (kappa = 0.37; \( p<0.01 \)) to substantial (kappa = 0.68; \( p<0.01 \)) agreement beyond that expected due to chance alone. The kappa statistics for the inter-panelist reliability testing also ranged from fair (kappa = 0.33; \( p<0.01 \)) to substantial (kappa = 0.68; \( p<0.01 \)) agreement beyond that expected due to chance alone. Observed agreement was never higher than 84.5%, and generally around 75% for both the inter- and intra-panelist reliability testing, only a satisfactory amount.

Based on the Kruskal-Wallis analysis of variance evaluation, we found that there were no statistically significant differences in the panelists’ categorization (\( p>0.95 \)) and intensity-scoring (\( p>0.25 \)) abilities of off-flavours.

2.3.2. Phase 2

Results of organoleptic testing of individual milk samples showed that all 9 samples collected before silage feeding were graded as “good” by both panels at both the first and second test sessions. Of the 18 milk samples taken after feeding the freshly opened baled silage, 16 were considered off-flavour positive by panel 1 and 2 during session 1, and 16
and 17 were considered off-flavour positive by panel 1 and 2 during session 2, respectively (Tables 4-6 & 4-7). Both panels had disagreement on two samples during the first test session, but only on one sample in test session 2. Overall, the two panels agreed on 16 (89.0%) and 17 (94.4%) of the 18 samples collected after silage feeding at the first and second test sessions, respectively (inter-panel reliability). Inter-panel agreement was therefore “almost perfect” with kappa values of 0.847 ($p<0.01$) and 0.922 ($p<0.01$) for the first and second sessions, respectively.

Similar results were obtained in the evaluation of intra-panel reliability that was assessed by comparing the evaluation records of each panel at the two test sessions (Table 4-8). Based on the alternative tests for inter- and intra-panel reliability proposed by Cicchetti & Feinstein [15], the results indicated highly satisfactory agreement (see Table 4-8). The observed proportion of agreement between the panels was 94.1% for the first test session, and 96.8% for the second session, both in the positive direction. Likewise, indexes of average proportional agreement were 90.0% and 95.7% for test sessions 1 and 2, respectively, in the negative direction. Intra-panel reliability assessment yielded almost identical results. However, for both the intra- and inter-panel reliability testing, slightly higher percentages of agreement were observed in the assessment of the positive (off-flavoured) samples compared with the assessment of control (non off-flavoured) samples. Values for the $AC_j$-statistic [16] for both inter- and intra-panel reliability were also within the perfect range ($> 0.81$). These results confirm the findings of the kappa statistic analyses.
2.4. Discussion

High levels of repeatability (intra-panelist and intra-panel reliability) of discriminative organoleptic analysis by trained graders both in analyzing BTM samples as well as individual cow samples suggest that this method can be used with confidence as a reliable method for pre-processing flavour quality assessment of raw milk. The subjective method was also shown to be highly valid, with individual panelists detecting acceptable and rejected milk with high accuracy, as determined the combined results of the assessments. While this does not give an assessment of reliability compared to an objective (i.e. instrument-based) measurement, it does demonstrate very good consistency of the organoleptic sensory method. Development of an appropriate objective off-flavour measurement tool is the subject of a subsequent paper in this thesis, as there is none currently accepted in the literature.

Phase 2 yielded “almost perfect” inter- and intra-panel agreements with all three of the methods of statistical analysis: Cohen kappa statistic[14], Cicchetti & Feinstein [15], and Gwet [16]. Cicchetti & Feinstein [15] argued that using a single measure such as Kappa, to express the level of reliability of graders may be less informative compared to when average positive agreement ($P_{pos}$) and average negative agreement ($P_{neg}$) are assessed separately. Gwet [16] on the other hand claims that, unlike the Cohen kappa, which is just as inconsistent as the other popular inter-rater agreement statistics, such as the $S$ coefficient [26] and $\pi$–statistic [27], the $AC_I$ statistic is not affected either by the graders’ classification probabilities (marginal probabilities) or the trait prevalence in the subject.
There are frequently situations where a second opinion (multiple organoleptic testing in series) is required before adequate measures are taken regarding the fate of a load of milk being tested. While this process may enhance accuracy, these additional assessments are usually not independent and may be biased by knowledge of the first assessment. Therefore, the highly reliable collaborative methods used in the second phase of the study were indicative of normal procedures determining the fate of milk samples.

While the methods in Phase 2 did mimic decisions on milk quality in the field (i.e. multiple assessments: farm- and plant-level), the generated off-flavour in the milk samples may have been more distinct in the individual cow milk samples than in normal BTM samples. This factor may explain the improvement registered in both the within- and between-grader reliability values in this phase compared to the discriminative assessments done in Phase 1.

Although overall agreement between the panelists in Phase 1 was high when differentiating milk of good quality from that of poor organoleptic quality, there was only a moderate level of agreement between the panelists in identifying "feed" flavoured samples and almost no agreement in samples that were assigned to other categories of off-flavour ("oxidized," "malty" and "rancid") or in scoring their intensity level. However, the relatively small sample size, especially samples representing certain intensity levels and categories of off-flavour, may have influenced not only the level of
agreement among and within the panelists, but also their significance level. Therefore, cautious interpretation of these descriptive assessment results is recommended. In an experimental study, one could remedy this problem by ensuring that there is a high number of samples within each represented category or intensity level of off-flavour, where the flavour defects could be artificially induced in order to control their authenticity and their intensity. A number of other researchers in the field of sensory evaluation of food products have reported that there were less difficulties for the panelists when the quality standards for acceptability of a product included only a few sensory attributes, such as “like/dislike,” or “sweetness/bitterness,” rather than assessments using multiple categories and intensities at once [13;28;29].

One could argue that the environmental conditions in which the graders conducted their assessments in both Phase 1 and 2 of this study were more desirable than those encountered during on-farm testing with respect to ambient air temperature, noise levels, barn smells, and the stress levels on the graders. These improved conditions may have lead to the enhancement of the ability of the graders to detect off-flavours in the present study. However, for practical reasons, the experiments could not be done on-farm. Furthermore, it is usually in an office or laboratory room of a milk processing plant where the second (and possibly third) opinions of other graders are obtained in order to confirm that milk samples are off-flavour, and therefore, the environmental conditions for Phase 2 were different from those encountered in the field.

The 95% confidence intervals around the kappa values indicate wide variation around the
estimates, primarily due to the relatively small sample sizes tested. Future studies could improve on this study by increasing the number of samples tested in order to make these confidence intervals narrow. Increasing the number of "normal" (good quality) samples would also be desirable so that the prevalence of off-flavoured samples is representative of what is encountered in the field.

2.5. Conclusions

This study established not only high reliability for discriminative organoleptic analysis of milk flavour quality, but also high validity, suggesting that it remains a valuable tool for quality control programs in the field, regardless of its subjective nature, especially in the absence of a more objective method. While our efforts to determine the validity of the discriminative organoleptic testing method yielded high \( S_e \) and \( S_p \), additional research with an objective gold standard (e.g., biochemical compound concentrations) is needed before conclusive reports on validity can be made.

The traditional qualitative and semi-quantitative scales used in this study (flavour defect description and ordinal flavour intensity scoring) did not appear to be reliable. Further research is needed to determine if the use of alternative scales with a much wider numerical spectrum and appropriate pre-study training of the panelists may result in the improvement of the graders' ability to reliably categorize and measure the intensity of off-flavours in milk. Furthermore, improvements may also be achievable in the categorization of flavour defects if so-called panel-generated descriptive analysis terms (simple sensory descriptive words) [7;30] are utilized instead of the traditional dairy
judging terminology (defect-oriented). However, because on-farm quality control of BTM is based on the assessment of the presence or absence of off-flavour, precision in the categorization or scoring of the intensity of off-flavour appears to be of low relevance for field practice at this point in time.
Acknowledgements

The authors would also like to extend appreciation to Theresa Andrews, Ricky Milton and Lloyd Dalziel at the Farm Service of the department of Health Management for their help in sample preparation before each test session. They would also like to thank ADL (dairy company) for their collaboration in the milk sampling, and the milk truck operators for their participation in the sensory analyses.
2.6. Reference List


66

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(21) Cohen J. "Weighted Kappa: nominal scale agreement with provision for scale disagreement or partial credit". Psychol Bull 1968; 70:213-220.

(22) Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977; 33:159-174.


Table 2-1. Weight matrix\(^a\) for the evaluation of the reliability of the panelists in assessing the intensity of off-flavours in Phase 1 – Individual panelist evaluations.

<table>
<thead>
<tr>
<th></th>
<th>“Very strong”</th>
<th>“Strong”</th>
<th>“Moderate”</th>
<th>“None”</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Very strong”</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Strong”</td>
<td>0.75</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Moderate”</td>
<td>0.25</td>
<td>0.50</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>“None”</td>
<td>0.00</td>
<td>0.00</td>
<td>0.25</td>
<td>1.00</td>
</tr>
</tbody>
</table>

\(^a\) A value of 1 corresponds to complete agreement while a value of zero corresponds to complete disagreement.
Table 2-2. Pair-wise comparison of the coefficients of reliability for discriminative organoleptic analysis of milk flavour quality in Phase 1 – Individual panelist evaluations.

A. Cohen’s kappa (95% Confidence Interval)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>“A”</th>
<th>“B”</th>
<th>“C”</th>
<th>“D”</th>
</tr>
</thead>
<tbody>
<tr>
<td>“A”</td>
<td>0.71(^1) (0.42, 1.0)</td>
<td>0.90(^2) (0.70, 1.0)</td>
<td>0.91(^2) (0.72, 1.0)</td>
<td>0.81(^2) (0.55, 1.0)</td>
</tr>
<tr>
<td>“B”</td>
<td>0.80(^1) (0.53, 1.0)</td>
<td>0.81(^2) (0.55, 1.0)</td>
<td>0.72(^2) (0.32, 1.0)</td>
<td></td>
</tr>
<tr>
<td>“C”</td>
<td></td>
<td>0.53(^1) (0.12, 0.72)</td>
<td>0.90(^2) (0.70, 1.0)</td>
<td></td>
</tr>
<tr>
<td>“D”</td>
<td></td>
<td></td>
<td>0.71(^1) (0.42, 1.0)</td>
<td></td>
</tr>
</tbody>
</table>

B. Corresponding \(P_{obs}\) - index of overall observed agreement (and \(P_{exp}\) = expected agreement due to chance alone)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>“A”</th>
<th>“B”</th>
<th>“C”</th>
<th>“D”</th>
</tr>
</thead>
<tbody>
<tr>
<td>“A”</td>
<td>85.7(^3) (50.3%)</td>
<td>95.2(^4) (50.3%)</td>
<td>95.2(^4) (49.9%)</td>
<td>90.5(^4) (49.7%)</td>
</tr>
<tr>
<td>“B”</td>
<td>90.5(^3) (52.4%)</td>
<td>90.5(^4) (49.9%)</td>
<td>85.7(^4) (49.0%)</td>
<td></td>
</tr>
<tr>
<td>“C”</td>
<td>76.2(^3) (49.9%)</td>
<td></td>
<td>95.2(^4) (50.3%)</td>
<td></td>
</tr>
<tr>
<td>“D”</td>
<td></td>
<td></td>
<td>85.7(^3) (50.3%)</td>
<td></td>
</tr>
</tbody>
</table>

C. Indexes of agreement: \(P_{pos}\) = proportion of agreement on off-flavour positive samples
(and \(P_{neg}\) = proportion of agreement on off-flavour negative samples)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>“A”</th>
<th>“B”</th>
<th>“C”</th>
<th>“D”</th>
</tr>
</thead>
<tbody>
<tr>
<td>“A”</td>
<td>90.9(^5) (84.2%)</td>
<td>95.2(^5) (94.7%)</td>
<td>95.2(^5) (95.2%)</td>
<td>90.0(^6) (90.9%)</td>
</tr>
<tr>
<td>“B”</td>
<td>92.3(^5) (87.5%)</td>
<td>90.9(^6) (90.0%)</td>
<td>85.7(^6) (85.7%)</td>
<td></td>
</tr>
<tr>
<td>“C”</td>
<td></td>
<td>76.2(^5) (76.2%)</td>
<td>90.9(^6) (84.2%)</td>
<td></td>
</tr>
<tr>
<td>“D”</td>
<td></td>
<td></td>
<td>84.2(^5) (90.9%)</td>
<td></td>
</tr>
</tbody>
</table>
D. Alternative chance-corrected statistic ($AC_j$) proposed by Gwet (2002)

<table>
<thead>
<tr>
<th>Panelist</th>
<th>“A” $^1$</th>
<th>“B” $^1$</th>
<th>“C” $^1$</th>
<th>“D” $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>“A”</td>
<td>0.72$^7$</td>
<td>0.91$^8$</td>
<td>0.91$^8$</td>
<td>0.81$^8$</td>
</tr>
<tr>
<td>“B”</td>
<td>0.82$^7$</td>
<td>0.90$^8$</td>
<td></td>
<td>0.71$^8$</td>
</tr>
<tr>
<td>“C”</td>
<td></td>
<td>0.52$^7$</td>
<td></td>
<td>0.91$^8$</td>
</tr>
<tr>
<td>“D”</td>
<td></td>
<td></td>
<td></td>
<td>0.72$^7$</td>
</tr>
</tbody>
</table>

$^1$ Intra-panelist agreement beyond that expected due to chance alone

$^2$ Inter-panelist agreement beyond that expected due to chance alone

$^3$ Intra-panelist index of observed agreement due to chance alone ($P_{obs}$)

$^4$ Inter-panelist index of observed agreement due to chance alone ($P_{exp}$)

$^5$ Intra-panelist index of agreement on off-flavour positive samples ($P_{pos}$)

$^6$ Inter-panelist index of agreement on off-flavour negative samples ($P_{neg}$)

$^7$ Intra-panelist index of the alternative chance-corrected statistic ($AC_j$)

$^8$ Inter-panelist index of the alternative chance-corrected statistic ($AC_j$)
Table 2-3. Sensitivity ($S_e$) and Specificity ($S_p$) of each of the panelists in the assessment of bulk tank milk samples in Phase 1 – Individual panelist evaluations.

<table>
<thead>
<tr>
<th>Panelist</th>
<th>Reliability parameter for each panelist</th>
<th>GOLD STANDARD = Initial on-farm assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>“A”</td>
<td>$S_e = a/n_1 = 90.9%$</td>
<td>$S_p = d/n_0 = 100%$</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>$a = 10$</td>
<td>$b = 0$</td>
</tr>
<tr>
<td></td>
<td>$c = 1$</td>
<td>$d = 10$</td>
</tr>
<tr>
<td></td>
<td>$n_1 = 11$</td>
<td>$n_0 = 10$</td>
</tr>
</tbody>
</table>

| “B”      | $S_e = a/n_1 = 81.8\%$                | $S_p = d/n_0 = 100\%$                |     |
|          | Positive                               | Negative                                | Total |
|          | $a = 9$                                | $b = 0$                                 | $m_1 = 9$ |
|          | $c = 2$                                | $d = 10$                                | $m_0 = 12$ |
|          | $n_1 = 11$                             | $n_0 = 10$                              | $N = 21$ |

| “C”      | $S_e = a/n_1 = 100\%$                | $S_p = d/n_0 = 100\%$                |     |
|          | Positive                               | Negative                                | Total |
|          | $a = 11$                               | $b = 0$                                 | $m_1 = 11$ |
|          | $c = 0$                                | $d = 10$                                | $m_0 = 10$ |
|          | $n_1 = 11$                             | $n_0 = 10$                              | $N = 21$ |

| “D”      | $S_e = a/n_1 = 100\%$                | $S_p = d/n_0 = 90\%$                |     |
|          | Positive                               | Negative                                | Total |
|          | $a = 11$                               | $b = 1$                                 | $m_1 = 10$ |
|          | $c = 0$                                | $d = 9$                                 | $m_0 = 11$ |
|          | $n_1 = 11$                             | $n_0 = 10$                              | $N = 21$ |
Table 2-4. Statistics for measuring overall agreement on each of the five categories of off-flavour in Phase 1 – Individual panelist evaluations

<table>
<thead>
<tr>
<th>Category</th>
<th>( \Sigma n_{ij} )</th>
<th>( P_j )</th>
<th>( T_j )</th>
<th>( K_j )</th>
<th>( \text{Var}(K_j) )</th>
<th>( \text{SE}(K_j) )</th>
<th>( K_j/\text{SE}(K_j) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. “Good”</td>
<td>158</td>
<td>0.50</td>
<td>0.92</td>
<td>0.84</td>
<td>0.09</td>
<td>0.30</td>
<td>2.8</td>
</tr>
<tr>
<td>2. “Feed”</td>
<td>99</td>
<td>0.37</td>
<td>0.73</td>
<td>0.57</td>
<td>0.067</td>
<td>0.26</td>
<td>2.2</td>
</tr>
<tr>
<td>3. “Oxidized”</td>
<td>7</td>
<td>0.060</td>
<td>0.13</td>
<td>0.07</td>
<td>0.051</td>
<td>0.23</td>
<td>0.31</td>
</tr>
<tr>
<td>4. “Rancid”</td>
<td>4</td>
<td>0.048</td>
<td>-0.003</td>
<td>-0.05</td>
<td>0.056</td>
<td>0.24</td>
<td>-0.21</td>
</tr>
<tr>
<td>5. “Malty”</td>
<td>2</td>
<td>0.024</td>
<td>-0.003</td>
<td>-0.03</td>
<td>0.082</td>
<td>0.28</td>
<td>-0.10</td>
</tr>
<tr>
<td><strong>OVERALL</strong></td>
<td><strong>270</strong></td>
<td>-</td>
<td>0.74</td>
<td>0.57</td>
<td>0.007</td>
<td>0.084</td>
<td><strong>6.8</strong></td>
</tr>
</tbody>
</table>

- \( \Sigma n_{ij} \) – Number of panelists who assigned \( i \)th sample to \( j \)th category;
- \( P_j \) – Proportion of all the panelists’ assignments to the \( j \)th category of off-flavour;
- \( T_j \) – Overall extent of agreement;
- \( K_j \) – kappa statistic;
- \( \text{Var}(K_j) \) – variance of \( K_j \);
- \( \text{SE}(K_j) \) – Standard Error of \( K_j \);
- \( K_j/\text{SE}(K_j) \) – Standardized kappa
Table 2-5. Pair-wise comparison of the coefficients of reliability of organoleptic scoring of the intensity of off-flavour in Phase 1 (semi-quantitative analysis) – Individual panelist evaluations.

<table>
<thead>
<tr>
<th></th>
<th>Panelist “A”</th>
<th>Panelist “B”</th>
<th>Panelist “C”</th>
<th>Panelist “D”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panelist “A”</td>
<td>0.60 (0.27, 0.99)</td>
<td>0.68 (0.40, 0.97)</td>
<td>0.41 (0.21, 0.62)</td>
<td>0.49 (0.21, 0.77)</td>
</tr>
<tr>
<td>Panelist “B”</td>
<td>0.48 (0.19, 0.78)</td>
<td>0.42 (0.14, 0.70)</td>
<td>0.33 (0.03, 0.63)</td>
<td></td>
</tr>
<tr>
<td>Panelist “C”</td>
<td></td>
<td>0.68 (0.34, 1.0)</td>
<td></td>
<td>0.42 (0.12, 0.73)</td>
</tr>
<tr>
<td>Panelist “D”</td>
<td></td>
<td></td>
<td></td>
<td>0.37 (0.07, 0.67)</td>
</tr>
</tbody>
</table>

A. Weighted kappa-statistic (95% Confidence Interval)

<table>
<thead>
<tr>
<th></th>
<th>Panelist “A”</th>
<th>Panelist “B”</th>
<th>Panelist “C”</th>
<th>Panelist “D”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panelist “A”</td>
<td>78.6% (46.7%)</td>
<td>83.3% (47.6%)</td>
<td>72.6% (53.3%)</td>
<td>75.0% (51.4%)</td>
</tr>
<tr>
<td>Panelist “B”</td>
<td>73.8% (49.3%)</td>
<td>75.0% (56.9%)</td>
<td></td>
<td>69.1% (53.5%)</td>
</tr>
<tr>
<td>Panelist “C”</td>
<td></td>
<td>84.5% (52.4%)</td>
<td></td>
<td>78.6% (62.9%)</td>
</tr>
<tr>
<td>Panelist “D”</td>
<td></td>
<td></td>
<td></td>
<td>72.6% (56.8%)</td>
</tr>
</tbody>
</table>

B. Corresponding index of observed agreement (and expected agreement due to chance alone)

1 Intra-panelist agreement beyond that expected due to chance alone
2 Inter-panelist agreement beyond that expected due to chance alone
3 Intra-panelist index of observed agreement
4 Inter-panelist index of observed agreement
Table 2-6. Distribution of samples by panel and response category for each test session.

<table>
<thead>
<tr>
<th></th>
<th>Panel “B”</th>
<th></th>
<th>Panel “A”</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Off-flavour positive</td>
<td>Off-flavour negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Off-flavour positive</td>
<td>15</td>
<td>1</td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Off-flavour negative</td>
<td>1</td>
<td>10</td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>11</td>
<td></td>
<td></td>
<td>27</td>
</tr>
</tbody>
</table>

**A. TEST SESSION 1**

<table>
<thead>
<tr>
<th></th>
<th>Panel “B”</th>
<th></th>
<th>Panel “A”</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Off-flavour positive</td>
<td>Off-flavour negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Off-flavour positive</td>
<td>16</td>
<td>1</td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Off-flavour negative</td>
<td>0</td>
<td>10</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>11</td>
<td></td>
<td></td>
<td>27</td>
</tr>
</tbody>
</table>

**B. TEST SESSION 2**

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Table 2-7. Distribution of samples by test session and response category for each panel

<table>
<thead>
<tr>
<th>Test session 2</th>
<th>Test session 1</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Off-flavour positive</td>
<td>Off-flavour negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Off-flavour positive</td>
<td>16</td>
<td>1</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Off-flavour negative</td>
<td>0</td>
<td>10</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>11</td>
<td></td>
<td>27</td>
</tr>
</tbody>
</table>

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Table 2-8. Evaluation of inter and intra-panel reliability of discriminative organoleptic analysis of individual cow samples – Phase 2 Panel evaluations.

### A – Intra-panel reliability

<table>
<thead>
<tr>
<th>Panel</th>
<th>Number of Samples</th>
<th>Expected agreement</th>
<th>Observed agreement</th>
<th>Kappa-statistic</th>
<th>95% confidence interval</th>
<th>Alternative statistics to kappa proposed by:</th>
<th>Cicchetti &amp; Feinstein (1990)</th>
<th>Gwet (2002)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original</td>
<td>Duplicate</td>
<td>Original</td>
<td>Duplicate</td>
<td></td>
<td>1 $P_{pos}$</td>
<td>2 $P_{neg}$</td>
<td>ACI statistic</td>
</tr>
<tr>
<td>“A”</td>
<td>27</td>
<td>27</td>
<td>51.71 %</td>
<td>92.59 %</td>
<td>+ 0.85</td>
<td>(0.65, 1.0)</td>
<td>93.75%</td>
<td>90.91%</td>
</tr>
<tr>
<td>“B”</td>
<td>27</td>
<td>27</td>
<td>52.40 %</td>
<td>96.30 %</td>
<td>+ 0.92</td>
<td>(0.76, 1.0)</td>
<td>96.97%</td>
<td>95.24%</td>
</tr>
</tbody>
</table>

### B – Intra-test (or Inter-panel) reliability

<table>
<thead>
<tr>
<th>Test</th>
<th>Number of samples</th>
<th>Expected agreement</th>
<th>Observed agreement</th>
<th>Kappa-statistic</th>
<th>95% CI confidence interval</th>
<th>Alternative statistics to kappa proposed by:</th>
<th>Cicchetti &amp; Feinstein (1990)</th>
<th>Gwet (2002)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>session</td>
<td>Original</td>
<td>Duplicate</td>
<td>Original</td>
<td>Duplicate</td>
<td></td>
<td>1 $P_{pos}$</td>
<td>2 $P_{neg}$</td>
</tr>
<tr>
<td>1</td>
<td>27</td>
<td>51.71 %</td>
<td>92.59 %</td>
<td>+ 0.85</td>
<td>(0.65, 1.0)</td>
<td>94.12%</td>
<td>90.00%</td>
<td>0.87</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>52.40 %</td>
<td>96.30 %</td>
<td>+ 0.92</td>
<td>(0.76, 1.0)</td>
<td>96.77%</td>
<td>95.65%</td>
<td>0.93</td>
</tr>
</tbody>
</table>

1 – Index of average proportional positive agreement;

2 – Index of average proportional negative agreement.
CHAPTER 3

GEOGRAPHICAL & TEMPORAL ASPECTS OF AN OUTBREAK OF OFF-FLAVOURS IN BULK-TANK MILK IN PRINCE EDWARD ISLAND, CANADA

Submitted to: Preventive Veterinary Medicine
Abstract

The geographical and temporal dynamics of an outbreak of off-flavours in bulk-tank milk that occurred between September 2000 and June 2002 in Prince Edward Island (PEI) dairy herds were examined descriptively using spatial, temporal and space-time scan statistics. Spatial and temporal analyses resulted in the identification of two and one cluster of off-flavour positive herds, respectively. The results of the space-time analysis indicated that there were 3 clusters in time and in space with the primary cluster, including six feed off-flavour case herds, located at the intersection of the Queens and Kings counties, and covering the period October 2000 to January 2001. The overall relative risk of a herd within this cluster to produce off-flavoured milk was 6.4 ($p<0.05$) compared to herds in the surrounding study areas. The two significant ($p<0.05$) secondary clusters, although located at different areas (in the Queens and Prince counties), had a relative risk similar to that of the most likely cluster ($RR = 6.4$), and were both comprised of 5 case herds. However, one was composed of case herds with a feed flavour defect identified between October 2000 and January 2001, and the other was predominantly composed of cases of rancid off-flavour, detected between December 2000 and March 2001. These findings suggested that the occurrence of off-flavour in bulk-tank milk was associated with periods of the year with the highest precipitation and lowest temperatures in some extent, and that some locations within PEI, for unexplained reasons, were more at risk than others.

Keywords: Milk, off-flavour, spatial, temporal, cluster.
Abbreviation keys: PEI = Prince Edward Island; RR = Relative risk; DM = Dry matter; CP = Crude protein.
3.1. Introduction

Off-flavour in milk is a quality defect in milk, which renders the aroma or the flavour of milk objectionable to consumers. The American Dairy Science Association’s (ADSA) Nomenclature, Standards, and Bibliography committee developed the standard classifications for off-flavours [1] based on the mechanism of flavour defect development. The standard categories are: “transmitted,” “lipolyzed,” “oxidized,” “microbial,” “heated,” “light-induced,” and “miscellaneous.” Over the years, other terms such “feed,” “barny,” “weed,” “fruity,” “cowy,” and many others have evolved around the primary categories and are mostly descriptive of either the flavour defects or their sources. Off-flavours occur unpredictably and in some cases occur as an outbreak in dairy producing regions.

During the late 1990s' outbreaks of off-flavours in bulk-tank milk in Prince Edward Island (PEI) prompted a two-year investigation of the associated risk factors [2]. This report presents the spatial and temporal distribution of the case herds for the period September 2000 - April 2002. Temporal / geographic distributions of case herds has not been taken into consideration in a formal way by earlier studies on the phenomenon of off-flavours in milk. Identifying clusters within space and time that have higher or lower risk for the problem of interest helps researchers generate hypotheses concerning causation. In the case of off-flavours, we were interested to know if the herds were more at risk of off-flavour incidents at certain times of the year and whether certain locations within PEI were more at risk than others, because this might suggest causal factors.
common to herds within the high-rate clusters.

Several statistical methods for testing the space-time interaction between the geographical and temporal distributions of case events have been developed [3-6]; however, they are mostly geared towards testing the presence of clusters and do not expand to the determination of their geographical locations. A comprehensive review of these methods has been published [7]. Ward and Carpenter [8] suggested the use of multiple techniques for the identification and description of spatial and temporal clustering of studied subjects, while Kulldorff and Hjalmars [9] offered an alternative method, known as the “Cluster-detection test,” which presents an advantage of not only detecting the cluster, but also identifying its geographical and spatial location and testing its level of significance.

The objective of the present study was to use the spatial, temporal and space-time scan statistics (SatScan v4.0, Maryland, USA, 2004) [10] to determine if the outbreak of off-flavours in bulk-tank milk in PEI was significantly clustered in space and time.

3.2. Material and Methods

3.2.1. Study area, study period and study herd definitions

The study area was the province of Prince Edward Island (PEI), located on the eastern coast of Canada. The province is divided roughly equally, Northwest to Southeast into three counties: Prince, Queens and Kings. The study period extended from September
2000 to April 2002. Off-flavour was diagnosed organoleptically by certified milk graders (milk transport personnel and milk receiving personnel at the processing plant): first by the milk transport personnel on-farm at the time of milk collection, then by the personnel at the receiving platform of the dairy processing plant who were given a 1-liter sample for reevaluation. The decision as to whether a bulk-tank load of milk was to be rejected was a collaborative assessment by at least two certified milk graders.

Within two hours following the rejection of a bulk-tank, telephone notification including the address of the herd, was made available to the author (A. Mounchili - principal investigator). The population at risk in the current study was the census of 307 dairy herds in PEI and herd was the study unit. Herds that did not experience off-flavour over the two-year study period were considered as control herds. Details on the assessment of risk factors for off-flavours can be found elsewhere [2]. Geographical data on dairy herd locations were provided by the PEI Department of Agriculture, Forestry, Aquaculture and Fisheries.

3.2.2. Data collection

The locations of the dairy herds in PEI were matched with their corresponding geographical coordinates for the mapping of the population of dairy herds in PEI. Herd management and nutritional data were collected from all the off-flavour positive (or case) herds and some off-flavour negative (control) herds within the framework of the study of risk factors; the other off-flavour negative herds that did not participate in this risk factor
study were assigned a random date within the time span from the first to the last outbreak dates as suggested by the authors of the software [10].

Temperature measurements at climatological stations in Canada are made from self-registering maximum and minimum thermometers set in a louvered, wooden shelter; whereas the precipitation is measured using a plastic graduated device [11]. Although officially there were 16 climatological stations in PEI, only 14 were operational and had complete data for the study period.

The maximum daily temperature was considered as the highest temperature recorded in a 24-hour period ending in the morning of the next day; and the minimum daily value was for a period of the same length, beginning in the evening of the previous day. The daily mean temperature in degrees Celsius (°C) was the average of the two, and the monthly value, the average of the corresponding daily mean records.

Total daily precipitation (mm) was defined as the sum of the total rainfall and the water equivalent of the total snowfall observed during the day and its corresponding monthly value was the sum of the daily records.

Both temperature and precipitation data were readily available on the official website of Environment Canada (www.climate.weatheroffice.ec.gc.ca/climate_normals/stnselect_e.html).
3.2.3. Analytical methods

3.2.3.1. Spatial, temporal and space-time clusters

The detection and evaluation of the statistically significant clusters were performed using the scan statistic (SaTScan v. 4.0, Maryland, USA), a software for spatial, temporal and space-time scan statistics. We used the Bernoulli model [12;13] as the data were in the format of cases and controls (“YES/NO”).

The spatial scan statistic was developed to test for geographical clusters and to identify their approximate location [14]. It imposes a circular window on the map and allows the center of the circle to move across the study region so that at different positions, the window includes different sets of neighbouring herds. The radius of this circular window was set to vary from zero to a maximum where at most 20% of the total population at risk was included. This allows the circles to be flexible both in location and in size. The method creates a very large number of distinct geographical windows, each capturing a different subset of neighbouring point locations (herds), and each being a potential candidate for a cluster of events (off-flavour cases) in the study region. The maximum possible spatial cluster size was set at 20% of the total population at risk (rather than 50% as recommended by Kulldorff [14]), because of the size and the shape of PEI; this prevented the spatial window from capturing large areas of ocean.

The temporal scan statistic uses a window that moves in one temporal dimension, defined
in the same way as the height of the cylindrical window used by the space-time scan statistic. Therefore, it is flexible at both the start and end date. In the present study, the temporal unit was expressed in months (i.e. Sept. 00, Oct. 00, etc) and the scanning window was set to vary from zero to 5% and 20% of the entire study period (which allowed the length of the temporal window to expand up to one and 4 months, respectively) in order to explore whether clustering was a monthly or seasonal issue.

The space-time scan statistic (which is an extension of the spatial scan statistic) is defined by a cylindrical window with a circular geographic base representing space and the height corresponding to time. Consequently, the cylindrical window is allowed to move in space and time, so that for each potential geographical location and size, each possible time period is covered. This process generates an infinite number of overlapping cylindrical windows of various sizes and shapes, jointly covering the entire study region, where each window reflects a possible cluster.

For each of the generated cylindrical windows (scanning windows in spatial, temporal or space-time analysis), the likelihood is calculated for observing the number of off-flavour positive cases occurring within the window. The window with the maximum likelihood, and with more than the expected number of cases is identified as the most likely (or primary) cluster, which is the cluster that is least likely to have occurred by chance alone. Details about the evaluation of the likelihood ratio tests are described elsewhere [14]. The distribution of the maximum likelihood under the null hypothesis is evaluated using Monte Carlo hypothesis testing [15]. The corresponding simulated \textit{p-value} results from
the comparison of the maximum likelihood from the actual data set with the maximum number generated in random replications (set to 999 for the present study) of the data under the null hypothesis.

In addition to the primary cluster, secondary clusters (which are clusters that do not overlap geographically with the primary cluster) are also reported if the likelihood ratio is larger than the likelihood ratio of the primary cluster for at least one dataset simulated under the null hypothesis.

3.2.3.2. Factors associated with herds in positive clusters

Low-rate space-time cluster analysis was conducted to identify off-flavour negative herds that were significantly clustered. Then, using either chi ($\chi^2$) square (for dichotomous variables) or $t$ test (for continuous variables) statistics, herd and nutritional management factors were compared between the high-rate (or off-flavour positive) space-time clustered herds and those that constituted the low-rate (or off-flavour negative) clusters. Also, descriptive statistics were used to compare the climate data (precipitation and temperature) for the geographical locations of identified high- and low-rate space-time clusters.

The point locations of all the dairy herds registered in PEI during the study period were plotted using the geographical information-system software ArcView 3.3 (ESRI, CA, USA, 2002). The inverse distance weighted interpolation function in the ArcView 3.3
extension software Spatial Analyst 2.0 (ESRI, CA, USA, 2000) was used to create the continuous surface maps from the point data layers for precipitation and temperature. Inverse distance weighted interpolation is a method used for interpolation of scatter points and assumes that the interpolating surface should be more heavily influenced by nearby points than distant ones. It determines cell values using a linearly weighted combination of a set of points (in the current study, the maximum number of neighboring points or climatological stations was set to eight). The weight is a function of inverse distance (i.e. the weight assigned to each scatter point diminishes as the distance from the interpolation point to the scatter point increases).

3.3. Results

3.3.1. Spatial and temporal analyses

Over the 20-month study period, of the 307 dairy herds that were registered in the province, 48 experienced at least one episode of off-flavour in milk. Figures 3-1 & 3-2 show the monthly dynamic of the off-flavour outbreak from September 2000 to April 2002 and the county-wise distribution of the case (and dairy) herds, respectively. Most cases occurred during the winter of the first year of the study (November 2000 – March 2001).

Results from analyses of spatial and temporal clustering are presented in Table 3-1. The purely spatial analysis identified two statistically significant clusters: a primary cluster (also known as the most likely cluster; $p<0.01$) comprised of five case herds located in
the western part of PEI in Prince county; and a marginally significant \((p=0.06)\) secondary cluster, comprised of four case herds. The overall relative risk within each of these clusters was 6.4 (Table 3-1).

Temporal analysis with the scanning window set at a maximum of 5\%, also yielded results similar to that with the scanning window set at a maximum of 20\% (Table 3-1). Both analyses identified the month of January 2001 as the most likely high-rate temporal clustered period.

3.3.2. Space-time clustering analysis

Space-time interaction analysis performed with the maximum temporal cluster size set at 5\% (equivalent to one month) and the maximum spatial cluster size at 20\% did not yield any evidence of monthly clustering of off-flavour positive herds. Conversely, the analysis with the maximum scanning windows for both spatial and temporal set at 20\% suggested that there was a significant space-time clustering of the occurrences of milk off-flavours in PEI over the 20-month study period (Table 3-2). Three significant clusters (one primary and two secondary) were detected, with the primary cluster located at the intersection of the central (Queens) and the eastern (Kings) counties (Figure 3-3). The primary cluster had a radius of 13.5 km and covered the period October 2000 to January 2001. It included 6 off-flavour cases out of a population at risk of 20 herds (while only 0.94 was expected); consequently the overall relative risk within the cluster was 6.4 \((p<0.01)\). The two significant \((p<0.05)\) secondary clusters had similar overall relative
risk (RR = 6.4) and were both comprised of 5 off-flavour positive herds; however, not only were they located in different counties, but they covered different time frames: the first, located in the central county (Queens) covered the period October 2000 to January 2001, while the second was located in the western county (Prince) and covered December 2000 to March 2001. The primary high-rate cluster and the first secondary cluster were characterized by herds that experienced feed off-flavour, while the second secondary cluster was characterized by herds that experienced rancid off-flavour.

Results presented in Table 3-2 showed evidence of marginally significant (p=0.10), low-rate (less likely) space-time clustering at two different geographical locations: Queens and Prince counties (Figure 3-4). These clusters were combined into a single group for the purpose of comparison with high-rate clusters of feed off-flavour cases on one hand (Table 3-3), and with high-rate cluster constituted predominantly of rancid off-flavour cases on the other hand (Table 3-4). Results indicated that the main forage in herds from feed off-flavour clusters was exclusively round-bale silage compared (p<0.01) to the low-rate clusters which fed different forages (from baled or chopped silages to dry hay). Also, there were significant (p<0.01) differences in the timing of feeding forage to lactating cows and in the adequacy of the ventilation system in the barn where the lactating cows were housed: in low-rate clustered herds, the forage was usually fed to the cows only after milking, whereas in the case herds of the high-rate clusters, forage was fed either before milking or as free-choice (i.e. the cows had unlimited access to the silage); and almost all the lactating cows’ barns (8 herds out of 11) in the high-rate clustered herds had inadequate ventilation systems based on the assessment of the
principal investigator.

The comparison of the herds in the secondary high-rate "rancid" cluster with those in the low-rate clusters (using chi square or t-test statistics) revealed that the proportion of stale cows (i.e. cows in lactation for more than 300 days) in the former (that varied from 30 to 61%) was significantly \( p<0.01 \) higher than in those in the latter (that varied from zero to 21%). Also, the herds in this high-rate cluster were fed mostly dry hay as the main forage (four of the five herds) and their ration was supplemented with soybeans (as a source of fat); whereas for the low-rate clustered herds, the main forage was either baled or chopped silage and soybean supplementation was uncommon (Table 3-4).

The exploration of climate data revealed that the geographic locations of the identified high-rate space-time clusters (Figures 3-5 & 3-6) tended to receive more precipitation than those areas where the low-rate clusters were detected (Figures 3-7 & 3-8). The amount of precipitation received by the high-rate clusters was at least 1.3 times higher than in the low-rate clusters. Also, although not conclusive, the temperature data showed some differences between the high-rate clusters of feed off-flavour positive herds and the primary low-rate cluster. The monthly average temperature in the latter was 8.2; whereas in the "feed" off-flavour clusters, it ranged from 5.6 to 5.9 °C (Table 3-5).

### 3.4. Discussion

The spatial (geographic) distribution (Figure 3-2) suggested that 97.9% of off-flavour
cases in bulk-tank milk occurred in the two western adjacent counties (43.7% and 54.2% for Prince and Queens counties, respectively). However, this observation was in almost perfect agreement with the distribution of the population of the dairy herds across PEI also shown in Figure 3-2.

The primary space-time cluster, as well as one of the statistically significant secondary clusters, was exclusively composed of cases of feed off-flavour. It appeared that in the case herds of the primary cluster and one secondary cluster, round-bale silage was not only the main forage for the lactating cows, but also it was fed either before milking or free choice (i.e. cows had unlimited access to the forage) at the time these herds experienced off-flavour in milk. Whereas in clustered control herds, the main forage was either chopped grass/corn silage or dry hay (about 70%), was fed primarily after the cows were milked (nine of the ten herds). Numerous researchers also established these relationships [16-20].

It was not surprising to find that all of the three high-rate clusters were identified during the first study year. Temporal (monthly) plotting of the case herds showed that there was a decline in the number of off-flavour occurrences over time; from 40 during the first year of the study (September 2000 / August 2001), the number of registered off-flavour case episodes decreased to 22 the second year (September 2001 / April 2002). As the study of the outbreak was being carried out, the producers were regularly provided with the generic knowledge on the multiple flavour defects in fresh raw milk and the preliminary results of the study in the form of recommendations.
The results from the space-time investigation setting the maximum temporal cluster size at 5% (one month) demonstrated no evidence of clustering, whereas those of the study with this parameter set at 20% (4 months) clearly indicated that the off-flavour outbreak was clustered in space and in time, suggesting that the outbreak was rather seasonal, not monthly focused.

It has been reported [21-23] that late lactation cows have greater tendency to produce milk susceptible to lipolysis (rancidity) than early lactation cows, although other authors [24;25] have argued that it is the reduced milk yield that is the important factor accounting for enhanced lipolytic susceptibility of milk produced by advanced lactation cows. In the current study, in secondary clustered positive herds with rancid off-flavour, dry hay as main forage and soybeans supplement were fed to the cows at the time they were identified as cases. A number of researchers [26-29] have established the association between feeding dry-feed diet (such as dry hay) and rancid off-flavour; whereas others [30-32] reported that because soybean meal was a source of polyunsaturated fatty acids, it could be responsible for rancidity in milk.

The seasonal pattern that was observed in this cluster corroborated with Fouts & Weaver’s report [33], which indicated that rancid off-flavour in milk was mostly registered during the coldest months of the year. However, some authors argued that this seasonal pattern per se is not the determining factor, but rather the stage of lactation of a relative majority of the cows and/or the quality of available feeds [27;34]. It is unclear
what the mechanism is for these cases of rancid off-flavour.

Farm management strategies to deal with unfavorable weather conditions (such as cooler temperatures, snowstorm, heavy rainfalls) may be potential sources of predisposing conditions to the production of milk tainted with feed flavour defects. The time frames captured by the case clusters include the period within which PEI experienced extremely bad winter weather conditions. Reports of 2002 made available by Environment Canada [35] and personal communications (with Gerard Morin at Environment Canada - Fredericton) indicated multiple snowstorms across the province over the period of December 2000 - January 2001. It is a common practice in PEI to keep the doors and windows of the barns closed whenever bad weather conditions are forecast; this is often done regardless of the adequacy of the ventilation system. Such practices can result in poor air circulation and accumulation of odours from not only the feedstuffs (particularly forages), but also from the bedding material contaminated with cows' manure and urine. Consequently, the cows can be exposed to these strong odours that are breathed and channeled from the respiratory tract to the milk via the blood. Evidence of the association of such conditions (poor air quality in the housing facilities of lactating cows) with transmitted (or feed) flavour defects in milk has been reported previously [2;17;36].

The associations seen here between the above-mentioned factors and the occurrence of off-flavours in bulk-tank milk are susceptible to confounding bias and therefore further research is needed to look at these factors together in a multivariable statistical model.
3.5. Conclusions

In summary, the present study revealed that there was some level of clustering of off-flavour case herds in PEI both in time (fall-winter months) and in space (Queens and Prince counties). Without controlling for confounding, feeding round-bale silage before milking, inadequate ventilation systems and infrequent cleaning of the bedding material were individual as risk factors associated with the occurrence of off-flavours. The observed seasonal (fall-winter) trends generated hypotheses regarding management and environmental differences that could explain these trends.
3.6. Reference List


Table 3-1. Spatial and temporal clustering analyses of an outbreak of off-flavoured milk in Prince Edward Island for a 20-month study period (Sept 2000 to Apr 2002).

A. Spatial clustering analysis using purely spatial scan statistic

<table>
<thead>
<tr>
<th>Category of cluster</th>
<th>Maximal cluster size</th>
<th>Type of cluster</th>
<th>County of location</th>
<th>Observed number of cases</th>
<th>Expected number of cases</th>
<th>Cluster radius</th>
<th>Relative risk</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-rate</td>
<td>5%</td>
<td>Primary</td>
<td>Prince</td>
<td>5</td>
<td>0.78</td>
<td>7.10 km</td>
<td>6.40</td>
<td>0.005</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Secondary I</td>
<td>Queens/Kings</td>
<td>4</td>
<td>0.63</td>
<td>1.91 km</td>
<td>6.40</td>
<td>0.036</td>
</tr>
<tr>
<td>&quot;</td>
<td>20%</td>
<td>Primary</td>
<td>Prince</td>
<td>5</td>
<td>0.78</td>
<td>5.41 km</td>
<td>6.40</td>
<td>0.009</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Secondary I</td>
<td>Queens/Kings</td>
<td>4</td>
<td>0.63</td>
<td>1.91 km</td>
<td>6.40</td>
<td>0.058</td>
</tr>
</tbody>
</table>

B. Temporal clustering analyses using purely temporal scan statistic

<table>
<thead>
<tr>
<th>Type of cluster</th>
<th>Maximal cluster size</th>
<th>Type of cluster</th>
<th>Time frame</th>
<th>Observed number of cases</th>
<th>Expected number of cases</th>
<th>Cluster radius</th>
<th>Relative risk</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-rate</td>
<td>5%</td>
<td>Primary</td>
<td>Jan. 2001</td>
<td>9</td>
<td>2.97</td>
<td>-</td>
<td>3.03</td>
<td>0.007</td>
</tr>
<tr>
<td>&quot;</td>
<td>20%</td>
<td>Primary</td>
<td>Jan. 2001</td>
<td>9</td>
<td>2.97</td>
<td>-</td>
<td>3.03</td>
<td>0.022</td>
</tr>
</tbody>
</table>
Table 3-2. Space-time clustering analyses of the outbreak of off-flavour in milk in Prince Edward Island for a 20-month study period (Sept 2000 to Apr 2002).

A. Maximum spatial and temporal cluster sizes set at 20%.

<table>
<thead>
<tr>
<th>Category of cluster</th>
<th>Type of cluster</th>
<th>County of location</th>
<th>Time frame</th>
<th>Cluster radius</th>
<th>Obs. case</th>
<th>Exp. case</th>
<th>Pop. at risk</th>
<th>Relative risk</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-rate</td>
<td>Primary</td>
<td>Queens/Kings</td>
<td>Oct. 2000 - Jan. 2001</td>
<td>13.50 km</td>
<td>6</td>
<td>0.94</td>
<td>20</td>
<td>6.40</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>Secondary I</td>
<td>Queens</td>
<td>Oct. 2000 - Jan. 2001</td>
<td>7.11 km</td>
<td>5</td>
<td>0.78</td>
<td>26</td>
<td>6.40</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>Secondary II</td>
<td>Prince</td>
<td>Dec. 2000 - Mar. 2001</td>
<td>10.84 km</td>
<td>5</td>
<td>0.78</td>
<td>13</td>
<td>6.40</td>
<td>0.047</td>
</tr>
<tr>
<td>Low-rate</td>
<td>Primary</td>
<td>Queens</td>
<td>Sept. 2001 - Dec. 2001</td>
<td>16.95 km</td>
<td>0</td>
<td>3.9</td>
<td>22</td>
<td>0.00</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>Secondary</td>
<td>Prince</td>
<td>Oct. 2000 - Jan. 2001</td>
<td>21.12 km</td>
<td>0</td>
<td>3.9</td>
<td>22</td>
<td>0.00</td>
<td>0.106</td>
</tr>
</tbody>
</table>

B. Maximum spatial cluster size set at 20% and temporal at 5%.

| High-rate           | Primary         | Queens             | Jan. 2001       | 9.77 km        | 3         | 0.47      | 39          | 6.40         | 0.158   |
|                     | Secondary       | Prince             | Jan. 2001       | 9.59 km        | 3         | 0.47      | 13          | 6.40         | 0.158   |

1 Observed number of cases within the cluster for the corresponding time frame; 2 Expected number of cases within the cluster for the corresponding time frame; 3 Population at risk; 4 More likely clusters of case herds; 5 More likely clusters of non-case (control) herds; 6 Intersection of Queens and Kings Counties.
Table 3-3. Comparison of high- and low-rate clusters using some herd-level risk factors for feed off-flavour in Prince Edward Island herds for a 20-month study period (Sept 2000 to Apr 2002).

<table>
<thead>
<tr>
<th>Risk factors for feed off-flavour in bulk tank milk</th>
<th>Proportion of clustered herds with the risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary &amp; Secondary high-rate cluster (Feed flavour cases)</td>
</tr>
<tr>
<td></td>
<td>Primary</td>
</tr>
<tr>
<td>Feeding round-bale silage</td>
<td>6/6</td>
</tr>
<tr>
<td>Feeding forage as free-choice (or before) milking</td>
<td>5/6</td>
</tr>
<tr>
<td>Inadequacy of the ventilation system in the cows' barn</td>
<td>4/6</td>
</tr>
<tr>
<td>Changing bedding material once a day (or less)</td>
<td>4/6</td>
</tr>
<tr>
<td>Not clipping hair on the lactating cows’ udder</td>
<td>4/6</td>
</tr>
</tbody>
</table>
Table 3-4. Comparison of herd-level risk factors in the 2nd secondary high-rate cluster (rancid off-flavour) with low-rate clusters for rancid off-flavour (*Literature review*).

<table>
<thead>
<tr>
<th>Risk factors for rancid off-flavour in bulk tank milk</th>
<th>Proportion of clustered herds with the risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2nd Secondary high-rate cluster</td>
</tr>
<tr>
<td></td>
<td>(Rancid flavour cases)</td>
</tr>
<tr>
<td></td>
<td>(N = 4/5)</td>
</tr>
<tr>
<td>A. Dichotomous variables (Chi-square statistic)</td>
<td></td>
</tr>
<tr>
<td>Feeding DRY HAY as main forage</td>
<td>4/4</td>
</tr>
<tr>
<td>Feeding soybean supplement</td>
<td>4/4</td>
</tr>
<tr>
<td>B. Continuous variable (t-test statistic)</td>
<td></td>
</tr>
<tr>
<td>Mean of the proportion (%) of stale cows in the clustered herds</td>
<td>39.4</td>
</tr>
</tbody>
</table>
Table 3-5. Climate data for the space-time clusters (high- and low-rate) detected in the study of the off-flavour outbreak in Prince Edward Island for the time frame within which these clusters were identified.

<table>
<thead>
<tr>
<th>Cluster category</th>
<th>Cluster Type</th>
<th>Geographic location</th>
<th>Corresponding clustered time frame</th>
<th>Monthly average precipitation (mm)</th>
<th>Monthly average temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH-RATE (^2)</td>
<td>Primary</td>
<td>Queens/Kings(^1)</td>
<td>Oct. 2000 – Jan. 2001</td>
<td>152.0</td>
<td>5.9</td>
</tr>
<tr>
<td>LOW-RATE (^1)</td>
<td>Primary</td>
<td>Queens</td>
<td>Sept. 2001 – Dec. 2001</td>
<td>66.2</td>
<td>8.2</td>
</tr>
</tbody>
</table>

\(^1\) Intersection of Queens and Kings Counties.

\(^2\) More likely clusters of case herds;

\(^3\) More likely clusters of non-case (control) herds.
Figure 3-1. Temporal distribution of the off-flavour positive herds in Prince Edward Island (Atlantic Canada) over the 20-month study period (September 2000 - April 2002).
Figure 3-2. Distribution of the dairy herds and off-flavour positive herds across PEI counties over the 20-month study period (September 2000 – April 2001).
Figure 3-3. High-rate (most likely) space-time clusters of off-flavours in -tank milk identified in PEI during a 20-month study period (September 2000 – April 2001)

- **Primary (p<0.05) HIGH-RATE Cluster** (constituted of cases of feed off-flavour)
- **Second Significant (p<0.05) Secondary HIGH-RATE Cluster** (mostly constituted of cases of rancid off-flavour)
- **First Significant (p<0.05) Secondary HIGH-RATE Cluster** (constituted of cases of feed off-flavour)

**Figure Legend**
- **DAIRY HERDS**
  - Control
  - Case
- **PEI COUNTIES**
  - Prince
  - Queens
  - Kings

**Scale**: 0 30 60 Kilometers
Figure 3-4. Low-rate (less likely clusters) space-time clusters of herds identified in the study of geographical and temporal aspects of milk off-flavours in PEI during a 20-month study period (September 2000 – April 2001).
Figure 3-5. Average monthly precipitation in Prince Edward Island from October 2000 to January 2001

Primary (most likely) significant (p<0.05) HIGH-RATE cluster constituted only of herds that experienced feed off-flavour - Queens/Kings County

Secondary significant (p<0.05) HIGH-RATE cluster constituted only of herds that experienced feed off-flavour - Queens County
Figure 3-6. Average monthly precipitation in Prince Edward Island from December 2000 to March 2001.

Secondary significant (p<0.10) HIGH-RATE cluster constituted predominantly of herds that experienced rancid off-flavour.

Legend:
- Precipitation (mm)
  - 70 - 80
  - 80 - 89
  - 89 - 99
  - 99 - 108
  - 108 - 117
  - 117 - 127
  - 127 - 136
  - 136 - 146

Map scale: 0-60 Kilometers

Compass directions:
Figure 3-7. Average monthly precipitation in Prince Edward Island from September 2001 to December 2001

Primary significant (p<0.10) LOW-RATE (less likely) cluster - Queens County
Figure 3-8. Average monthly precipitation in Prince Edward Island from October 2001 to January 2001.
CHAPTER 4

RISK FACTORS FOR MILK OFF-FLAVOURS IN DAIRY HERDS
FROM PRINCE EDWARD ISLAND, CANADA

In Press: Preventive Veterinary Medicine
Abstract

A sudden increase in the incidence of milk off-flavours in bulk-tank milk from Prince Edward Island (Canada) dairy farms in the late 1990s prompted an investigation of potential herd-level risk factors. A prospective case-control study was conducted from 2000 to 2002. Data on herd management were obtained by questionnaire and field investigation from all the 62 identified off-flavour positive farms (cases) and 62 loosely matched (for data-collection convenience) off-flavour negative farms (controls). Forty-three of the 62 cases (69%) of milk off-flavours identified during the study period were classified as "transmitted" (feed) off-flavours, and 9 (15%), 6 (10%), and 4 (6%) as "rancid," "oxidized" and "malty" off-flavours, respectively. Given this evidence and the relatively low incidence of other flavour defects in milk, only transmitted-flavour cases were considered in the analyses of risk factors. Poor air quality in the lactating cows' barn (OR = 40.8), using baled silage as the main forage (OR = 10.6), as well as feeding roughage before milking (OR = 253.3) or as a free choice (OR = 3.2) all were significantly (p<0.05) associated with the incidence of transmitted flavours in bulk-tank milk. Clipping the hair on the cows' udder (OR = 0.07) and changing the bedding material more than once a day (OR = 0.12) were protective. The finding about feeding baled silage before milking has generated etiological hypotheses about silage composition (in particular the off-flavour compounds or their precursors) and also about the process of silage making itself.
**Key words:** Milk off-flavour, feed, transmitted, oxidized, rancid, malty, silage, organoleptic.

**Abbreviation keys:** PEI = Prince Edward Island; OR = Odds Ratio; BIC = Bayesian Information Criterion; PAF = Population Attributable Fraction.
4.1. Introduction

Flavour is of paramount importance to the dairy industry because of the impact it has on the acceptance of milk and other dairy products [1]. Milk of good quality is a very bland food with a slightly sweet taste, very little odour, and a smooth, rich feel in the mouth. Milk's bland taste makes it susceptible to flavour defects (off-flavours) from a variety of sources [2]. Milk off-flavours are a common problem particularly in the Northern Hemisphere where the use of stored forages and other food supplements is common. Although most forms of off-flavoured milk have not been shown to be harmful to public health, flavour-quality assessment of non-pasteurized milk was made mandatory on Prince Edward Island (PEI), to enhance consumers' confidence in milk products. The PEI dairy industry reported considerable economic loss due to a sudden increased incidence of off-flavours in non-pasteurized milk in the 1990s. Approximately 16.5% of the dairy herds on the island were reported to have experienced at least one episode of milk off-flavour during the 1999-winter season (unpublished data from the dairy industry).

Based on the nomenclature developed by the American Dairy Science Association (ADSA), milk off-flavours can be classified using the following categories: "oxidized," "rancid," "malty," "transmitted" and "chemical" [3;4].

Transmitted flavours, also known as "feed" off-flavours, are described as defects caused by the transfer of aromatic substances either from the cows' feed or their surroundings through the respiratory or digestive systems to the bloodstream and then into the milk [4;5]. A review of available literature regarding flavour defects of bulk-tank milk [2;5-
10] provides very little insight as to why sudden increases in the incidence of these off-flavours occur in certain localities, and how best to predict and prevent them. While many herd- and farm-level factors have been associated with milk off-flavours in the literature, these associations have never been based on rigorously designed epidemiological studies. Most extensive studies have been focused on oxidation [11-16] and rancidity [17-20] in milk, with less attention paid to “transmitted” off-flavours, which have become the most-pressing problem in PEI dairy herds.

It was hypothesized that the observed increase in the incidence of milk off-flavours in PEI dairy farms was related to specific nutritional, farm management or environmental factors. The objectives of the current study were:

1) to identify the categories of milk off-flavour that were most common in PEI;

2) to determine the differences in herd management practices associated with the occurrence of milk off-flavours on PEI dairy farms.

4.2. Materials and methods

4.2.1. Farm identification and selection

Herd was the study unit, and the study area was the province of PEI, Canada. Over a 2-year study period (September 2000 – April 2002), one hundred and twenty-four PEI dairy farms (62 case farms and 62 matched control farms) were registered for the study. A farm
was considered a case if an off-flavour was identified in its bulk-tank milk, resulting in
the condemnation of that tank-load; the same farm could be considered in the case group
more than once if, between two consecutive milk condemnations, there was at least a 1
month interval. We used computer-generated random numbers to select control herds
from those dairy herds with the same telephone exchange index (1st three digits of the 7-
digit telephone number), and which had not had an off-flavour in the previous month.
The pool of eligible controls was expanded to the county level if there was no farm with
the same exchange index. Consequently, it was a loose matching, which was considered
only for data collection convenience, because the principal investigator (A. Mounchili)
usually scheduled farm visits for both case and control farms on the same day.

4.2.2. Farm classification of “off-flavour”

Certified milk graders (milk-truck drivers and the milk receiving personnel at the dairy
processing plant) routinely assess the flavour quality of bulk-tank milk in PEI. The
drivers perform the assessment on-farm prior to collection by sniffing and tasting a
sample obtained from the producer’s bulk-tank. This is usually done in the milk-tank
room (or outside the barn if the milk-tank room appeared not to be odour-free). Only milk
classified as “acceptable” (milk without any objectionable taste or odour) is transferred
from the farm’s bulk-tank to the truck and transported to the processing plant. When milk
in a bulk-tank is suspected or classified as off-flavoured, it is not pumped into the truck’s
tank. A representative 1-liter sample is submitted in a sealed plastic container to a
receiver at the plant for reassessment. If the receiver confirms the suspect off-flavour, the
milk is rejected. But, if the suspect sample is found to be acceptable by this receiver, then a third qualified milk grader at the plant is asked to assess the sample for final classification (i.e. simple majority vote). Occasionally, an entire truckload of milk is rejected for off-flavour at the plant even though all component milk collected on the trip (derived from 1-4 producers) was classified as “acceptable” by the driver at the farm. In these cases, milk samples from the individual bulk tanks are retrospectively reassessed as above by up to 3 certified graders and if one is found to be off-flavoured, that farm is classified as a case for the purpose of the study.

4.2.3. Data and sample collection

Within ≤2 hours after identification of a case of milk off-flavour, dairy company personnel notified the principal investigator. The latter immediately scheduled an on-farm investigation based on the availability of the herds’ (case and control) owners within the following 24 to 72 hours. The order of farm-visits for these two farms depended on the schedule agreed upon with the case herd owner. There were instances where the visit to control herds was rescheduled for another day when the owners were not available on the specified day. Using a ten-page closed questions questionnaire (completed by A. Mounchili during the farm visit), data were recorded on approximately 50 variables related to the herd’s characteristics, management, nutritional management, health status and forage cropping management (complete questionnaire available as Appendix A). The main forage fed to lactating cows at the time when the problem occurred and the water in the barn, were sampled and stored at -20°C until laboratory analyses were completed.
The forage was sampled using a core sampler and for water, it was allowed to run for approximately 2 min prior to taking a sample in a sterile plastic container.

4.2.4. Chemical analyses

Laboratory analyses were carried out by the Soil and Feed Testing Laboratory of the PEI Department of Agriculture and Forestry (Charlottetown PEI, Canada). Forages were analyzed for moisture, pH, crude protein (CP) and bound protein (BP) using CHN-2000 and CHN-600 Elemental Analyzers (LECO Corp., St Joseph: MI, USA), and mineral content (Ca, P, Mg, K, Cu, and Zn) [21] using Inductively Coupled Argon Plasma Spectrometer (Genesis Laboratory Systems, Inc. Colorado, USA). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an ANKOM Fiber Analyzer (ANKOM Technology, Fairport, NY) according to the methodology supplied by the company, which is based on the methods described by Van Soest and co-workers [22]. A full range of chemical analyses of water was performed, but only three parameters (Cu, Fe & Zn), known as pro-oxidants that can contribute to the development of oxidized off-flavour in milk [15;23;24], were of major interest for the present study.

4.2.5. Statistical analyses

4.2.5.1. Data screening and transformation

Questionnaire data and laboratory data (results of forage and water analyses) were
managed using a spreadsheet and descriptive statistical analyses were carried out with STATA 7.0 [25]. Only 43 case herds (for which the flavour defect was characterized as “transmitted” off-flavours) and their controls were considered for subsequent analytical statistics. Following data-quality checks, performed using descriptive statistics, some variables likely to be subject to a recording bias were excluded from further investigation (for example the weather conditions the day before and the day of the occurrence of the off-flavour problem, manure consistency, changes in milk production and in milk components, and the presence of unusual components in the feed).

The variable representing “forage fed to lactating cows” was dichotomized into herds feeding round-bale silage and those that were not (combination of dry hay and chopped silage), because relatively few farms were using these other types of forage. The schedule of feeding forage to lactating cows was also converted into a three-level variable: feeding forage <2 hours before milking, feeding forage as a free choice (i.e. the animals had access to forage continuously) and feeding forage only after milking. Also, in the process of laying out the causal-web model [26], variables describing (measuring) the same management procedures were grouped together and their internal reliability was established using Cronbach’s reliability test [27]. Cronbach’s Alpha measures the level of correlation between a set of independent variables recorded at the same time. The widely accepted cut-off of Cronbach’s alpha for a set of variables to be combined as a block (composite variable) is 0.70 or higher [28]. Three blocks were identified: “cow hygiene,” “air quality in the barn” and “dairy hygiene management,” but only “cow hygiene” yielded a Cronbach’s alpha greater than 0.70 (Table 4-1); therefore, the predictors that constituted the other blocks were considered in the analyses as initially recorded.
4.2.5.2. Univariable analyses

Potential risk factors were screened using univariable statistical methods for association with the outcome under investigation. A total of 64 variables (48 from the questionnaire, 13 and 3, respectively, from the results of laboratory analyses of silage and water samples) were screened. Continuous and categorical predictors were assessed using the $t$-test statistic and the $\chi^2$-test of independence, respectively. Only predictors showing an association with the occurrence of "transmitted" off-flavour at $p<0.20$ were candidates for subsequent multivariable analyses. These predictors were assessed for missing values and only two predictors (poor air quality in the barn and poor air quality in the milking-tank room) had one missing value each.

Prior to univariable assessment, continuous predictors were categorized into three or more levels to investigate the linearity of their relationship with the outcome of interest to check whether these predictors were to be used in the analysis as recorded or if they needed to undergo some transformation before being used. Following univariable screening analysis, all pair-wise correlations among unconditionally significant ($p<0.20$) predictors were examined using Kendall's rank correlation coefficients (dichotomous predictors), and, for any highly correlated (correlation coefficients greater than 0.70) pair of variables, only the variable for which the association with the outcome made the most biological sense was considered for further statistical analyses.
4.2.5.3. Multivariable analyses

Variables that were associated in univariable analyses at a significance level $p<0.20$ were entered in ordinary logistic regression models for multivariable model building. A backward stepwise elimination [29] was adopted. The outcome variable indicated whether the farm produced milk with a transmitted flavour defect or not. The predictor or a subset of dummy variables (representing one predictor) that had lowest significance was removed sequentially, based on the Wald’s test or the likelihood ratio test, and only predictors significant at $p<0.05$ were retained in the final model. Due to the fact that air quality in the lactating cows’ barn was assessed using either the experience of the principal investigator and the adequacy of the ventilation system, two separate statistical models were considered: one with each of the two predictors.

Predictors were evaluated for confounding by following the principle of adding and removing variables [30]. A predictor was kept in the final models if its removal resulted in a fluctuation of $>25\%$ in the magnitude of one or more other coefficients [29]. Interaction was examined by the addition of biologically meaningful, two-way interaction terms between main effect variables in the final multivariable model and statistically evaluating their effect. Interaction terms that provided a significant reduction in model deviance as measured by likelihood ratio test statistic ($\chi^2: p<0.05$) were retained.
4.2.5.4. Post-fit diagnostics on final multivariable models

The fit of the final multivariable models was assessed using the Hosmer-Lemeshow goodness-of-fit of test. The final models were assessed for their robustness by inspecting the standardized residuals, leverage values, delta-deviances and delta-betas. The models were refitted following sequential exclusion of the observations with the largest delta-beta (i.e. those observations whose exclusion were predicted to have the largest influence on the fit of the model) [31]. Models were considered stable and robust when removal of the observations had no substantial effect on any of the coefficients or their level of significance.

The likelihood-ratio test and the Bayesian information criterion (BIC) index [32] were used to compare nested and non-nested models, respectively.

Because of the case definition, which allowed the inclusion of repeated episodes as new cases (if there was one month interval between consecutive episodes), the effect of these repeated cases was assessed by refitting an additional multivariable logistic regression model without the episodes and their respective controls.

4.3. Results

Over the 20-month study period starting from September 2000, 104 individual bulk-tank loads were rejected at the farm level (73 the first year and 31 the following year (Figure
2-1). These 104 loads represented 149,217 liters of milk. Also, a total of 17 truckloads of milk were rejected during the same season, representing 173,663 liters. Rejected truckloads represented cases of off-flavour that could not be identified at the farm level (false negative). Consequently, considerable reduction in milk rejections was observed during the second year of the study (from 17 truck loads rejected the first year to none, and from 73 individual-farm rejections to 31). Overall, for the study period, there were 92,100 bulk-tanks at risk of off-flavour, with the total number of recorded rejections at 121 (bulk-tanks and truckloads). Thus the incidence rate was about 1.3 per 1000 bulk-tanks. This value is relatively underestimated as its computation included only the rejections that were recorded by the processing industry; and there were situations where the milk was disposed (because of off-flavour) by the producers, but not recorded by the industry.

The discrepancy between the number of rejected loads (104) of milk and the number of registered cases (62) is explained by the fact that some herds registered repeated episodes of milk off-flavours within a relatively short period (<1 month apart). A total of four herds had multiple (13) cases: two were registered as cases four times each, and the two others, twice and thrice, respectively. Also, only three herds initially considered as controls, were reported as off-flavour positive during the study period (after they had served as controls).

During the study, 97 % of case and control herd owners agreed to participate. The most-frequently reported type of flavour defect in milk during the study was “transmitted” off-
flavour (43 of 62 cases; 69 %). There also were 9 (14 %) “rancid” off-flavour, 6 (10 %) “oxidized” and 4 (6.5 %) “malty” off-flavours. Given the relatively low incidence of rancid, oxidized, and malty off-flavours, we restricted the analyses to “transmitted” off-flavours (43 cases and their respective controls).

The median herd size of milking cows on the farms involved in the study was 34 (9 to 166 lactating cows) for case herds and 39 (11 to 147 lactating cows) for control herds. Milk production ranged from 8 to 32 liters per cow per day for case farms, and 7 to 39 liters per cow per day for control farms. The lactating cows were housed mostly in tie-stall barns; 31 (72 %) for case farms and 33 (77 %) for the controls. Twelve predictors were unconditionally associated \( (p<0.20) \) with “transmitted” off-flavour in PEI dairy herds: ten management factors (Table 4-2) and two laboratory parameters of the forages (calcium and protein solubility levels). Pair-wise correlation analyses suggested that two (air quality in the lactating cows’ barn and air quality in the milk-tank room were highly correlated; consequently, only the predictor representing air quality in the lactating cows’ barn was considered for multivariable analysis.

Multivariable statistical analyses resulted in a model with five significant \( (p<0.05) \) variables (Table 4-3): timing of feeding stored forage, feeding baled silage as the main forage, poor air quality in the barn, clipping udders and the frequency of changing the bedding material. Multicollinearity among these variables in the final model was assessed. The highest variance inflated factor (VIF) was 1.35 and the lowest was 1.08,
suggesting that multicollinearity was not a problem. Also, no interactions between these variables were observed, but this may have been due to the limited power in the study. Because we believed that the measurement of “working status of the ventilation system” was less subjective than the assessment of air quality, another statistical model with the variable “air quality in the lactating cows’ barn” replaced by the variable “working status of the ventilation system” was built for comparative purposes. This latter variable was dichotomized into adequate vs. inadequate. In the resultant model, the effect of the variable representing the frequency of changing bedding material each day became only half strong as in the previous model (OR = 0.30) and lost statistical significance (Table 4-3). All the other predictors in the model remained statistically significant, although the magnitude of their individual effects changed (Table 4-3). These two models were compared using the BIC. The BIC difference of +8.28 was a strong indication that the model, including the subjective measure of air quality (not the ventilation system), was more likely to have generated the observed data.

Population-attributable fraction (PAF) for the variable representing “timing of feeding roughage to lactating cows” was estimated [33], and the results suggested that 70% of the cases of “transmitted” off-flavours would not have happened, had all the lactating cows in the studied herds been fed roughage only after milking.

Statistical analyses on the reduced dataset (i.e. did not include repeated cases and their respective controls) yielded a multivariable model structurally similar to that of the full dataset, but with the variables having different magnitude (Table 4-5).
The variable representing changing bedding material more than once a day was identified as a confounder as it was associated with both the outcome of interest and another explanatory variable (air quality in the lactating cows’ barn), and its removal from the final multivariable statistical model resulted in substantial changes in the magnitude of the remaining variables. No two-way interaction terms were found statistically significant; and all post-fit diagnostics yielded results that suggested no lack of fit of the statistical models.

4.4. Discussion

The impact of the phenomenon of off-flavours goes beyond the resulted low incidence of bulk-tank rejection (1.3 per 1000); while this would appear to be a rather low incidence, it does not reflect the potential psychological effect on individual dairy producers (especially small scale producers whose monthly income depends largely on the milk production) and most importantly on the consumers, who may lose confidence in milk and milk products. It has also impacted the relationship between the milk-tank operators and the dairy producers, who have not always been cooperative with the negative evaluations of the former (because of the subsequent condemnation without compensation). Therefore, although economically, the off-flavour outbreak seemed of less importance, it had a potential of generating a substantial negative effect on the industry as a whole.
The strong association between feeding baled silage and milk off-flavours has been established already in numerous publications [10;34-38]. However, none of those studies addressed the effect of different forms of silage under commercial dairy farming conditions. Approximately 81% of the farms in the present study were feeding round-bale silage. In this respect, the studied population was typical of PEI dairy herds, because VanLeeuwen and Keefe [39] reported that approximately 70% of PEI dairy herds were using round-bale silage as the main forage during the fall-winter period when pasture becomes unavailable.

Although none of the chemical parameters of forage and water was retained in the final multivariable model, it is important to point out that silages from both case and control herds had intermediate levels of calcium [40], suggesting that they were made of a mixture of grass and legumes. This finding was in agreement with the assessments of both the producers (during data collection) and the PEI Soil and Feed Testing Laboratory. The most-common mixture for silage used by these herds was the so-called “triple-mix:” timothy (Phleum pratense), alsike clover (Trifolium hybridum) and red clover (Trifolium pratense L.). Silages from the control herds had significantly higher calcium levels than those from the case herds suggested that mixed grass-legume silages with higher legume content were (unconditionally) less likely to be associated with off-flavour; so were silages with lower solubility (Table 4-4).

Spoilage of silage (as would be indicated by elevated pH) was not observed and consequently did not appear to be associated with off-flavour. Silages from case herds
had higher solubility levels than those from control herds, which might suggest that the fermentation process in those silages was slower than in silages from control herds. This may have allowed other microorganisms to compete with lactic acid bacteria, leading to the formation of alcohols such as ethanol (known to be a source of off-flavour in milk [41], ketones, esters and acids). A prolonged fermentation may also allow microorganisms to extensively degrade plant proteins to highly soluble components such as short chain peptides, amino acids and ammonia. Complete elucidation of this process requires additional research concerning the microbial populations and fermentation products in round-bale silages in a similar study.

As early as 1938, it was recognized that inhalation of aroma-active compounds by lactating cows resulted in transference to the mammary gland [6]. Also, several other authors [2;2;35] demonstrated that exposure to odours in closed buildings results in the transfer of the volatile compounds that generate these odours to the mammary gland. Consequently, the finding of our study that poor air quality in the barn was associated (OR=41) with “transmitted” off-flavours was not surprising. Feeding baled silage to cows prior to milking in barns with poor air quality leads to a high risk of off-flavour in milk.

It appeared that herds in which the producers were clipping hair on the cow’s udder and changing the bedding material more than once a day were less likely to produce off-flavoured milk. These management practices could be viewed as surrogate measurements of hygiene management.
Neither milk production nor bulk-milk somatic cell counts were significantly different between the groups \( (p>0.20) \). Herd size and breed were not associated with "transmitted" off-flavour. Holstein Friesian was the only breed in the study herds.

Unlike oxidized off-flavour in milk, supplementation with vitamin E (and/or selenium) was not preventive \([12;42;43]\) for transmitted off-flavours; suggesting that the compounds and/or the mechanism involved in the development of oxidized off-flavour in milk are different from those involved in transmitted off-flavour.

The incidence of transmitted flavours in bulk-tank milk was plotted over time; 88\% of the cases of transmitted off-flavour were recorded during fall-winter season (Figure 2-1). We also realized that there was a substantial decrease (of about 57\%) in the number of rejected bulk-tank loads during the second year of the study. We believed that this might have been largely due to the series of oral and written communications on generic knowledge about the causes and types of off-flavours, combined with our preliminary study findings, which were made available to the dairy producers after the 1st study year.

The investigator was not blind to the case-control status of the herd when he performed the assessment of factors such as air quality in the barn or the level of cleanliness of the cows. This might have led to the introduction of a non-differential misclassification bias, which might have biased the observed effects toward or away from the null.

The identification of case herds was solely based on the judgment of the milk graders. As a result, this process might be susceptible to 2 forms of misclassification bias because of
the subjective nature of the organoleptic assessment. First, some cases may have been missed, while other so-called cases may have been non-cases; however, verification at the processing plant would minimize but not eliminate false positive [44]. In the first year of the study, a substantial number of truck-loads (17) of co-mingled bulk-tank milk (from more than one farm), which had been deemed to be free of any off-flavour at the farm level, tested positive to milk off-flavour at the platform of the dairy processing plant at reception. This did not greatly affect the results of the study because the problem farms were identified in all the 17 cases by re-evaluating, organoleptically, the individual milk samples from the bulk-tank that contributed to those condemned truckloads. The truck testing (upon arrival at the dairy processing plant) acted as a second level of case detection, giving fewer false negative and misclassification bias. This misclassification bias may have resulted in either over or underestimation of the coefficients of the predictors in the final model, because only “more severe” cases would have been included in the study. On the other hand, any misclassification of non-cases (off-flavour negative) would have resulted in the reduction in the OR of the predictors in the final model.

Another potential source of misclassification bias was the categorization of off-flavours. Misclassification of some off-flavours as “transmitted” when they were, in fact other types, or vice versa, would have reduced the power of the study by making the case-group less homogenous. Consequently, the estimated effects of risk factors might be conservative estimates.
However, the magnitude and significance of the associations between the predictors (such as “feeding roughage before milking” and “feeding round-bale silage”) in the final models and off-flavour were so strong that we believe the impact of the above-mentioned biases could not have played an important role in the results of this study.

Based on personal communications (dairy industry), the findings about low incidence of other flavour defects in bulk-tank milk were consistent with previous-years’ industry records. The association of this outbreak of off-flavours in bulk-tank milk with feeding baled silage to lactating cows was less surprising because there has been a marked increase in the use of round-bale silage in PEI over the last 15 – 20 years [39]. Chopped silage and dry hay used to be the primary forages of choice.

4.5. Conclusions

Results of the current study (prompted by a substantial increase in the incidence of milk off-flavours observed on PEI dairy farms during the years 1999 – 2000) lend strong support to the findings of the study of the temporal and geographical distribution of the registered cases that hypothesized that the outbreak was a product of changes in management (herd and nutritional) and environmental factors. It revealed that most flavour defects in bulk tank milk were those known as transmitted (or “feed”) off-flavours; and that among the associated risk factors, the strongest associations were: timing of feeding forage (<2 hours before milking or feeding forage as a free choice), feeding baled mixed timothy silage to lactating cows, poor air quality in the lactating cows’ barn. The study determined that the relative measures of dairy hygiene
management (clipping hair on the cows’ udder and changing the bedding material more than once a day) were protective against these flavour defects.
Acknowledgements:

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4.6. Reference List


Figure 4-1. Registered cases of milk off-flavour categories during the two-year study period (September 2000 – April 2002)

- Malty - 4 cases
- Oxidized - 6 cases
- Rancid - 9 cases
- Transmitted - 43 cases

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Table 4-1. Evaluation of Cronbach’s Alpha (coefficient of collinearity) for the predictors describing the same factor (three blocks of explanatory variables).

<table>
<thead>
<tr>
<th>Within-block factor (= item)</th>
<th>Obs.</th>
<th>IRC ^1</th>
<th>AIC ^2</th>
<th>Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Block 1: COW HYGIENE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soiling above the fetlock</td>
<td>86</td>
<td>0.83</td>
<td>0.72</td>
<td>0.89</td>
</tr>
<tr>
<td>Soiling above the hocks</td>
<td>86</td>
<td>0.88</td>
<td>0.70</td>
<td>0.88</td>
</tr>
<tr>
<td>Soiling on the flank</td>
<td>86</td>
<td>0.70</td>
<td>0.83</td>
<td>0.94</td>
</tr>
<tr>
<td>General cleanliness of cow</td>
<td>86</td>
<td>0.86</td>
<td>0.72</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>TEST SCALE</strong></td>
<td></td>
<td></td>
<td>0.74</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>Block 2: DAIRY HYGIENE MANAGEMENT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clean stall</td>
<td>86</td>
<td>0.18</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>Stall bedded</td>
<td>86</td>
<td>0.23</td>
<td>0.04</td>
<td>0.13</td>
</tr>
<tr>
<td>Clipped hair on udders</td>
<td>86</td>
<td>0.10</td>
<td>0.12</td>
<td>0.29</td>
</tr>
<tr>
<td>Changing bedding material more than once</td>
<td>86</td>
<td>0.08</td>
<td>0.13</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>TEST SCALE</strong></td>
<td></td>
<td></td>
<td>0.09</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Block 3: AIR QUALITY IN LACTATING COWS’ BARN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air quality in the barn (as recorded initially)</td>
<td>85</td>
<td>0.44</td>
<td>0.24</td>
<td>0.38</td>
</tr>
<tr>
<td>Good ventilation system</td>
<td>85</td>
<td>0.59</td>
<td>0.07</td>
<td>0.13</td>
</tr>
<tr>
<td>Feeding forage in the barn</td>
<td>85</td>
<td>0.17</td>
<td>0.63</td>
<td>0.77</td>
</tr>
<tr>
<td><strong>TEST SCALE</strong></td>
<td></td>
<td></td>
<td>0.31</td>
<td>0.57</td>
</tr>
</tbody>
</table>

^1 Number of observations;
^2 Item-rest correlation;
^3 Average inter-item correlation.

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Table 4-2. Management factors unconditionally associated \( p<0.20 \) with “transmitted” off-flavour in milk from PEI dairy herds from 2000 to 2002.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Case (n = 43)</th>
<th>Control (n = 43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor air quality in the housing area</td>
<td>Yes 25 58.1</td>
<td>Yes 4 9.3</td>
</tr>
<tr>
<td>Poor air quality in the milk tank room</td>
<td>Yes 11 55.8</td>
<td>Yes 0 0.0</td>
</tr>
<tr>
<td>Component feeding system (vs. total mixed ration)</td>
<td>43 100</td>
<td>38 88.4</td>
</tr>
<tr>
<td>Feeding baled silage as main forage</td>
<td>Yes 42 97.7</td>
<td>Yes 28 65.1</td>
</tr>
<tr>
<td>Working status of the mechanical ventilation system</td>
<td>17 39.5</td>
<td>2 4.7</td>
</tr>
<tr>
<td>Vitamin/Selenium supplement to lactating cows</td>
<td>Yes 7 16.3</td>
<td>Yes 1 2.3</td>
</tr>
<tr>
<td>Time of feeding roughage to lactating cows</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- After milking</td>
<td>Yes 8 18.6</td>
<td>Yes 34 79.1</td>
</tr>
<tr>
<td>- &lt; 2 hours before milking</td>
<td>Yes 21 48.8</td>
<td>Yes 2 4.7</td>
</tr>
<tr>
<td>- Free-choice feeding</td>
<td>Yes 14 32.6</td>
<td>Yes 7 16.3</td>
</tr>
<tr>
<td>Apparent soiling of the udders</td>
<td>Yes 12 27.9</td>
<td>Yes 4 9.3</td>
</tr>
<tr>
<td>Clipped hair on cows’ udders</td>
<td>Yes 9 20.9</td>
<td>Yes 21 51.2</td>
</tr>
<tr>
<td>Changing bedding material &gt; 1 time a day</td>
<td>Yes 16 37.2</td>
<td>Yes 9 21.0</td>
</tr>
</tbody>
</table>

\* Percentage of herds with the risk factor
Table 4-3. Logistic regression models that resulted from the multivariable analyses of risk factors for “transmitted” off-flavour using the full dataset (that included 43 cases) collected from September 2000 to April 2002.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Model with the subjective measurement of air quality</th>
<th>Model with “adequacy of the ventilation system” as the measure of air quality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Feeding baled silage as main forage</td>
<td>11</td>
<td>0.8, 148</td>
</tr>
<tr>
<td>Time of feeding roughage to lactating cows</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- After milking (Baseline)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- Before (&lt; 2 hours) milking</td>
<td>253</td>
<td>12, 518</td>
</tr>
<tr>
<td>- As free choice</td>
<td>3.2</td>
<td>0.5, 20</td>
</tr>
<tr>
<td>Clipping hair on cows’ udders</td>
<td>0.07</td>
<td>0.01, 0.6</td>
</tr>
<tr>
<td>Changing bedding material &gt; 1 time a day</td>
<td>0.12</td>
<td>0.01, 0.2</td>
</tr>
<tr>
<td>Poor air quality in the lactating cow barn</td>
<td>41</td>
<td>4.7, 352.4</td>
</tr>
<tr>
<td>Working status of the ventilation system</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> p-value for overall assessment of the variable time of feeding forage to lactating cows (with feeding after milking considered as the baseline).
Table 4-4. Descriptive and comparative analyses of the laboratory results of silages that were used by the herds enrolled in the study of risk factors for off-flavour in milk on PEI dairy herds from September 2000 to April 2002.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases (n = 42)*</th>
<th>Controls (n = 27)*</th>
<th>p(t)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>95% CI</td>
<td>Mean</td>
</tr>
<tr>
<td>PH</td>
<td>5.2</td>
<td>5.1, 5.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Dry matter (%)</td>
<td>51</td>
<td>48, 53</td>
<td>51</td>
</tr>
<tr>
<td>Crude protein (% DM(^a))</td>
<td>13.4</td>
<td>13, 14</td>
<td>13.6</td>
</tr>
<tr>
<td>Bound protein (%DM)</td>
<td>8.3</td>
<td>7.0, 9.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Acid detergent fiber (%DM)</td>
<td>32</td>
<td>31, 33</td>
<td>33.4</td>
</tr>
<tr>
<td>Neutral detergent fiber (%DM)</td>
<td>52</td>
<td>50, 54</td>
<td>53.7</td>
</tr>
<tr>
<td>Solubility (%CP(^b))</td>
<td>50</td>
<td>46, 54</td>
<td>44</td>
</tr>
<tr>
<td>Calcium (% DM)</td>
<td>0.60</td>
<td>0.52, 0.70</td>
<td>0.71</td>
</tr>
<tr>
<td>Phosphorus (% DM)</td>
<td>0.27</td>
<td>0.25, 0.29</td>
<td>0.26</td>
</tr>
<tr>
<td>Magnesium (%DM)</td>
<td>0.20</td>
<td>0.18, 0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>Potassium (% DM)</td>
<td>2.1</td>
<td>2.0, 2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Copper (ppm)</td>
<td>6.9</td>
<td>5.1, 8.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>24</td>
<td>22, 26</td>
<td>23</td>
</tr>
</tbody>
</table>

\(^a\) Dry matter; \(^b\) Crude protein; *42 case herds vs. 27 were feeding round-baled silage
Table 4-5. Logistic regression models that resulted from the multivariable analyses of risk factors associated with “transmitted” off-flavour in 35 case herds (no repeated cases) from September 2000 to April 2002.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Model with the subjective measurement of air quality</th>
<th>Model with “adequacy of the ventilation system” as the measure of air quality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Feeding baled silage as main forage</td>
<td>34.9</td>
<td>0.8, 148</td>
</tr>
<tr>
<td>Time of feeding roughage to lactating cows</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- After milking</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- Before (&lt; 2 hours) milking</td>
<td>446.1</td>
<td>8.6, 23051.3</td>
</tr>
<tr>
<td>- As free choice</td>
<td>3.2</td>
<td>0.10, 16.7</td>
</tr>
<tr>
<td>Clipping hair on cows’ udders</td>
<td>0.28</td>
<td>0.002, 0.40</td>
</tr>
<tr>
<td>Changing bedding material &gt; 1 time a day</td>
<td>0.16</td>
<td>0.001, 0.60</td>
</tr>
<tr>
<td>Poor air quality in the lactating cow barn</td>
<td>218.1</td>
<td>4.7, 352.4</td>
</tr>
<tr>
<td>Working status of the ventilation system</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> p-value for overall assessment of the variable time of feeding forage to lactating cows (with feeding after milking considered as the baseline).
CHAPTER 5

GAS CHROMATOGRAPHIC CHARACTERIZATION OF VOLATILE COMPOUNDS IN MILK TAINTED WITH OFF-FLAVOUR

Submitted to: *International Dairy Journal*
Abstract

Milk samples collected from 9 healthy mid-lactation Holstein cows were analyzed organoleptically by two sensory panels, and chromatographically using mass spectrometry/flame ionization (MSD/FID) and olfactometric detectors. The sensory panels found milk sampled after the cows were forage-starved for approximately 12 hours to be of good flavour quality; and at least 89% of those collected after the cows were fed baled grass silage to be tainted with “feed” off-flavour. The corresponding MSD/FID chromatograms revealed that 30 min post-feeding samples had significantly \( (p<0.05) \) higher concentrations of ethanol, propane-2-one, dimethyl sulfide, butane-2-one, hexanal, heptanal, octane-2,3-dione, and marginally \( (p<0.10) \) lower concentrations of butane-2,2,3,3-tetramethyl, pentane-2-methyl, and pentane-2,3,4-trimethyl than pre-feeding samples. Whereas 3 hour post-feeding samples showed higher concentrations of only 4 of these compounds (propane-2-one, dimethyl sulfide, butane-2-one and hexanal). Olfactometric analysis performed on 5 milk samples (4 off-flavoured and 1 of good flavour quality) detected approximately 75 aroma-active compounds (AACs) among which 62 were identified. Nearly all of these AACs were common to all the analyzed milk extracts, despite having different flavours, suggesting that off-flavour is caused by the concentration differences of a common set of compounds rather than the presence of any specific compound(s).

Keywords: Off-flavour; volatile (flavour impact) compound; milk; silage; feeding; solid phase micro-extraction; gas chromatography; mass spectrometry; flame ionization
detector; sensory analysis; and olfactometry.

**Abbreviation keys:** HS-SPME = Headspace solid phase micro extraction; GC-MS = Gas chromatography-Mass spectrometry, GC-FID = Gas chromatography-flame ionization detector; GC-O = Gas chromatography-Olfactometry; VOC = Volatile organic compound; AAC = Aroma-active compound.
5.1. Introduction

Undesirable flavours in raw milk associated with the feeding of ensiled forages have been a problem in the dairy industry for many years [1-7]. Identifying the volatile organic compounds (VOCs) responsible for these off-flavours has been technically challenging because of limitations in technology and the vast number of volatile compounds produced during the ensiling process [2;8-10]. Relatively recently, new developments in instrumentation and methods for extracting volatile and semi-volatile compounds from complex food systems have enhanced our ability to identify flavour components [11;12].

Few studies have attempted to correlate gas-chromatographic data to “feed” off-flavours in raw milk [2;8-10], and none provides a comprehensive analysis of volatile compounds present in milk from the same cow, sampled before and after silage feeding, under controlled conditions. This information would be important to further our understanding of the volatile components of silage responsible for feed off-flavours and could provide the basis for more objective and reliable instrument-based diagnostic test, which might replace or complement traditional organoleptic testing of raw milk.

The objective of the present investigation was to develop a reliable on-farm model for recreating “feed” off-flavours in milk from cows fed grass silage and more importantly, to use headspace solid phase micro-extraction (HS-SPME) gas chromatography techniques to fingerprint the VOCs associated with these flavour defects in fresh milk.
5.2. Materials and methods

5.2.1. Herd, cow and sample acquisition

Milk samples were obtained from 9 lactating Holstein Friesian cows in mid-lactation (100–250 days in milk) without any mastitis or udder abnormalities; and the cows were selected from 3 commercial dairy farms from Prince Edward Island (PEI). The farms from the list of the clients of the ambulatory farm service of the Atlantic Veterinary College, because these farms were typical of PEI dairy herds in regards to farm management practices and nutrition. Although from 3 different farms, the cows, which were all healthy throughout the study, were kept on similar rations and under similar housing conditions (tie stall barn).

The selected cows (three from each farm) were initially forage-starved from 07:00 PM until 07:00AM. During this time they received their regular portion of barley-based concentrate. Before they were fed freshly opened round bale grass silage at approximately 7:00 AM, the first series of milk samples (approximately 500 ml) was collected by hand. Thirty min after the cows were given silage the second series of milk samples was collected ("30 min" samples), followed by the third series 2½ hours later ("3 hour" samples). After collecting the first series of milk samples, the cows were completely milked (by the producer) using the milking machine. Consequently, the second and third milk samplings were milk newly formed. Collected milk samples were then split into five aliquots: one of 100-150 ml stored in a glass bottle at -20° C, and four of 70-80 ml each stored in semi-transparent plastic containers at +4° C. The former was
submitted three weeks later for HS-SPME GC analyses; while the latter samples were assessed for their flavour quality by two panels of trained and experienced milk graders within 48 hours of collection.

The main forages used in the selected herds were all mixed grass-legume, round bale silages, predominantly composed of grass (timothy – *Phleum pratense*) and about 10-15% clover (alsike clover – *Trifolium hybridum* and red clover – *Trifolium pratense L*.), and harvested around June-July 2002 (first-cut). They were typical of silages that were used in off-flavour positive herds in the risk factor study [13]. Samples of these silages were taken using a "PUSH TYPE" Multi-Forage Sampler (Star Quality Samplers, Edmonton, AB, Canada) from 8 to 10 sites in the wrapped bale, and thoroughly mixed to get a homogeneous sample.

5.2.2. Laboratory analyses

Forage samples were divided into two aliquots: one was put in a sealable plastic bag and was destined for routine nutrient analyses, while the other one, packed in a natural HDPE 500 ml plastic jar with screw-top, was analyzed for volatile organic compounds (VOC) using HS SPME-GC. Both aliquots were stored at +4º C immediately after collection and transferred 6 to 8 hours later to a -20º C freezer until submission for analyses. Routine chemical analyses were performed 48 hours after sample collection and gas chromatography approximately three weeks later.
5.2.2.1. **Organoleptic and nutrient analyses of silage samples**

The silage samples were organoleptically examined by the author (colour, odour, consistency and general appearance) for suitability of consumption. Silages were also analyzed by the Soil and Feed Testing Laboratory of the PEI Department of Agriculture and Forestry (Charlottetown PEI, Canada) for moisture and pH-value (AOAC, 2000), and for crude protein (CP) and bound protein (BP) [14] using, respectively, CHN-2000 Elemental Analyzer and CHN-600 Elemental Analyzer (LECO Corp., St Joseph, MI, USA). Mineral content (Ca, P, Mg, K, Cu, and Zn) [14] was measured using Inductively Coupled Argon Plasma Spectrometer (Genesis Laboratory Systems, Inc. Colorado, USA). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an ANKOM220 Fiber Analyzer (ANKOM Technology, Fairport, NY) according to the methodology supplied by the company, which is based on the methods described by [15].

5.2.3. **Organoleptic evaluation of milk flavour.**

Two sensory panels of five male milk graders, each with extensive experience (> 2 years) in detecting and characterizing off-flavours in milk, carried out discriminative ("accepted" / "rejected") organoleptic assessment of the 27 milk samples (i.e. pre-feeding, "30 min," and "3 hour" samples) and their respective replicates in two test sessions within a period of 48 hours. Milk samples graded "accepted" were considered to be of good flavour quality and those graded as "rejected", off-flavoured.
The panelists were blinded to sample identification; they had no knowledge of the number of pre- and post-feeding samples or number of cows enrolled in the study. They were given only general information about the study design, specifically on how the milk samples were collected.

To ensure a better organoleptic evaluation, milk samples were conditioned at room temperature for 2-3 hours before the scheduled time for the test to allow the serving temperature to be higher (around 14-16° C) than what is generally considered as normal drinking temperature for milk [16]. At the beginning of each test session and after the assessment of two consecutive samples, the panelists were recommended to eat unsalted crackers and filtered water to rinse their mouth and aid removing any residual flavors from the palate [16]. A simple randomization was used to determine the serving order of the samples to be tested. A copy of a simple questionnaire was given to the head of each panel to fill in the panel’s answers during the test session. Each of the three panelists evaluated the aroma-active compounds of five milk samples, chosen randomly from three different pools: two from pre-feeding and “30 min” samples, and one from “3 hour” samples.

5.2.4. Headspace solid phase micro extraction procedures for milk & silage

Aliquots of 15 g from each of the 27 milk samples were collected in a crimp-top headspace vial sealed with a teflon-lined silicone rubber septum. The septum was pierced
with a sharp needle to allow the SPME syringe (Supelco, Bellefonte, PA) to be inserted into the headspace, followed by the extension into the vial of the 2 cm sampling fiber coated with Divinylbenzene-Carboxen-Polydimethylsiloxane. The vial was placed on a magnetic stirrer with a SPME holder suspended above and held in position by a clamp. The volatile compounds were allowed to equilibrate for 90 min at 45°C, after which the SPME fiber was withdrawn and injected into a gas chromatograph-mass spectrometry equipped with a mass selective detector (MSD) as well as a flame ionization detector (FID). The extraction time for HS-SPME was 45 min at 45°C.

5.2.5. Instrumentation and operating conditions of gas chromatography mass spectrometry/Flame ionization detection for milk and silage

The analyses of VOCs in the milk samples were carried out with a Hewlett-Packard HP 5890, Series II gas chromatography equipped with a 0.75 mm I.D. inlet liner (Supelco SA, CH-1196 Gland). The SPME fiber was conditioned in the hot injector portion of the gas chromatograph according to the instructions provided by the supplier. SPME desorption was done at 260°C during the first 3 min, then the fiber was left for another 14 min in the injection port with split set “ON” to further clean the fiber. The injector mode was splitless. Peak separation was carried out on a 60 m x 0.32 mm I.D. x 1 μm DB-1 column (Supelco). The oven temperature, initially held at 35°C for 3 min, was programmed to 260°C at a heating rate of 3°C/min, and then was finally held at 260°C for 12 min. Helium was used as carrier gas at a constant inlet pressure of 110 kPa. Two detectors were mounted in parallel by splitting the flow at the end of the capillary column.
into two streams: one to a flame ionization detector (FID), and the other to a Hewlett-Packard mass selective detector (MSD model HP 5971) which recorded the signals. MS detection used for the qualitative analysis was performed on a quadropole mass spectrometer operating in full scan EI (Electron Impact) ionization mode (70 eV). The FID signal was utilized for the semi-quantitative evaluation of the peak heights (arbitrary units) of the isolated compounds.

The GC injection port and the FID were maintained at a temperature of 260° C for thermal desorption. Peak heights and peak areas were integrated by Agilent ChemStation software rev. A.09.03. The VOCs detected were identified using the Wiley MS library. Confirmation of the identity of the volatile compounds was achieved by comparing the GC retention indices and mass spectra of individual components with those of authentic reference compounds injected under the same operating conditions.

Similar HS-SPME analytical (GC-MS and GC-FID) techniques were applied for the gas chromatographic analysis of the silage samples.

5.2.6. GC-Olfactometry instrumentation and operating conditions for milk

Gas chromatography-olfactometry was applied on 2 pre-feeding and 3 post-feeding milk samples for the identification of the aroma-active compounds potentially associated with feed flavour in milk. It was carried out on the same above-mentioned Hewlett Packard HP 5890 equipment using a panel of 3 trained subjects. The VOCs were collected using the same HS-SPME techniques and type of fiber as for GC-MS/FID at 55° C for 60 min.
while stirring the sample. Gas chromatographic conditions were identical to those described for GC-MS/FID, except the oven temperature program was initially held at 260° C for only 2 min (instead of 12 min). Olfactometric data acquisition was performed with the Gerstel ODP recorder (sniffing port) for Agilent and MSD Chemstation software version 1.7.4. The chromatographic effluent from the column was mixed with non-humidified airflow to cool the carrier gas in order to avoid drying of the mucous membranes of the nose of the subject who sniffed the effluent for approximately 45 min. The panelists were asked to breathe normally while sniffing the effluent and to record both the description of the detected odour and the time of appearance and disappearance of this odour. Such analysis was repeated over several sessions until no additional odours could be detected. A series of n-alkanes were then analyzed under similar conditions but with the column connected to the FID to calculate the retention indices [17]. A detailed procedure for GC-O is described by Marsili [18].

5.2.7. Statistical analyses

Cohen’s kappa statistics [19], indices of observed agreement in positive and negative directions [20], as well as the AC_f statistic recently introduced by Gwet [21] were used to evaluate the reliability within and between the two panels. A 2 X 2 contingency table was used to compute these statistics, with details provided elsewhere [22].

The corresponding FID-peak heights of the VOCs identified using MSD were used to compare the abundance of these compounds across the three different sampling periods.
The mean value and the standard deviation of the mean of the relative concentration (FID-peak height) of each compound identified were calculated and matched pair analyses were used to monitor the variation in the concentrations of the major recovered VOCs over the three sampling periods (pre-feeding samples vs. 30 min samples and pre-feeding samples vs. 3 hour samples).

Univariable analyses were conducted to screen VOC variables for their relationship to off-flavour \( (p \leq 0.20) \); whereas, using backward elimination, multivariable logistic regression \( (p \leq 0.05) \) on a random subset of about 75.0 % (i.e. 20 observations) of the data was used to study the relationship between the concentration of these VOCs and the overall sensory characteristic of the milk with the dependent variable being “off-flavour” or not. The remaining 25.0% of the dataset were then used for cross-validation of the resulting statistical model. The process was repeated three times, and each time using a new 75.0% random subset of the data. Confounding and interactions, as well as post-fit diagnostics were assessed as described in the risk factor study [13].

The sensory data used in this statistical computation were pooled from the combination of the four evaluations of the two panels using Cronbach’s alpha techniques [23], which is briefly described elsewhere [13]. Kappa statistic and logistic regression were assessed using the statistical software package STATA 8.0 [24], whereas other statistics (indices of positive and negative agreements and \( AC_1 \) statistic) were calculated manually as described by the corresponding authors [20;21].
5.3. Results

5.3.1. Organoleptic and chemical analyses of the silages

Sensory assessment of the 3 silages suggested that they were of good quality (color, odour, consistency and general appearance), and therefore fit for animal feeding. The chemical compositions (Table 5-1) of these silages were within the range of good quality mixed grass silages, and mirrored the values obtained by Mounchili and co-workers [22], suggesting that they were representative of the silages made in PEI.

5.3.2. Organoleptic analysis of the milk samples

The sensory panels agreed that all the 9 milk samples obtained from the cows before feeding of baled grass silage were of good quality (i.e. free of off flavour). For samples collected after the feeding of freshly unwrapped silage, the two panels agreed that 7 of 9 and 8 of 9 samples were off-flavoured, respectively, 30 min and 3 hours post-feeding (Table 5-2). Consequently, Cohen's kappa [19], indices of positive and negative agreement proposed by Cicchetti & Feinstein [20], and $AC_I$ statistic [21] all yielded highly satisfactory results in the reliability assessment. Cohen kappa and $AC_I$ statistic were within the perfect range ($>0.81$) [25] for both intra-panel agreement (comparison of the evaluation scores of original and duplicate samples for each panel) and inter-panel agreement (comparison of the evaluation scores of both panels for each test session), and values of $\geq 90.0\%$ for Cicchetti & Feinstein's indices [20] of average proportional...
positive and negative agreements (Table 5-3).

5.3.3. Gas chromatographic analyses

5.3.3.1. GC-MS/FID of milk samples

Gas chromatographic analysis resulted in separation of a series of VOCs contained in the analyzed milk samples. Table 5-4 lists those found in at least 8 of the 27 samples. Hydrocarbons (pentane, pentane-2-methyl, butane-2,2,3,3-tetramethyl, hexane, pentane-2,3,4-trimethyl, toluene, pentane-4-methyl, pentane-2,4-dimethyl, octane-4-methyl, benzene-1,2-dimethyl), followed by ketones (propane-2-one, butane-2-one, octane-2,3-dione) and aldehydes (pentanal, hexanal, heptanal), alcohols (ethanol and pentane-1-ol) and one sulphur containing compound (dimethyl sulfide) were the compounds regularly detected. Twenty-three additional peaks were isolated occasionally (less than 6 times in a total of 27 analyzed samples) and identified either by using mass selective detector (MSD) or flame ionization detector (FID) (Table 5-5). However, they were not used in the statistical analyses, due to their relatively low frequency of detection. Figures 5-1 (A, B & C) show a series of typical chromatograms of headspace volatile compounds obtained with HS-SPME from 15 g of raw milk, sampled from a cow before, 30 min after and 3 hours after feeding silage. They illustrate the patterns of the volatiles recovered before and after silage feeding. It is important to keep in mind that the differences in FID-peak heights are interpreted as the variation in intensities of VOCs following silage consumption. Results obtained suggested that the peak heights of ethanol, propane-2-one, dimethyl sulfide, butane-2-one, hexanal, heptanal and octane-2,3-dione found in the
chromatograms of 30 min (post-feeding) milk samples were significantly ($p<0.05$) higher than those found in the chromatograms of the pre-feeding samples, whereas pentane-2-methyl, butane-2,2,3,3-tetramethyl and pentane-2,3,4-trimethyl had marginal ($p<0.10$) decreasing FID-peak heights after silage was fed (Table 5-4). Also, the comparison of the chromatograms of 3 hours post-feeding samples to those of pre-feeding samples suggest that propane-2-one, dimethyl sulfide, and butane-2-one had significantly ($p<0.05$) higher peaks in the former and a marginally significant ($p<0.10$) higher peak of hexanal.

The summative sensory ratings, generated from the combination of the four sets of the organoleptic results of both panels using Cronbach’s alpha technique, were combined with chromatographic data to study the VOCs associated with the development of objectionable “feed” off-flavour characteristics in the milk. The former represented the dependent binary variable (with two levels: “good” = 0 and “off-flavour” = 1), while the latter were the predictors. Primary screening (ordinary logistic regression) of the relationship between each of the 20 regularly identified compounds by HS-SPME GC and the occurrence of milk off-flavour suggested that four compounds (propane-2-one, butane-2-one, ethanol, and dimethyl sulfide) were unconditionally ($p<0.20$) associated with the detection of off-flavour (Table 5-6). The exploration of pair-wise associations between these compounds revealed a considerably higher correlation ($p>0.8$) between dimethyl sulfide and butane-2-one; consequently, in order to avoid multicollinearity, one of these two explanatory variables was dropped from multivariable statistical analysis, which yielded a final model with only one predictor: dimethyl sulfide or butane-2-one (peak #4 & 6 in Figures 5-1 & 5-2). The resulting statistical model for each of the three
runs of 75% of the data (Table 5-6) perfectly predicted the organoleptic outcome (off-flavour versus non off-flavour) of the remaining 25%.

5.3.3.2. Gas chromatographic analysis of the silage samples

Gas chromatographic analysis of the silage samples indicated the presence of a large number (over 100) of volatile compounds including all of the 19 major VOCs detected in the milk samples. Figure 5-2 shows a typical chromatogram of silage.

5.3.3.3. Gas chromatography-Olfactometric analysis of the milk samples

Approximately 75 aroma-active compounds (AACs) were detected in all the five analyzed milk extracts by GC-O, which has often a much lower detection threshold than the GC-MS and GC-FID. Sixty-two of these compounds were identified by electron impact MS and linear retention indices (Table 5-7). Thirty-one AACs were found in all five analyzed milk samples. Only four of the 70 compounds showed specific distinction in the occurrence or absence in either off-flavoured samples or samples of good quality: two unidentified compounds, somehow were sniffed only in off-flavoured samples, whereas acetic acid ethyl ester and toluene could be detected only in milk samples of good quality. Twelve of the AACs were also detected by GC-MS/FID: five of the major VOCs (pentanal, pentane-1-ol, toluene, hexanal, benzene-1,2-dimethyl) and seven of the irregular ones (acetic acid ethyl ester, octane, ethyl benzene, benzene-1,3-dimethyl, hexanoic acid, octanoic acid and decanoic acid).
Results suggested that there were some strong similarities in the terminology that the
olfactometric panel used for the description of the odour released by 23 of 75 detected
AACs (Table 5-7) and that used by the sensory panel to describe the flavour defect in
milk: terms such as fruity, sweet, strawberry, silage, grassy or floral were commonly
used by both panels.

The validity of the logistic regression was checked (post-fit diagnostics) and results
suggested no lack of fit of the resulted statistical models.

5.4. Discussion

Headspace solid phase micro extraction used for the extraction of the volatiles is known
not only for its ability to detect low molecular weight compounds, but also for its ability
to minimize contamination of the sample or artifacts due to sample manipulations [26]. It
is also recognized as an ideal technique for the analysis of biological samples because it
reduces interference from high molecular-mass, non-volatile components, such as
proteins, which consequently results in much cleaner extracts [27].

The striking feature of the HS-SPME GC-MS/FID results was that, with the exception of
only one compound (ethanol), the identified volatile compounds in milk samples of good
organoleptic quality were similar to those of milk tainted with feed flavour. Organoleptic
differences between the milk samples were related not to the compositional (qualitative)
differences, but rather to the magnitudes of the peak heights (semi-quantitative) of certain
compounds: ethanol, propane-2-one, dimethyl sulfide, butane-2-one, hexanal, heptanal and octane-2,3-dione, which were much more abundant in off-flavoured milk (which appeared to be the milk sampled during the post-feeding period) than in milk of good quality (pre-feeding samples). These compounds were also found by several authors who studied the volatile compounds, characteristic to “feed” (or transmitted) off-flavoured milk [2;8-10;28] or those who simply profiled the VOC content of milk from cows receiving different diets [29-31].

Statistical screening of all of the 19 major VOCs of the analyzed milk samples revealed that, only four (ethanol, propane-2-one, dimethyl sulfide and butane-2-one) were unconditionally associated with the detection of the “feed” off-flavour in milk.

While studying sensory characteristics of some compounds of the volatile fraction of milk, a group lead by Shipe [2] found that ethanol was imparting a sweet (vanilla-like) pleasant flavour in milk. Randby and co-workers [32] reported that, although the findings of their work pointed out that the off-flavour in milk produced by cows fed grass silage mixed with ethanol could not be attributed solely to the ethanol transmitted to the milk, precautions were to be taken as silages classified as well-fermented with a pleasant appearance and aroma may still contain ethanol in such amounts that a feed flavour might be imparted to milk. The present study could not determine whether the flavour accompanying (or generated by) the increased concentration of ethanol or any of the recovered VOCs was pleasant or not. However, we did note that the GC VOC profile of milk sampled before feeding silage to the cows did not indicate the presence of ethanol; it
appeared only in the chromatograms of seven of the nine 30 min samples and in one of the 3 hour samples.

The association of dimethyl sulfide with milk flavour established in this study has also been reported by numerous authors [2;8;33]. All reported that dimethyl sulfide was among the principal contributors in the development of off-flavours in milk. They claimed that it had a very low threshold of olfactory perception in fresh milk of good quality, and that very small differences in its concentration could change the pleasant aroma of milk to a strong “unpleasant feed-like,” “malty” or “cowy” odour. This fact was also confirmed by the work of Bosset and co-workers [34], who investigated sulfur-containing compounds (dimethyl sulfide and dimethyl disulfide) on different heat-treated milks, such as pasteurized and UHT milk.

Potts and Kessler [35] indicated no apparent relationship between the concentrations of ketones in the milk of grass silage-fed cows and the flavour of the milk. However, the results of the current study were in agreement with those of many other authors [2;8;29] who found increased amounts of propane-2-one and butane-2-one in milk from cows fed silage shortly before milking. They all agreed that these compounds were likely imparting “cowy” or “feed-like” flavour to the milk. The work of Shipe and coworkers [2] demonstrated that the four above-mentioned compounds (ethanol, propane-2-one, dimethyl sulfide and butane-2-one) were capable of imparting off-flavours to milk.
Statistical exploration of the chromatographic data also revealed that the increase observed in the concentrations of volatile compounds such as ethanol, hexanal, heptanal, and octane-2,3-dione, right after silage feeding was significant \((p<0.05)\) only in 30 min samples. However, the lack of statistical significance in the variation of the concentrations of these VOCs in the post-feeding milk cannot be necessarily interpreted as an insignificant sensorial contribution in the development of off-flavour, as small differences in some compounds (other than the above-mentioned) were reported as sources of off-flavours in milk or in other food products [2;35]. The current investigation used much more advanced analytical techniques (HS-SPME and GC-O) than those applied in the earlier studies carried out between 1962 and 1972 by the above-mentioned authors [2;8-10;29;32;33;35].

Just like in GC-MS/FID, the GC-O results (the AAC profiles) for off-flavoured milk and milk of good flavour quality were similar, suggesting that off-flavour is primarily caused by the concentration differences of a common subset of AACs. Bendall [37] reached the same conclusion in his work on the aroma compounds of fresh milk (of good organoleptic quality) from two New Zealand cows on different diets (one on TMR diet and another on pasture diet). One could hypothesize that the subset of AACs whose odour descriptions (by the GC-O panel) matched that of the flavour defect generated may be responsible for the off-flavour. However, within the scope of the current study, it would be over interpretation, because the sensory panel’s goal is the (instantaneous) assessment of the aroma resulting from the combination of all the AACs present in the
tested milk, while the olfactometric panel (GC-O) focuses on the sequential assessment of the odour released by individual compounds.

Although dimethyl sulfide appeared to be statistically associated with the detection of off-flavour, it was not detected by GC-O, suggesting that by itself, at this concentration in milk, it is not an odour-active compound as claimed by other authors [2;8;33]. Other correlates include butane-2-one, propane-2-one. This finding illustrates the value of including GC-O in addition to GC-MS/FID.

It is important to note that GC-O does not have the same detection threshold as the GC-MS/FID. The latter methods are adequate for the identification and/or quantification of volatile and semi-volatile compounds (which are not necessarily aroma-active) present in the analyzed extract at levels equal to or greater than $10^{-5}$ g/L. Conversely, the former method has been reported to be capable of detecting odour-active compounds at levels as low as parts per trillion [38]. Therefore, lack of statistically significant differences in the concentration of a VOC may not necessarily indicate an insignificant sensorial contribution in the development of off-flavour.

5.5. Conclusions

The present study showed that feeding lactating cows with freshly opened baled silage up to 3 hours before milking can give rise to objectionable flavours in milk produced by these cows. Furthermore, four VOCs (ethanol, propane-2-one, butane-2-one, and
dimethyl sulfide) were strongly associated with the detection of off-flavour in the milk extracts. Of these, dimethyl sulfide showed the strongest relationship (statistically), sufficient to serve as a proxy indicator for the presence or absence of off-flavour. These findings support the feasibility of the development of a potential instrument-based diagnostic assay as a valuable alternative or complement to traditional organoleptic testing for unacceptable flavours in raw milk. However, to fully assess the potential for dimethyl sulfide as a diagnostic test, a large study (using absolute concentrations of VOCs versus relative abundance) would need to be performed.

Interestingly, nearly all of the identified AACs were present in milk samples obtained both before and after the feeding of baled silage. This suggests that, when considering the chemical cause of “feed” off-flavour in milk, relative concentrations of AACs are likely more important than the simple presence or absence of one or more of these compounds. Given the complexity of the chemical composition of milk, it is important to note that the difference in the concentrations of these compounds may be statistically not significant, but chemically sufficient to render the flavour of milk objectionable. Consequently, further work that would include a precise quantification of the AACs, and the determination of the concentration threshold (odour value) above which they, either singly or in combination, appear to be important in the development of off-flavour in the milk, is necessary.
Acknowledgements

This study was conducted in part at the Swiss Federal Dairy Research Station, Liebefeld, Switzerland, and we would like to express our gratitude to this institution. The authors would also like to extend appreciation to Gerard Toole, the Hooper and Jewell families, to have graciously agreed to enroll their cows in this study, to Yogi Fell, Theresa Andrews, and Lloyd Dalziel for their help in samples handling and shipping. We would also like to thank Bryan Grimmelt at the AVC toxicology laboratory, as well as Mark Grimmet from Agriculture Canada & Agr-Food Canada for their tutoring in gas chromatography.
5.6. Reference List


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Table 5-1. Chemical composition of the silages that were fed to the cows in the study of the compounds associated with the development of off-flavour in milk.

<table>
<thead>
<tr>
<th>Analysis performed</th>
<th>Farm “A”</th>
<th>Farm “B”</th>
<th>Farm “C”</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td>4.4</td>
<td>4.7</td>
<td>5.2</td>
</tr>
<tr>
<td>Dry Matter (DM) %</td>
<td>43.1</td>
<td>55.4</td>
<td>56.8</td>
</tr>
<tr>
<td>Solubility (%CP)</td>
<td>34.9</td>
<td>52.5</td>
<td>39.5</td>
</tr>
<tr>
<td>Crude Protein (CP) (% DM)</td>
<td>11.0</td>
<td>16.1</td>
<td>12.5</td>
</tr>
<tr>
<td>Bound Protein (BP) (% DM)</td>
<td>15.4</td>
<td>7.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Acid detergent fiber (ADF) (% DM)</td>
<td>41.0</td>
<td>34.2</td>
<td>29.4</td>
</tr>
<tr>
<td>Neutral detergent fiber (NDF) (% DM)</td>
<td>58.8</td>
<td>55.0</td>
<td>46.4</td>
</tr>
<tr>
<td>Calcium (% DM)</td>
<td>0.60</td>
<td>0.56</td>
<td>0.83</td>
</tr>
<tr>
<td>Phosphorus (% DM)</td>
<td>0.20</td>
<td>0.31</td>
<td>0.25</td>
</tr>
<tr>
<td>Magnesium (% DM)</td>
<td>0.08</td>
<td>0.21</td>
<td>0.18</td>
</tr>
<tr>
<td>Potassium (% DM)</td>
<td>1.7</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Copper (mg/kg DM)</td>
<td>4.9</td>
<td>6.3</td>
<td>5.9</td>
</tr>
<tr>
<td>Zinc (mg/kg DM)</td>
<td>19.5</td>
<td>21.8</td>
<td>17.5</td>
</tr>
</tbody>
</table>

1 Silage from farm “A”  
2 Silage from farm “B”  
3 Silage from farm “C”
Table 5-2. Proportion of milk samples on which the two panels (A) or each panel (B) agreed.

### A. BETWEEN PANELS ASSESSMENT

<table>
<thead>
<tr>
<th>Test session</th>
<th>Pre-feeding milk samples</th>
<th>Post-feeding (&quot;30 min&quot;) milk samples</th>
<th>Post-feeding (&quot;3 hour&quot;) milk samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non off-flavoured</td>
<td>Off-flavoured</td>
<td>Non off-flavoured</td>
</tr>
<tr>
<td>1</td>
<td>100%</td>
<td>0.0%</td>
<td>11.0%</td>
</tr>
<tr>
<td>2</td>
<td>100%</td>
<td>0.0%</td>
<td>11.0%</td>
</tr>
</tbody>
</table>

### B. WITHIN PANEL ASSESSMENT

<table>
<thead>
<tr>
<th>Panel</th>
<th>Pre-feeding samples</th>
<th>Post-feeding (&quot;30 min&quot;) milk samples</th>
<th>Post-feeding (&quot;3 hour&quot;) milk samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non off-flavoured</td>
<td>Off-flavoured</td>
<td>Non off-flavoured</td>
</tr>
<tr>
<td>&quot;A&quot;</td>
<td>100%</td>
<td>0.0%</td>
<td>11.0%</td>
</tr>
<tr>
<td>&quot;B&quot;</td>
<td>100%</td>
<td>0.0%</td>
<td>11.0%</td>
</tr>
</tbody>
</table>
Table 5-3. Summary of the results of inter- (A) and intra-panel (B) reliability of discriminative organoleptic testing of 27 milk samples.

**A - Intra-panel reliability**

<table>
<thead>
<tr>
<th>Panel</th>
<th>Number of Samples</th>
<th>Expected agreement</th>
<th>Observed agreement</th>
<th>Kappa-statistic</th>
<th>P-value</th>
<th>Alternative statistics to kappa proposed by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original</td>
<td>Duplicate</td>
<td></td>
<td></td>
<td></td>
<td>$^{1} P_{pos}$ $^{2} P_{neg}$ AC1 statistic</td>
</tr>
<tr>
<td>“A”</td>
<td>27</td>
<td>27</td>
<td>51.71 %</td>
<td>92.59 %</td>
<td>+ 0.85</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>“B”</td>
<td>27</td>
<td>27</td>
<td>52.40 %</td>
<td>96.30 %</td>
<td>+ 0.92</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

**B - Inter-panel (or Intra-test) reliability**

<table>
<thead>
<tr>
<th>Test session</th>
<th>Number of samples</th>
<th>Expected agreement</th>
<th>Observed agreement</th>
<th>Kappa-statistic</th>
<th>P-value</th>
<th>Alternative statistics to kappa proposed by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$^{1} P_{pos}$ $^{2} P_{neg}$ AC1 statistic</td>
</tr>
<tr>
<td>1</td>
<td>27</td>
<td>27</td>
<td>51.71 %</td>
<td>92.59 %</td>
<td>+ 0.85</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>27</td>
<td>52.40 %</td>
<td>96.30 %</td>
<td>+ 0.92</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

$^{1} P_{pos}$ – Index of average proportional positive agreement;

$^{2} P_{neg}$ – Index of average proportional negative agreement
Table 5-4. Major volatile organic compounds detected (by HS-SPME-GC-MS/FID) in the volatile fraction of the 27 milk samples and the variation of their relative concentration (peak height) over time after silage feeding.

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Volatile organic compound</th>
<th>Mean Retention index</th>
<th>Range of retention time</th>
<th>Comparison of post-feeding against pre-feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\mu_{1/2} - \mu_0$</td>
</tr>
<tr>
<td>1</td>
<td>Ethanol</td>
<td>444</td>
<td>6.337 - 6.500</td>
<td>322.8</td>
</tr>
<tr>
<td>2</td>
<td>Propane-2-one (=Acetone)</td>
<td>471</td>
<td>6.947 - 7.042</td>
<td>1193.3</td>
</tr>
<tr>
<td>3</td>
<td>Pentane</td>
<td>501</td>
<td>7.667 - 7.720</td>
<td>633.3</td>
</tr>
<tr>
<td>4</td>
<td>Dimethyl sulfide</td>
<td>509</td>
<td>7.850 - 7.995</td>
<td>517.2</td>
</tr>
<tr>
<td>5</td>
<td>Pentane-2-methyl</td>
<td>566</td>
<td>9.788 - 9.817</td>
<td>-617.2</td>
</tr>
<tr>
<td>6</td>
<td>Butane-2-one</td>
<td>574</td>
<td>9.959 - 10.071</td>
<td>7151.8</td>
</tr>
<tr>
<td>7</td>
<td>Hexane</td>
<td>601</td>
<td>10.865 - 10.967</td>
<td>-221.6</td>
</tr>
<tr>
<td>8</td>
<td>Pentanal</td>
<td>675</td>
<td>14.138 - 14.196</td>
<td>-43.9</td>
</tr>
<tr>
<td>9</td>
<td>Butane-2,2,3,3-tetramethyl</td>
<td>691</td>
<td>14.819 - 14.860</td>
<td>-503.78</td>
</tr>
<tr>
<td>10</td>
<td>Pentane-1-ol</td>
<td>751</td>
<td>17.530 - 17.565</td>
<td>-68.6</td>
</tr>
<tr>
<td>11</td>
<td>Pentane-2,3,4-trimethyl*</td>
<td>753</td>
<td>17.670 - 17.687</td>
<td>-277.4</td>
</tr>
<tr>
<td>12</td>
<td>Toluene</td>
<td>759</td>
<td>17.930 - 17.965</td>
<td>-110.1</td>
</tr>
<tr>
<td>13</td>
<td>Heptane-2-methyl</td>
<td>767</td>
<td>18.269 - 18.294</td>
<td>-205.3</td>
</tr>
<tr>
<td>14</td>
<td>Hexanal</td>
<td>779</td>
<td>18.811 - 18.842</td>
<td>3450.3</td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td>RT (min)</td>
<td>Tm (°C)</td>
<td>∆Tm (°C)</td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>15</td>
<td>Heptane-2,4-dimethyl</td>
<td>825</td>
<td>20.877 - 20.917</td>
<td>-309.4</td>
</tr>
<tr>
<td>16</td>
<td>Octane-4-methyl</td>
<td>866</td>
<td>22.681 - 22.696</td>
<td>-130.3</td>
</tr>
<tr>
<td>17</td>
<td>Heptanal</td>
<td>882</td>
<td>23.373 - 23.393</td>
<td>214.0</td>
</tr>
<tr>
<td>18</td>
<td>Benzene-1,2-dimethyl</td>
<td>888</td>
<td>23.660 - 23.677</td>
<td>-36.9</td>
</tr>
<tr>
<td>19</td>
<td>Octane-2,3-dione</td>
<td>961</td>
<td>26.655 - 26.684</td>
<td>108.7</td>
</tr>
</tbody>
</table>

1. Mean difference between the peak height of VOC ½ hour after silage feeding and that of before silage feeding.

2. Mean difference between the peak height of VOC 3 hours after silage feeding and that of before silage feeding.

3. Only one sample exhibited a peak for ethanol 3 hours after silage feeding;

* Tentative identification using MSD
Table 5-5. Volatile organic compounds (VOCs) detected by HS-SPME-GC-MS/FID in less than 6 of the 27 milk samples analyzed.

<table>
<thead>
<tr>
<th>Volatile organic compound (VOC)</th>
<th>Mean RI (using FID)</th>
<th>Frequency of detection ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butane-2,3-dimethyl</td>
<td>563</td>
<td>3/27</td>
</tr>
<tr>
<td>Pentane-3-methyl</td>
<td>582</td>
<td>3/27</td>
</tr>
<tr>
<td>Acetic acid ethyl ester</td>
<td>600</td>
<td>2/27</td>
</tr>
<tr>
<td>Heptane</td>
<td>700</td>
<td>1/27</td>
</tr>
<tr>
<td>Hexane-2,5-dimethyl*</td>
<td>733</td>
<td>5/27</td>
</tr>
<tr>
<td>Hexane-2,4-dimethyl*</td>
<td>735</td>
<td>5/27</td>
</tr>
<tr>
<td>Pentane-2,3,3-trimethyl*</td>
<td>759</td>
<td>1/27</td>
</tr>
<tr>
<td>Hexane-2,3-dimethyl*</td>
<td>762</td>
<td>5/27</td>
</tr>
<tr>
<td>Heptane-4-methyl*</td>
<td>768</td>
<td>2/27</td>
</tr>
<tr>
<td>Heptane-3-methyl*</td>
<td>774</td>
<td>2/27</td>
</tr>
<tr>
<td>Octane</td>
<td>800</td>
<td>3/27</td>
</tr>
<tr>
<td>Dodecane-2-ethyl*</td>
<td>842</td>
<td>1/27</td>
</tr>
<tr>
<td>Ethyl Benzene</td>
<td>856</td>
<td>2/27</td>
</tr>
<tr>
<td>Benzene-1,3-dimethyl</td>
<td>864</td>
<td>2/27</td>
</tr>
<tr>
<td>Hexanoic acid*</td>
<td>949</td>
<td>4/27</td>
</tr>
<tr>
<td>Limonene*</td>
<td>1034</td>
<td>3/27</td>
</tr>
<tr>
<td>Nonanal</td>
<td>1087</td>
<td>1/27</td>
</tr>
<tr>
<td>Undecane</td>
<td>1100</td>
<td>1/27</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>1140</td>
<td>1/27</td>
</tr>
<tr>
<td>Benzoic acid ethyl ester</td>
<td>1153</td>
<td>1/27</td>
</tr>
<tr>
<td>Benzaldehyde ethyl*</td>
<td>1209</td>
<td>3/27</td>
</tr>
<tr>
<td>Undecane-2-one</td>
<td>1279</td>
<td>1/27</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>1337</td>
<td>1/27</td>
</tr>
</tbody>
</table>

* Tentative identification using MSD ¹Ratio of milk samples containing the VOC.

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Table 5-6. Volatile organic compounds associated with the development of “feed” off-flavour in the raw milk samples.

A. Unconditional association (p < 0.20)

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Predictor</th>
<th>Odds ratio</th>
<th>SE</th>
<th>p-value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethanol</td>
<td>6</td>
<td>7.1</td>
<td>&lt; 0.12</td>
<td>0.61, 59.3</td>
</tr>
<tr>
<td>2</td>
<td>Propane-2-one_1 = (&lt; 2897) Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Propane-2-one_2 = (2898, 4814)</td>
<td>7</td>
<td>7.5</td>
<td>0.07</td>
<td>0.9, 56.9</td>
</tr>
<tr>
<td>-</td>
<td>Propane-2-one_3 = (&gt; 4815)</td>
<td>28</td>
<td>37.2</td>
<td>0.01</td>
<td>2.1, 379.2</td>
</tr>
<tr>
<td>4</td>
<td>Dimethyl sulfide (&gt; 267)</td>
<td>150</td>
<td>220.8</td>
<td>0.001</td>
<td>8.4, 2685.0</td>
</tr>
<tr>
<td>6</td>
<td>Butane-2-one (&gt; 3771)</td>
<td>150</td>
<td>220.8</td>
<td>0.001</td>
<td>8.4, 2685.0</td>
</tr>
</tbody>
</table>

B. Multivariate logistic regression (p < 0.05) on 75% random subset of the data

<table>
<thead>
<tr>
<th>Random subset</th>
<th>Predictor</th>
<th>Odds ratio</th>
<th>SE</th>
<th>p-value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>№ 1</td>
<td>Dimethyl sulfide (&gt; 267)</td>
<td>77</td>
<td>115.1</td>
<td>0.004</td>
<td>4.11, 1441.0</td>
</tr>
<tr>
<td>№ 2</td>
<td>Dimethyl sulfide (&gt; 267)</td>
<td>90</td>
<td>133.83</td>
<td>0.002</td>
<td>4.88, 1659.4</td>
</tr>
<tr>
<td>№ 3</td>
<td>Dimethyl sulfide (&gt; 267)</td>
<td>84</td>
<td>125.33</td>
<td>0.003</td>
<td>4.51, 1564.2</td>
</tr>
</tbody>
</table>

C. Multivariate logistic regression (p < 0.05) on 100% of the data

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Predictor</th>
<th>Odds ratio</th>
<th>SE</th>
<th>p-value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full (100%)</td>
<td>Dimethyl sulfide</td>
<td>150</td>
<td>220.8</td>
<td>0.001</td>
<td>8.4, 2685.0</td>
</tr>
</tbody>
</table>
Table 5-7. Summary of the aroma-active compounds that were detected by gas chromatography-olfactometry of 5 (with and without off-flavour) of the 27 collected milk samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>Descriptive terms used by three-person GC-O panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, ethyl ester</td>
<td>601</td>
<td>Slightly roast-like, burnt, caramel-like</td>
</tr>
<tr>
<td>Unknown</td>
<td>622</td>
<td><em>Slightly smoky, burnt, vegetable-like, not specific</em></td>
</tr>
<tr>
<td>Butanal, 3-methyl</td>
<td>634</td>
<td>Slightly sweet, alcoholic, fruity</td>
</tr>
<tr>
<td>Butane-1-ol and/or</td>
<td>651</td>
<td>Slightly unpleasant, cooked vegetable, pungent, dusty</td>
</tr>
<tr>
<td>Benzene</td>
<td>653</td>
<td></td>
</tr>
<tr>
<td>Pentane-2,3-dione and/or</td>
<td>672</td>
<td>Fruity, slightly burnt, cardboard-like, cabbage-like</td>
</tr>
<tr>
<td>Pentanal</td>
<td>675</td>
<td></td>
</tr>
<tr>
<td>2-ethylfuran and/or</td>
<td>691</td>
<td>Slightly burnt, vegetable soup, aromatic, balsamic,</td>
</tr>
<tr>
<td>Propanoic acid, ethyl ester</td>
<td>692</td>
<td>floral</td>
</tr>
<tr>
<td>Butanoic acid, methyl ester</td>
<td>708</td>
<td>Fruity, leather-like</td>
</tr>
<tr>
<td>2-methyl-2-butenal and/or</td>
<td>722</td>
<td>Pudding-like, fruity, peach, weak dung-like, burnt</td>
</tr>
<tr>
<td>4-methyl-2-pentanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2(E)-Pentenal and/or</td>
<td>731</td>
<td>Fruity, apple-like, roasted/caramel-like</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>733</td>
<td></td>
</tr>
<tr>
<td>Propanoic acid, 2-methyl and/or</td>
<td>744</td>
<td>Slightly sour, slightly caramel-like, slightly vinegar-like</td>
</tr>
<tr>
<td>Pentane-1-ol</td>
<td>751</td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>761</td>
<td>Burnt, bread/caramel-like, sulfur-like, sour-like, rancid</td>
</tr>
<tr>
<td>Hexane-2-one</td>
<td>770</td>
<td>Animal smell, fecal, not specific</td>
</tr>
<tr>
<td>Hexanal and/or</td>
<td>780</td>
<td>Sweet, grass-like, intense, persistent, slightly floral,</td>
</tr>
<tr>
<td>Butanoic acid ethyl ester</td>
<td>785</td>
<td>vegetable soup, cut grass,</td>
</tr>
<tr>
<td>Unknown</td>
<td>791</td>
<td><em>Fruity, bouillon-like, bakery-like, not specific</em></td>
</tr>
<tr>
<td>Unknown and/or</td>
<td>794</td>
<td><em>Fresh/floral, fruity, not specific</em></td>
</tr>
<tr>
<td>Component</td>
<td>Octane</td>
<td>Characteristic Description</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------</td>
<td>--------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Octane</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>Pentanoic acid, methyl ester</td>
<td>807</td>
<td>Slightly sweet, burnt milk, weak floral</td>
</tr>
<tr>
<td>(E)-2-hexenal</td>
<td>825</td>
<td>Burnt, caramel, slightly chemical, medicinal, sour milky</td>
</tr>
<tr>
<td>Furfuryl alcohol and/or</td>
<td>833</td>
<td>Caramel, fruity-like, rancid, earthy, not specific</td>
</tr>
<tr>
<td>Butanoic acid, 3-methyl-</td>
<td>835</td>
<td></td>
</tr>
<tr>
<td>Butanoic acid, 2-methyl-</td>
<td>849</td>
<td>Fruity, sweaty, rancid, burnt, sour</td>
</tr>
<tr>
<td>Hexane-1-ol</td>
<td>853</td>
<td>Fragrant, slightly sweet, grass-like, woody, candy-like, floral/fruity caramel-like, vegetable, dried onion</td>
</tr>
<tr>
<td>Ethyl benzene and/or</td>
<td>858</td>
<td></td>
</tr>
<tr>
<td>Methylsulfone</td>
<td>865</td>
<td>Pungent, slightly caramel-like, paper-like</td>
</tr>
<tr>
<td>Pentanoic acid and/or</td>
<td>867</td>
<td></td>
</tr>
<tr>
<td>Benzene-1,4-(or 1,3-)-dimethyl-</td>
<td>870</td>
<td>Intense potato soup, cooked potato,</td>
</tr>
<tr>
<td>Methional and/or</td>
<td>872</td>
<td></td>
</tr>
<tr>
<td>Heptane-2-one</td>
<td>878</td>
<td><em>Intense, stale, cooked potato, slightly sour</em></td>
</tr>
<tr>
<td>Unknown</td>
<td>886</td>
<td>Bouillon-like, floral, intense smoky, pleasant, fruity, fresh, potato-like, earthy</td>
</tr>
<tr>
<td>Heptane-2-ol and/or</td>
<td>888</td>
<td></td>
</tr>
<tr>
<td>Benzene-1,2-dimethyl-</td>
<td>891-900</td>
<td>Intense, roasted breadcrumbs, bake house, cooked rice, biscuits, cooked milk</td>
</tr>
<tr>
<td>Pyrazine-2,6-dimethyl and/or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrazine-2,3-dimethyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexanoic acid, methyl ester</td>
<td>906</td>
<td>Pungent, fruity, unpleasant, sweaty, musty, intense</td>
</tr>
<tr>
<td>Unknown</td>
<td>910-915</td>
<td><em>Creamy, pleasant, floral/fruity, caramel-like</em></td>
</tr>
<tr>
<td>1-octene-3-one</td>
<td>950</td>
<td>Intense and persistent mushroom-like</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>952</td>
<td>Unpleasant, chemical, caramel-like</td>
</tr>
<tr>
<td>1-octene-3-ol</td>
<td>963</td>
<td>Intense, persistent mushroom-like, medicinal, earthy</td>
</tr>
<tr>
<td>dimethyltrisulfide and/or</td>
<td>967</td>
<td></td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene-1,3,5-trimethyl</td>
<td>Pleasant bouillon-like, Maggie sauce, short and intense, intensely pungent, burnt caramel, forest-like</td>
</tr>
<tr>
<td>octan-2-one and/or</td>
<td>972</td>
</tr>
<tr>
<td>Terpene</td>
<td>976</td>
</tr>
<tr>
<td>Hexanoic acid, ethyl ester and/or</td>
<td>980^ 3 Pleasant, fragrant, slightly acetic acid-like, earthy,</td>
</tr>
<tr>
<td>Octanal</td>
<td>985^ 2 floral, grass-like, fruity</td>
</tr>
<tr>
<td>Trimethylpyrazine and/or</td>
<td>984</td>
</tr>
<tr>
<td>Beta-pinene</td>
<td>988</td>
</tr>
<tr>
<td>Unknown</td>
<td>994-1010 Roasted potato, pungent</td>
</tr>
<tr>
<td>1-hexanol, 2-ethyl- and/or</td>
<td>1015^ 2 Honey, vegetable, green, moist</td>
</tr>
<tr>
<td>Benzene acetaldehyde</td>
<td>1017^ 2</td>
</tr>
<tr>
<td>Unknown</td>
<td>1025-1043 Stable, burnt</td>
</tr>
<tr>
<td>Ethanone, 1-phenyl</td>
<td>1045</td>
</tr>
<tr>
<td>2H-pyran-2-one, tetrahydro-6-methyl-(lactone)</td>
<td>1057 Fecal, slightly animal, cardboard- or paper-like, butyric acid-like,</td>
</tr>
<tr>
<td>Pyrazine, tetramethyl-</td>
<td>1066 Slightly sour/rancid, burnt wood, fatty, not specific</td>
</tr>
<tr>
<td>Nonane-2-one and/or</td>
<td>1074^ 2 Candy-like, slightly sweet, mouldy, musty, pleasant,</td>
</tr>
<tr>
<td>Heptanoic acid, ethyl ester</td>
<td>1077^ 2 fruity, ripe fruit, slightly vineagar-like, persistent</td>
</tr>
<tr>
<td>Ethanol, 2-phenyl-</td>
<td>1095^ 2 Fragrant, candy, fruity, grass/floral -like, fresh, pleasant,</td>
</tr>
<tr>
<td>Unknown</td>
<td>1100 Roasted nuts, cooked cabbage</td>
</tr>
<tr>
<td>Heptanoic acid, 4-hydroxy-,(gamma)-lactone</td>
<td>1123 Cooked vegetable, potato-like</td>
</tr>
<tr>
<td>2(E)-nonenal</td>
<td>1135^ 2 Potato, cucumber/vegetable, floral, fragrant, hay</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>1145 Burnt milk or pudding, intense</td>
</tr>
<tr>
<td>Unknown</td>
<td>1158 Spicy/citrus, floral, not specific</td>
</tr>
<tr>
<td>Compound</td>
<td>Retention Index</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Unknown</td>
<td>1172</td>
</tr>
<tr>
<td>Octanoic acid, ethyl ester</td>
<td>1179$^2$</td>
</tr>
<tr>
<td>Unknown</td>
<td>1195$^2$</td>
</tr>
<tr>
<td>Unknown</td>
<td>1204</td>
</tr>
<tr>
<td>Octanoic acid, 4-hydroxy-</td>
<td>1211</td>
</tr>
<tr>
<td>(gamma)-lactone</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1240-1244$^2$</td>
</tr>
<tr>
<td>Decanoid acid</td>
<td>1352</td>
</tr>
</tbody>
</table>

$^1$ Retention index reported in the literature

$^2$ Aroma-active compounds (AACs) that the GC-O panel described using terminologies similar to those used by the sensory panel for the description of the off-flavour ("feed") studied in the present investigation.
Figure 5-1. Typical chromatograms of headspace volatile compounds of milk sampled over time from a cow


A. Pre-feeding milk sample (after 12 forage-starvation); B. milk sampled 30 min after the cow was fed silage; C. Milk sampled 3 hours after the cow was fed silage.
Figure 5-2. Typical chromatograms of headspace volatile compounds of silage

N.B. The scale on the Y-axis ("abundance") was magnified to 2.5 times bigger than the one in the chromatograms of milk samples, and still 5 of the detected peaks (No 1, 7, 14, and two unidentified) were over the printing limit.)
CHAPTER 6

DEVELOPMENT OF AN INSTRUMENT-BASED DIAGNOSTIC TEST FOR OFF-FLAVOURS IN MILK
6.1. Introduction

The importance of milk grading lies in the fact that dairy products are only as good as the raw materials from which they are made [1], and flavour and aroma are important aspects of consumer acceptance criteria. An incorrect diagnosis of off-flavour automatically translates into loss of income for the dairy producer and, to a certain extent, for the dairy processing company. On the other hand, an incorrect decision to process off-flavoured milk can result in loss of product and possible loss of consumers' confidence in milk and other milk products.

The only accepted testing method for the monitoring of the flavour quality of raw milk is organoleptic assessment by a trained sensory panel. However, it is a subjective method that cannot be standardized or easily validated, and depends on many factors that cannot always be controlled, including physiological status of the graders and environmental conditions under which the testing takes place. Various factors, such as respiratory infections, allergies, medications, pregnancy, chewing gum, and eating or drinking shortly (about ½ hour) prior to testing, have been reported to interfere with normal functions of taste and smell [2]. Also, using a trained panel is time consuming, and presents a health hazard for the panelists because of a potential exposure to pathogenic microorganisms in the milk. Thus, there is a need to find a more objective, consistent and practical alternative (or complementary) diagnostic tool for the detection of objectionable flavours in raw milk.
In the past two decades, substantial progress has been achieved toward developing sophisticated sensing (taste/smell) systems for rapid and reliable assessment of the quality of food and beverage systems [3]. These systems, generally referred to as “electronic” or “artificial noses,” are composed of an array of chemical gas sensors coupled with multivariate data processing methods. They are claimed to have a certain similarity in the measurement concept with the human olfactory system. During the assessment of a flavoured sample by a human panelist, the volatile odoriferous chemicals reach the olfactory receptors (in the nasal cavity) which generate electrical signals that are transferred to the brain for odour recognition [4-7]. Similarly, the interaction between the sensors of an electronic nose with the flavour compounds result in the formation of electrical signals that are then interpreted numerically and graphically by multivariate pattern recognition techniques: consequently, samples with similar flavour or aroma usually generate similar sensory response patterns and those with different flavours exhibit differences in the corresponding graphs.

The objective of the present trial was to use traditional organoleptic assessment (by a trained panel of milk graders) as a “gold standard” to develop a (proprietary) headspace sensor array instrument (αFOX, Alpa MOS, Toulouse, France) for the detection of off-flavours in milk and investigate its reliability.
6.2. Material and Methods

6.2.1. Sample collection and organoleptic testing

Within a period of one month in 2002, a total of 14 samples of bulk-tank milk found to be tainted with feed off-flavour during routine flavour quality control were collected by commercial milk truck operators, each in a 500 ml sterile glass bottle. In order to confirm the status of the samples, two additional milk graders retested at the processing plant in a blind manner (i.e. not knowing whether there was an off-flavoured sample or not). The blinding of the graders was accomplished by serving an off-flavour sample with two samples of good quality in similar 30 ml sterile semi-transparent plastic containers. Only samples that were rejected by all graders were retained for this study. Each off-flavoured sample was matched with a control (milk of good quality) on the date of pick-up. Similarly, only samples that were graded as acceptable by both graders were used as control samples.

6.2.2. Sample handling

The samples were stored at +4°C at the processing plant. Within 24 hours, they were transported to the Atlantic Veterinary College in a cooler packed with ice and then stored at -80°C until submitted for analysis to Alpha MOS France. In order to ensure adequate refrigeration during transportation (by air) to France, the 28 milk samples were packaged in cardboard boxes packed with dry ice. The samples arrived in France in good condition.
6.2.3. Laboratory analyses

Because of the proprietary and confidential nature of the unique “data treatments” developed and used by Alpha MOS, it was not possible to obtain the description of detailed analytical procedures and the raw data on which statistical analyses (discriminant factorial analysis – DFA and principal component analysis – PCA) were based. For this reason, the results of the analyses performed in the Alpha MOS laboratory were included in the thesis as an appendix (Appendix B) in recognition that the analytic work was performed by Alpha MOS and not by the University of Prince Edward Island.

Twenty of the 28 milk samples, labeled either with the prefix “FF” (feed off-flavour positive) or “RS” (control), were destined for the calibration (or “training”) of the aFOX system for pattern recognition and correlation with feed off-flavour, the remaining 8 samples with undisclosed off-flavour status (or unknown samples), labeled with the prefix “SOS”, were used for the determination of the discriminative ability and repeatability of the calibrated aFOX system.

6.2.4. Statistical analyses

Multivariate statistical analyses (PCA and DFA) were performed on the data generated by the selected subset of sensors that constituted the aFOX system and the organoleptic data of the standards (“FF” and “RS” samples):

- DFA was performed on both datasets to determine whether a reliable
An identification model was built; and PCA was applied on the sensor data to determine the discriminative ability of the αFOX system. PCA transforms complex data such as those generated by the multiple sensors of the αFOX system into a less complex data; it provides a view into a 2-dimensional graph than can group sets of data into population clusters.

6.3. Results

In summary, it appeared that the calibrations that the αFOX system developed, based on the provided known milk samples (standards), were successful at classifying the unknown samples. Results presented in appendix B are summarized in Table 6.1. The αFOX classification of seven of the eight unknown samples perfectly matched that of the trained sensory panel. Replicates of these samples, analyzed under the same conditions, yielded similar results. Because of the lack of a suitable gold standard, it was not possible to identify which of the two tests (the αFOX system and the sensory panel) correctly evaluated the misclassified samples (three standards and one “unknown”).

6.4. Discussion and Conclusions

Pattern recognition is not only based on the odour-active compounds, but also on odourless volatile compounds contained in the analyzed sample. Ampuero and Bosset [8] indicated that electronic noses could be trained using an efficient sensory panel to
recognize new patterns and associate them with new flavours. Miniature commercial portable sensing systems for food products, such as cereals or fish [9-11], have already been developed; and to date, a number of research institutions and organizations (http://www.nose-network.org/review) are involved in the development of novel sensor technologies and the implementation of new data processing algorithms (and a combination thereof) in various fields. Numerous publications [8,12-16] have indicated successful application of these technologies (at the experimental stage) to a series of dairy products, including off-flavoured milk; however, these works were not followed through for an eventual validation of the methods and the systems.

Although the results from the assessment using αFOX system provided strong evidence on the suitability of this system for routine milk flavour quality control programs, it is necessary to revalidate the entire procedure in a well-controlled fashion. This includes accurate selection and appropriate handling of multiple standards and unknowns, and appropriate selection of the gas sensors to be mounted together. It is hypothesized that the use of standards with various intensity of off-flavour (or non off-flavour) would result in the improvement of the sensitivity and specificity of the resulting system.

Given the diversity of the volatile compounds involved in the different classes of milk flavour defects (feed, oxidized, rancid, malty, chemical, etc.), the manufacturer of the αFOX system and others may have to face the challenge of developing specific electronic noses instead of a broad selective sensor.
6.5. Reference List


(14) Schaller E, Bosset JO, Escher F. Electronic noses and their application to food: a review. Lebensm Wiss u Technol 1998;305-316.


Table 6-1. Assessment of 28 milk samples by a sensory panel and αFOX system.

<table>
<thead>
<tr>
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<th>Sensory panel</th>
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<td>“Control”</td>
<td>Total</td>
<td></td>
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<tr>
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<th>“Control”</th>
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<td>“Control”</td>
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<tr>
<td>Total</td>
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CHAPTER 7

GENERAL DISCUSSION
7.1. Introduction

At the onset of this research program in 2000, the dairy industry in PEI was faced with a serious increase in the incidence of milk rejection because of reported objectionable flavours in bulk-tank milk. Past outbreaks in the Maritime provinces pointed to milk oxidation as the most likely cause. During the course of the studies, research techniques that have not previously been applied to milk quality problem, were used:

- Diagnostic test evaluation to assess the reliability of the milk graders (Chapter 2),
- cluster analysis for the study of geographical and temporal aspects of this increased incidence of objectionable flavours in milk (Chapter 3),
- controlled epidemiological study for the determination of risk factors associated with these objectionable flavours (Chapter 4),

As a result, this thesis contains perhaps the most thorough documentation and analyses for an off-flavour outbreak to date. Strong evidence has been provided in this thesis that the major off-flavour implicated in the PEI outbreak was derived from round-bale silage (Chapter 4). An outbreak of feed off-flavour of this magnitude has not previously been reported.

Recognizing the difficulties inherent to any organoleptic assay to identify milk flavour defects, research was conducted to identify the volatile compounds that might be responsible for the objectionable flavours. The ultimate goal of this line of research was to assess the feasibility of an instrument-based assay to reliably and objectively identify
feed and other off-flavours. These studies, using HS-SPME GC analyses and live-cow model for producing off-flavoured in milk, were successful and a number of compounds were identified that showed promise as markers for feed off-flavour in milk derived from cows fed baled grass silage (Chapter 5). In parallel with the HS-SPME GC studies, a proprietary sensor assay instrument was developed and also tested to determine its suitability for discriminating unacceptable from acceptable samples (Chapter 6). The results of this pilot study, contained herein, showed great promise and will form the basis of future studies in off-flavours in PEI. A brief summary of the methods and results from each step of this program of research follows below.

7.2. Reliability of sensory analysis of milk flavour

The evaluation of intra- and inter-grader reliability of a sensory panel in the assessment of milk flavour quality produced results ranging from fair to almost perfect depending on the task performed. When two panels of five certified milk graders each were asked to perform only the discriminative sensory analysis (assessment of the presence or absence of off-flavour in the tested sample), the test yielded almost perfect results, with kappa values and $AC_1$ statistics for both intra- and inter-panel agreements over 0.85 ($p<0.01$). The proportion of agreement between the two panels ranged from 94% to 97% on off-flavoured milk samples and on samples of good quality, it was slightly lower: from 91% to 96%. The assessment of the intra-panel reliability yielded similar results. However, when individual panelists were asked not only to differentiate good samples from off-flavoured, but also to assess the type of off-flavour (qualitative analysis) and its intensity
level (semi-quantitative analysis), the results were not as consistently high as those obtained in the discriminative analysis performed by the panels of five graders each. Kappa values and $AC_j$ statistics suggested that pair-wise inter-panelist agreement ranged from substantial (0.71) to almost perfect (0.91), whereas for intra-panelist agreement, the range was from 0.52 to 0.82 (i.e. from fair agreement to almost perfect).

As for the semi-quantitative sensory analysis, the level of agreement was significantly lower; it ranged from fair (kappa = 0.33) to moderate (kappa = 0.68) for both inter- and intra-panelist reliability. Whereas qualitative sensory analysis (classification of the tested sample in one of the following categories: “good,” “feed,” “oxidized,” “rancid,” and “malty”) yielded almost perfect (kappa = 0.84) and moderate (kappa = 0.57) levels of agreement, respectively for the first two categories only. It can be speculated that the lack of agreement between the panelists on samples representing other flavour defect categories (“oxidized,” “rancid,” or “malty”) was due to their under-representation in the overall sample size; very few assessed samples were assigned one of these categories. However, given the fact that qualitative and semi-quantitative sensory analyses are of lesser importance in quality control monitoring program, it would be legitimate to conclude that organoleptic assessment of flavour quality of bulk-tank milk by trained panelists is an appropriate tool for milk flavour quality monitoring, particularly in the absence of a more objective instrument-based method.
7.3. Geographical and temporal aspects of the outbreak

Clustering analyses indicated that registered cases of off-flavour in bulk-tank milk in PEI for the 20-month study period were clustered in time (fall-winter season) and in space (Queens and Prince counties). This analysis also suggested that there was a statistically significant \( p<0.05 \) space-time clustering effect. Results showed that the two high-rate clusters of transmitted (or feed) off-flavour had on average higher monthly precipitation levels than the areas of location of the low-rate clustered herds. Temperature data were not as conclusive, because it appeared that only the location area of the primary low-rate cluster was warmer than those of the high-rate and secondary low-rate cluster, which had similar average monthly temperature \( (5.6 - 5.9 \, ^\circ C) \). Also, high-rate clustered herds that experienced transmitted off-flavour had poorer air quality in the barn housing lactating cows (because of inadequacy of ventilation system) than herds in low-rate clusters, used round-bale silage as the main forage for lactating cows, and fed this forage either shortly before milking or as free-choice. Conversely, in low-rate clustered herds, the main forage was either chopped grass or corn silage, which were usually fed to the lactating cows only after milking.

7.4. Risk factors for the major off-flavour detected in the studied outbreak

The findings presented in this thesis (Chapter 2) indicated that, unlike the situation experienced by the adjacent province of New Brunswick more than a decade ago, where
oxidized off-flavour was the major flavour defect [1], the most frequently encountered off-flavour in bulk-tank milk in the PEI outbreak was feed off-flavour with the following associated risk factors:

- Poor air quality in the barn for lactating cows, with an odds ratio (OR) of 41,
- Feeding baled silage to lactating cows, which exhibited an OR of 11,
- Feeding baled silage or other type of forage before milking (OR = 253) or as free choice (OR = 3.2),
- Not clipping the cows' udders (OR = 14.3)
- Changing bedding material less than or only once a day (OR = 8.3).

Interestingly, a marked decrease in the number of bulk-tank milk rejection due to off-flavour was observed during the period when the dairy producers were being provided with recommendations elaborated on the basis of the knowledge of these above-mentioned factors. The declining pattern of the outbreak was still noted more than a year after the study ended (Personal communications with the of the PEI dairy companies).

It is important to note that the pressing demand for practical and quick answers following the onset of the outbreak led to the prioritization of the risk factor study over the study of the geographical and temporal aspects of the outbreak (clustering analysis). Normally, the latter approach should have been the primary step as it would have generated valuable hypotheses about known or potential risk factors and regions of priority for subsequent more controlled studies.
Also, because of the lack of an objective measurement tool for subjective parameters such as air quality or the adequacy of the ventilation system, it would have been preferable to assign their assessment to a “blinded” assessor not to the principal investigator who had knowledge of whether the herd in which the data were being collected was off-flavour positive or negative. This would have prevented (or minimized) potential biases.

Numerous publications [2;3] have indicated that off-flavours described as “stale,” “barny,” “bitter,” “rancid,” “unclean” or even “feed” could be of microbial origin; consequently, it might have been advisable to investigate the bacterial counts of each herd involved in risk factor study similar to that in chapter 2. Such an approach was considered at the primary stage of the study as there were suspicions of simultaneous development of feed and oxidized off-flavours. The total antioxidant capacity of 20 bulk-tank milk samples [10 feed off-flavoured and 10 controls (determined by the sensory panel during routine monitoring)] was analyzed using ORAC (oxygen Radical Absorbance Capacity) assay. Results indicated no significant difference between off-flavoured samples and control samples, suggesting that a difference in the antioxidant capacity of the milk was not associated with the development of this off-flavour. This investigation was not carried out on all the collected bulk-tank samples because of shortage of funds and because this preliminary study did not support the hypothesis of milk oxidation as the cause of off-flavour. However, this approach to the milk flavour defects could be useful in outbreaks of oxidized or/and rancid flavours if the ORAC test could be further validated with milk.

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7.5. Development of a model for a reliable reproduction of feed off-flavour

The multipurpose experimental study that involved 9 Holstein Friesian cows from three PEI commercial dairy farms and two sensory panels resulted in the successful development of a model that could reliably reproduce feed off-flavour in milk under typical dairy farming conditions. Milk samples collected from the cows after they were forage-starved for approximately 12 hours, and those collected 30 min and 3 hours, respectively, after they were fed round-bale silage, were submitted for flavour quality assessment to two sensory panels of certified graders. The two panels found that milk samples collected prior to silage feeding were good quality, whereas, of the nine samples of 30 min post-feeding, one was of good quality. The two panels found that eight of the nine samples from 3 hour post-feeding were off-flavoured. Consequently, kappa values, ACI statistics and the other measurement of agreement level between the different panels, such as the indices of positive and negative agreement, were all within the “almost perfect” range as defined by Landis and Koch (1977). It was concluded, based on this evidence, that feeding freshly opened baled silage to lactating cows 30 min to 3 hours prior to milking causes objectionable feed off-flavour.

7.6. Profiling of the associated flavour compounds

In order to identify the volatile compounds that were associated with feed flavour defect, aliquots of above-mentioned samples (section 7.5), as well as the silage samples from the
three farms were analyzed chromatographically (gas chromatography-mass spectrometry/flame ionization detection – GC-MS/FID and gas chromatography-olfactometry – GC-O) using headspace solid phase micro extraction (HS-SPME) techniques. Nineteen volatile organic compounds (VOCs) were detected in at least 8 of the 27 milk samples and 23 compounds in less than seven samples. Much similar chromatographic analysis of the analyzed silages in the study revealed the presence of all the 19 major VOCs detected in the milk samples, but in much higher concentrations. Given that there was a substantial increase in the relative concentrations of some of these compounds in post-feeding milk samples, it was hypothesized that these compounds (as a set) were potential markers for feed flavour defect of timothy baled silage origin. Statistical analyses using both GC-MS/FID data (as predictors) and sensory data (as outcome) suggested that the occurrence of feed off-flavour in milk could be significantly \( p<0.01 \) predicted by the relative concentration of either Dimethyl sulfide or Butane-2-one. These two parameters showed evidence of high correlation in the multivariable logistic regression model building, and to prevent multicollinearity it was recommended to drop one [4]. However, because minute elevation above the concentration threshold of volatile compounds may be sufficient to render a milk sample off-flavoured and not sufficient to exhibit a statistically significant association, caution is recommended for the interpretation of the results of statistical analyses.

Olfactometric analysis (HS-SPME GC-O), which was applied on 2 pre-feeding and 3 post-feeding milk samples, offered a different perspective, which is the identification of the aroma-active compounds contained in the analyzed samples. It was applied on a
limited number of samples because it was time-consuming process. It resulted in the
detection of a total of 75 aroma-active compounds (AACs), of which 23 were described
with terminologies similar to those usually used for the description of feed off-flavour. A
total of 70 AACs were detected in all the analyzed samples, suggesting that perhaps off-
flavour is caused by the concentration differences of a set of aroma-active compounds
rather than just the presence of certain compounds. However, it would not be justified to
conclude that these compounds were the only ones responsible for the generated flavour
defect. The fundamental difference between sensory and olfactometric analyses is the fact
that in the sensory analysis the panelists simultaneously assess the overall flavour quality
of a food system (milk in this case); whereas in the GC-O, they proceed with a sequential
determination of individual aroma-active compounds. Nevertheless, these results can be
viewed as the primary step toward elucidating the mystery of flavour defects in complex
food systems such as milk. Any further consideration toward pursuing such investigation
would require the use of only fresh (< 1 month) and well-preserved milk samples as the
preliminary chromatographic analyses performed on long-term stored (> 3 months at -80
°C) samples yielded misleading results.

7.7. Development of an alternative (or complementary) diagnostic
assay for the detection of off-flavours.

Although the assessment of the discriminative sensory analysis in chapter 4 yielded
satisfactory results, the routine monitoring of the flavour of bulk-tank milk has
sometimes been fiercely challenged, usually by the dairy producers whose milk has been
condemned because of off-flavour. On a few occasions, it has degenerated to serious altercations between the producers and the milk graders. Also, there have been reports of truckloads of milk (which represent multiple farm pick-ups) being tainted with off-flavour, which means that either one of the bulk-tank milk loads picked up by the milk grader (who is usually the milk truck driver) was off-flavoured (and he couldn’t identify it) or the off-flavour fully developed during transportation to the processing plant. Such situations have been possible because of the inherent variation of the subjectivity of the sensory panel whose accuracy depends on numerous physiological (uncontrollable) and environmental factors [5], such as the health status of the graders, the meal they ate prior to the assessment of the samples, and the air quality in the surrounding environment where the grading is performed. Thus, there is a need for a more objective and consistent instrument-based diagnostic assay for off-flavours. To foster this idea, collaborative work was initialized with Alpha MOS (Toulouse, France) to begin the development of an instrument-based diagnostic tool (αFOX system). Evaluations by a certified sensory panel of a batch of 20 bulk-tank milk samples (10 off-flavoured and 10 of good quality) were used to produce a set of standards αFOX system. This system was then applied to 8 unknown samples, and results suggested that the developed αFOX system had not only very good discriminative ability but also very good repeatability. Of the 8 “blinded” samples, the results yielded by the αFOX system on 7 were similar to those of the sensory panel of the investigative team at the Atlantic Veterinary College. At this stage of the study, it is difficult to speculate on which of the two tests (the sensory panel or the αFOX system) correctly classified the sample on which they disagreed.
7.8. Recommendations for future research.

With satisfactory results obtained in the preliminary step of the feasibility study of an instrument-based diagnostic assay, future development with a much larger sample size is recommended. The use of not only the sensory panel as standard, but also other analytical techniques such as gas chromatography could improve detection accuracy. However, the success of the αFOX system (gas-sensor array) depends on the selection of accurate standards and validation of the calibration. Unlike classical gas chromatography (GC/MS or GC-O), which is a multistage analytical technique (extraction, pre-concentration, injection in the chromatograph for separation, then qualitative and quantitative detection of the volatile molecules), the technology of electronic noses allows the analysis, simultaneously of the flavour characteristic (aroma) of a product in its original matrix. On the other hand, because of the thousands of sensitive nerve endings active in the human nose and retronasal system, it would be extremely challenging under ideal conditions for an electronic nose, equipped with few sensors (4 to 64) to outclass or level up with the extraordinary capacities of the human brain (coupled with memories from long-term cultural and social training) in analyzing flavour stimuli [6]. However, given the extreme dependency of the performance of the human brain on factors such as age and physiological status of the assessor, the testing technique and the environment, and other difficulties related to the quantification of human senses, the technology of electronic noses seems to be the ultimate universal alternative for the future of quality control of milk flavour and other food products.
7.9. Concluding Remarks

The research performed within the framework of this thesis was unique in that, it provided strong insight for a better understanding of the phenomenon of off-flavours in general and transmitted off-flavour in particular. It is undoubtedly to our knowledge the first time that an outbreak of off-flavour in milk has been studied at such an advanced level. Novel analytical approaches were used different stages of the research, starting from the assessment of the reliability of organoleptic assessment of milk flavour to a potential development of a diagnostic assay for similar purposes. It was shown that the use of the former analytical technique was appropriate for the screening of raw milk for off-flavours. Preliminary findings on the use of an instrument-based diagnostic as an alternative or a complementary tool to organoleptic assessment yielded satisfactory results. Miniaturization of such device may revolutionize milk flavour quality control.

Most importantly, this research achieved its primary goal, which was the identification of the factors associated with increase occurrence of off-flavours in bulk-tank milk, followed by the elaboration of control measures to minimize the problem. It was found that transmitted off-flavour was the driving force of the PEI outbreak and feeding stored forage to lactating cows before milking or as a free-choice was the most important associated risk factor (with a population attributable fraction of 0.70) and that fall-winter period was the major risk period. Control strategies recommended based on our findings seemed to produce a significant positive effect as its application coincided with a sharp and continuous decrease in the incidence of off-flavours.
7.10 Reference List


8. APPENDIX A.

QUESTIONNAIRE FOR THE RISK FACTORS STUDY (Chapter 4)
Milk Off Flavour Project - Check List

ON FARM

Questionnaire completed?
Red top blood samples: 5 < & 5 >= 150 DIM; record cow ID and DIM on data sheet.
Milk samples, from cows as above.
Body condition score (out of 5), from cows as above, record on data sheet.
Water sample, free flow from tap in tank room, freeze.
Feed Samples: (describe as labeled for freezing)
Grain 1 _________________  Forage 1 _________________
Grain 2 _________________  Forage 2 _________________
Other _________________
Feed Data Sheet completed?  Copy ADLIC Report from last test?
Feed tags as required?

ON RETURN
Fill in mileage and account number (624022) in vehicle log.
Spin bloods and freeze serum and milk -20C labeled with ID, date and owner.
Split forage samples (if not done on farm), label and freeze one half of sample in screw top container; other half in zip lock bag to go to feed lab (see Feed Data Sheet for what analyses to order).

FOR CONTROL HERDS:
Notify Dairy Lab - Wendy 368-4480
Request that bulk tank milk sample, day of problem, and 2 pick-ups preceding, be kept by dairy lab (if problem herd, she will do this automatically).
Notify Les Halliday - 569-7639 or ljhalliday@gov.pe.ca (home 892-5331)
Let him know that feed samples will be arriving for this herd.

FOR BOTH CONTROL & PROBLEM HERDS:
Notify Ron Sampson - 368-5600 or rtsampson@gov.pe.ca
Let him know that milking equipment evaluation will be required for this herd.

Copy questionnaire and feed data sheet and send copies in envelope to both Les and Ron (drop off at research Station with feed samples)

FOLLOW-UP

Report received - from nutritionist
Report received - from milking technologist
Follow-up call at 3 weeks - date completed _________________
Questionnaire for Milk Off-Flavor Herds

Section 1 - Problem Overview

1) Producer Name __________________ ADLIC No. __________________
   Farm Name __________________ Producer Shipping No. __________

2) Mailing Address _____________________________________________
   Postal Code __________ Community Name _______________________
   Phone __________ Road or Route and Civic No. __________________

3) Date Problem Identified _______________________________________

4) Location problem was identified: __Farm __Plant (ADL / Montague / Purity?)

5) Was milk pick-up refused? __Yes __No

6) How many shipments were dumped? ___

7) Type of off-flavor according to driver: e.g. oxidized, feed, rancid, flat, salty, malty,
   unclean, chemical. ___________________________________________

8) Was off-flavor confirmed by second test? __Yes __No
   If so, by who? _______________________________________________

9) Has this herd had a previous problem? __Yes __No
   If so, when and what sort? _____________________________________

Visit Type: Problem / Control

Survey Date: ______ Mileage: ______ Vehicle Used: ______

Atlantic Veterinary College
University of Prince Edward Island
550 University Avenue, Charlottetown PEI C1A 4P3, (902) 566-0993

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10) Has there been problems with delayed milk pick-up (e.g. storm)?  Yes  No
   If so, describe? ________________________________________________________________

11) Please describe the weather conditions on the day before, and the day on, the
    off-flavor problem occurred. ______________________________________________________

12) Has there been anyone working up the problem so far?  Yes  No
    If yes, please list: ______________________________________________________________

13) Has there been any action taken or changes implemented as a result of the off-flavor
    problem?  Yes  No
    If so, please describe: __________________________________________________________

Section 2 - General Herd Characteristics

1) What breed primarily exists on this operation?  Holstein  Ayrshire  Guernsey
   ________ Jersey  Brown Swiss  Other (please describe)

2) What type of housing are the milk cows housed in?  Free Stalls  Tie Stalls
   ________ Other

3) What is the air quality in the housing area?  Satisfactory  Poor  V.Poor

4) Is this a recorded herd?  Yes  No

5) What is the herd size?  Lactating Cows  Dry Cows

6) How many lactating cows are first lactation?  ________

7) Has there been any recent changes , or trends developing in per cow milk production
   and average peak milk?  Yes  No
   If so, detail: ____________________________________________________________________

   Please report the volume (litres of milk) of the refused pick-up ________ Litres
   or if unknown, the pick-up just prior to the rejection ________ Litres

8) At the time of the off-flavor milk problem, was there any recent changes or trends in
   milk components?  Yes  No
   If so, detail: __________________________________________________________________

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9) What is the general appearance of the cows? (E.g.: cow comfort, nervousness, etc.)

_____________________________________________________________________

10) Please describe the general cleanliness of the cows, feed areas, housing areas, milk parlor, and milk room. ________________________________________________________________
_____________________________________________________________________

Total bacterial count problems? ____________________________________________

11) A. Manure consistency: very firm / firm / normal / loose / very loose

B. Does soiling of hair extend
   a) above fetlocks __Yes __No
   b) above hocks __Yes __No
   c) on flanks __Yes __No

12) Is soiling of udders apparent? __Yes __No

13) Is hair clipped from udders? __Yes __No

14) Are stalls adequate size? __Yes __No

15) Are stalls clean? __Yes __No

16) Are stalls well bedded? __Yes __No ____________ Bedding type

17) Does the milk tank room have a strong silage smell prior to milking?
   ______ Satisfactory ______ Poor ______ Very Poor

   If unsatisfactory, describe location of silage (storage and feeding areas). _______
_____________________________________________________________________

18) Do cows have an exercise area? __Yes __No

   If yes, how many hours per day are spent outside of the barn? ____________

19) Describe the maintenance schedule for bedding? __Daily __Twice-daily __Other (describe: ___________________________)

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Section 3 - Nutritional Management

1) What feeding system do you use for your milking cows?
   Component feeding  TMR  Base TMR + supplements
   Are computer feeders used?  Yes / No

2) The main forage at the time of the off-flavor milk was:
   Dry hay  Round bale  silage  Chopped Grass/Legume silage  Corn Silage
   Specific description of main forage(s) fed to milkers

3) Do you feed Rumensin (Monensin)?  Yes  No

4) How many times (a day) are cows fed silage?
   once  twice  three  four  more

5) When is silage first fed?  before milking  after milking?

6) What is the delay between unwrapping a bale and feeding?
   minutes, hours.

7) Are open bales in the milking cow barn between opening and feeding?  Yes  No

8) Please detail the feeding routine for a 24 hour period beginning with the first feeding:
   5:00 a.m.
   6:00 a.m.
   7:00 a.m.
   8:00 a.m.
   9:00 a.m.
   10:00 a.m.
   11:00 a.m.
   12:00 noon
   1:00 p.m.
   2:00 p.m.
   3:00 p.m.
   4:00 p.m.
   5:00 p.m.
   6:00 p.m.
   7:00 p.m.
   8:00 p.m.
   9:00 p.m.
   10:00 p.m.
   11:00 p.m.
   12:00 Midnight
   1:00 a.m.
9) Has there been any recent changes in feeding routine? Yes No
If so, describe: ____________________________________________________________

10) Has there been any recent employee changes or changes in who feeds or milks the cows? Yes No
If so, detail: ______________________________________________________________

11) Are there any unusual components in the feed (weeds, etc.)? Yes No

12) Do you typically inject your cows with selenium/vitamin E? Yes No
If so, when?(please check appropriate area as well as indicating dose)
- during dry period (____ times)(dose _____), at dry-off (dose _____),
- during lactation period (____ times), at calving (dose _____),
- bred heifers prior to calving(____ times)(dose _____)

13) Is selenium typically added to the ration or diet of the milk cows? Yes No
If so, how is the selenium added to the milking cow diet?
- mineral premix added to concentrate
- included in commercial feed
- free-choice mineral salts
- mineral salt block with selenium
- other (please describe) ___________________________________________________

14) Is vitamin E typically added to the ration or diet of the milk cows? Yes No
If so, how is the vitamin E added to the milking cow diet?
- mineral premix added to concentrate;
- included in commercial feed
- other (please describe) ___________________________________________________

15) Had you taken steps to increase the selenium and/or vitamin E content of the diet before the current problem? selenium / vitamin E / both / neither

16) Did you use a silage additive (e.g. an inoculant) Yes No
If yes: Which product did you use: ___________________________________________

17) If possible please give a brief history of the silage that was being fed when the off-flavour problem occurred.
- Field (if named) __________________________ Date made ______________________
- Cropping history of field _____________________________
- Fertilizer application? ___________________________________________
- Manure application? ___________________________________________
- Weather during silage harvest _____________________________

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- Delay between cutting and baling: _______ hours;  
- Between baling and wrapping: _______ hours 

Other comments concerning silage making problems ________________________________

18) Do you use roasted or extruded soybeans or added fat/oils in your rations? 
   __Yes  __No If yes which: ____________________________________________

19) Milk urea nitrogen (MUN) herd average value if recorded: ____________________
   (Attach most recent ADLIC reports if a recorded herd)

Section 4 - Disease Incidence

1) Has there been any cases of ketosis/acidity in the herd in the month previous to the off-flavor problem?  __Yes  __No  If yes, how many? _____

2) Has there been any clinical mastitis cases in the month previous to the off-flavor problem?  __Yes  __No  If so, how many cases? _____

3) What is the most recent bulk tank average somatic cell count? __________________

4) Have there been any cases of retained membranes in the month previous to the off-flavor problem?  __Yes  __No  If yes, how many? _____

5) Has there been any cases of metritis in the month previous to the off-flavor problem?  __Yes  __No  If so, how many? _____

6) Has there been any cases of displaced abomasum in the month previous to the off-flavor problem?  __Yes  __No  If so, how many? _____

7) Has there been any trends in disease that you have noticed in the six months previous to the off-flavor milk problem? (E.g. pneumonia, lameness, etc.)  
   __Yes  __No  If so, describe: ____________________________________________
FOLLOW-UP NOTES
include notes on repeat rejections
Milk Off-Flavor Project

Producer Release

This is to certify that Drs. Wichtel, Keefe and Van Leeuwen, of the Atlantic Veterinary College, may have access to production and milk quality data generated from my farm (under the names and numbers on the front of this survey) and contained in databases held by ADLIC, the Provincial Dairy Lab and any one of the PEI dairy companies. All information will remain strictly confidential and will be used only for the research project in which I am participating.

Producer Name _______________ ADLIC No. _______________

SIGNED _______________ DATE _______________
Milk Off-Flavour

Feed Data Collection Sheet

1. Sample silage(s), mix well and divide into 2, send one to the feed lab (F7 package), other stored in plastic contained and frozen for analysis at later date.

   For round bales - minimum of 3 cores for large bore 5 for small bore (1/bale)
   For bunker 10 grab samples from open face
   Upright Silo 4-5 grab samples from shoot when unloading.

   **Note which have caused a problem (if any).**
   Silage label used: _______________ Type of odour: _______________
   Silage label used: _______________ Type of odour: _______________
   Hay label used: __________________ (Lab package F8)

   For round bales note bale density (hard or soft) ________, condition of plastic (holes y,n) ______ amount of plastic used (3-4 layers or 5-6 layers used) ______________________

   For bunker - note amount of spoilage on top and sides (none, slight or excessive) ______

2. For on farm mixed ration -- sample whole grain - label used: ______ (Lab F5 package)

   Grain processing (rolled, crimp, ground) ________________________________
   Type of supplement used - Trade name (Feed Tag): _________________________
   Type of mineral used - Trade name (Feed Tag): ___________________________
   Type of topdress used - Trade name (Feed Tag): ___________________________ 
   Other feed (protein, yeast, etc) _______________________________________
   List any extras added to supplement (Vit E, etc) _________________________

   Mix components (kg/lb)- Grain ______, supplement _______, mineral ____ Other: ______
   If unsure take 1-2 kg sample, send to lab (F4 package)

3. Commercial complete ration - name and Feed Tag: ________________________

   List any extras added to ration: ________________________________________

4. Feeding rate based on production - (eg 30 kg milk early lactation - how much feed offered) ____________________________________________________________

5. How is grain fed computer (y/n) ____, rail feeder (Rovibec) # of times/day ______, scoop feed in barn (y/n) __ # of times/day ______, in parlour (y/n) ____ how much ______

6. For TMR mix list ingredients; Feed #1 ______ weight: ______

   Feed #2 ______ weight: ______
   Feed #3 ______ weight: ______
   Feed #4 ______ weight: ______
   Feed #5 ______ weight: ______
9. APPENDIX B.

REPORT FROM ALPHA M.O.S. ON THE DEVELOPMENT OF

αFOX SYSTEM (Chapter 6)
Analysis Report 981

Milk Analysis

Performed for:
University of Prince Edward Island
Dept. of Health Management

To the attention of
Leigh Gao

March 2003
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Olfactory Study of Milk with the $\alpha$ FOX

I. INTRODUCTION

The Alpha MOS $\alpha$FOX System utilizes a total headspace, Sensors technique. The information gathered by the analysis of the samples is evaluated based on a correlation of the Fox data with the data generated by traditional analysis techniques such as GC and GC/MS and/or sensory panel evaluation. The goal of this feasibility is to utilize known samples to select the best data combinations, thus establishing a calibration, which enables the prediction of the unknown samples. The use of the Fox provides the user a method to qualify and quantify complex sample matrices with greater speed than traditional techniques by predicting the quality of samples compared to the standard set or to determine the quantity of a chemical or the intensity of an olfactive attribute such as taste or smell.

Based on the samples and the calibration data provided, the goal of the feasibility study was to determine the qualitative capabilities of the Alpha MOS $\alpha$FOX (Sensor Array) on different qualities of milk samples.

**Time spent for the analysis:** 3 days

**Time spent writing of the report:** 3 days

**Application performed by:** Xavier Bredzinski
II. ANALYSIS WITH αFOX

a. Equipment

Alpha M.O.S: αFOX Instrument and αPrometheus Software

Alpha M.O.S: Odorscanner HS100 Automatic Sampler

All analyses were performed using the same instrumental configuration.

c. Sensors used: 18 Metal Oxide Sensors (MOS)

Sensor chamber 1: Chamber CL (High Performance Controlled in temperature)
LY/LG, LY/G, LY/AA, LY/GH, LY/gCTL, LY/gCT

Sensor chamber 2: Chamber A (High Performance Controlled in temperature)
T30/1, P10/1, P10/2, P40/1, T70/2, PA2

Sensor chamber 3: Chamber B (High Performance Controlled in temperature)
P30/1, P40/2, P30/2, T40/2, T40/1, TA2
## II. RAW MILK SAMPLE LABELS

<table>
<thead>
<tr>
<th>UPEI Labels</th>
<th>Quality</th>
<th>Sensory Panel Score</th>
<th>Decision</th>
<th>Alpha-MOS Labels</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOOD SAMPLES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS11 A</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_A</td>
</tr>
<tr>
<td>RS22 B</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_B</td>
</tr>
<tr>
<td>RS33 C</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_C</td>
</tr>
<tr>
<td>RS44 D</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_D</td>
</tr>
<tr>
<td>RS55 E</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_E</td>
</tr>
<tr>
<td>RS66 F</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_F</td>
</tr>
<tr>
<td>RS77 G</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_G</td>
</tr>
<tr>
<td>RS88 H</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_H</td>
</tr>
<tr>
<td>RS99 I</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_I</td>
</tr>
<tr>
<td>RS00 J</td>
<td>Good</td>
<td>1</td>
<td>Accepted</td>
<td>G_J</td>
</tr>
<tr>
<td>OFF-FLAVOURED SAMPLES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF11 K</td>
<td>Strong</td>
<td>3</td>
<td>Rejected</td>
<td>B_K</td>
</tr>
<tr>
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<td>4</td>
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<td>B_L</td>
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<td>Rejected</td>
<td>B_M</td>
</tr>
<tr>
<td>FF44 N</td>
<td>Strong</td>
<td>3</td>
<td>Rejected</td>
<td>B_N</td>
</tr>
<tr>
<td>FF55 O</td>
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<td>2</td>
<td>Rejected</td>
<td>B_O</td>
</tr>
<tr>
<td>FF66 P</td>
<td>Strong</td>
<td>3</td>
<td>Rejected</td>
<td>B_P</td>
</tr>
<tr>
<td>FF77 Q</td>
<td>Strong</td>
<td>3</td>
<td>Rejected</td>
<td>B_Q</td>
</tr>
<tr>
<td>FF88 R</td>
<td>Strong</td>
<td>3</td>
<td>Rejected</td>
<td>B_R</td>
</tr>
<tr>
<td>FF99 S</td>
<td>Strong</td>
<td>3</td>
<td>Rejected</td>
<td>B_S</td>
</tr>
<tr>
<td>FF00 T</td>
<td>Moderate</td>
<td>2</td>
<td>Rejected</td>
<td>B_T</td>
</tr>
<tr>
<td>UNKNOWN SAMPLES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOS11</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_1</td>
</tr>
<tr>
<td>SOS22</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_2</td>
</tr>
<tr>
<td>SOS33</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_3</td>
</tr>
<tr>
<td>SOS44</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_4</td>
</tr>
<tr>
<td>SOS55</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_5</td>
</tr>
<tr>
<td>SOS66</td>
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<td>Unknown</td>
<td>Unknown</td>
<td>U_6</td>
</tr>
<tr>
<td>SOS77</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_7</td>
</tr>
<tr>
<td>SOS88</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>U_8</td>
</tr>
</tbody>
</table>
In the following data plots, a number follows the Alpha MOS references. This number indicates the place of the sample in the autosampler tray.

### III. MILK ANALYSIS

#### A. Analytical conditions

<table>
<thead>
<tr>
<th>Carrier gas</th>
<th>Synthetic dry air</th>
</tr>
</thead>
</table>

**Sample preparation**
- Quantity of sample in the vial: 1 ml
- Total volume of the vial: 10 ml

**Headspace generation**
- Headspace generation time: 10 min
- Headspace generation temperature: 80°C
- Agitation speed: 500 rpm

**Headspace injection**
- Injected volume: 2500 µl
- Injection speed: 2500 µl/second
- Total volume of the syringe: 2.5 ml
- Syringe temperature: 85°C

**Acquisition parameters**
- Acquisition time: 120 seconds
- Time between two injections: 20 min.

These analytical conditions could be optimized and five minutes of delay (time between two injections) can be obtained.

All samples were analyzed using the same conditions.
B. Analysis of the Sensor Responses

Figure 1: Comparison between two samples, B_M (Strong FF33) on the left and G_H (Good RS88) on the right.

Figure 1 is an example of the raw data generated by all of the sensors for two samples shown side-by-side. The measurement is a function of change induced by the volatiles present in the sample ($\Delta R/R_0$ vs. time (s)). The intensity given by the Sensor Array System should be correlated with sensory panel scores or chemical analysis to select samples of significant difference and to calibrate the system using the multivariate data processing with statistics.
C. Statistical analysis

1. Discrimination of different milk qualities

   a) **Principal Component Analysis (PCA)**

   The data was compiled into a format for further analysis by taking the raw values of the sensors at the maximum of each sensor response curve. To determine if the samples are well discriminated, a Principal Component Analysis has been performed on the data. The Principal Component Analysis reduces the information generated by the 18 sensors into coordinates that can be plotted on two axes. The function of the PCA is to calculate the coordinate that best separates the samples. Samples that are similar in quality will be grouped close together; samples where differences can be found will be discriminated.

![Figure 2: All samples PCA](image-url)
All of the samples submitted for analysis are displayed in Figure 2. By looking at the positions of the samples, several observations can be made regarding the data.

- The reproducibility is good. This is determined by looking at the proximity of the replicates.
- GA, G_C and G_H are described as the same quality (accepted) and appear to be very similar. They will be considered as equivalents (See Section 2-b).
- A “rejected” sample area can be defined with the samples related to moderate, strong and very strong on the left and the “accepted” samples shown on the right side of the map.
- Moderate samples (B_O and B_T) are projected on the boundary of the rejected area however; these samples are close to the “accepted” area indicating a possible borderline quality.
- The “Very Strong” sample (B_L) is projected at the bottom of the “rejected” area.

Three samples G_G (RS77), B_R (FF88) and B_K (FF11) do not follow the same pattern, as the other samples with similar quality description. Some observations regarding these samples include:

- G_G and B_R have been conditioned in a different type of bottle than the others...
- B_K (FF11) labeled “strong” is not grouping with the other bad samples; this sample is located near the “moderates” samples of the “rejected” area.

b) Discriminant Factorial Analysis (DFA)
To test if a reliable discrimination has been obtained and a correlation between “accepted” and “rejected” samples by the sensory panel is provided from the data, a Discriminant Factorial Analysis (DFA) was performed. A DFA is a multivariate statistical analysis method, which finds the best combination of variables that separate the various clusters according to the provided labels.

The DFA in Figure 3 uses the information given by all 18 sensors.

![Figure 3: General DFA by sensory panel groups](image)

In Figure 3, all the samples are displayed. The data presented on the graph indicates that the system is able to discriminate “rejected” (red) from “accepted” (blue) samples. This model was constructed with the samples that appear to be “ambiguous” in the description (G_G, B_R and B_K). By including these samples the model will not provide maximum
discrimination between strong and good samples.

In order to further optimize the calibration of the predictive model, samples labeled G_G, B_K and B_R, have been excluded and the recalculation of the DFA shown in Figure 4. This model takes into account the data collected on the nine “accepted” milk samples and the eight “rejected” milk samples.

In this DFA, a sensor optimization was performed to select the sensors that are best at defining the differences between the groups. Nine sensors were selected: LY/G, LY/AA, LY/Gh, LY/gCT, T30/1, T70/2, PA2, P30/1 and T40/2.

Figure 4 presents the optimized discrimination between “Acceptable” and Rejected.”

Figure 4: Optimized DFA by sensory panel groups
qualities obtained with this calculation. A larger dispersion related to the "rejected" milks than as seen with the "accepted" milks. This is typical due to the amount of variation seen within the off flavored samples.

2. Identification of Unknown Samples:

a) Identification of unknown samples

As shown in the previous Figures, a calibration model was developed to identify quality of the unknown samples as "Rejected" or "Accepted". The optimized sensors were selected as part of the calibration procedure: LY/G, LY/AA, LY/Gh, LY/gCT, T30/1, T70/2, PA2, P30/1 and T40/2: With the optimized identification model calibrated, it is then possible to predict the quality of the unknown samples as shown in Figure 5.

Figure 5: Prediction groups with unknown samples projected
b)Selected identification of unknown samples

In the development of the prediction calibration discussed previously, it was found that for some of the samples were close to the opposite quality groups. To further resolve the differences between these “borderline” qualities (Figure 6) additional optimization was conducted. This DFA was calculated on a smaller range of qualities and required that the sensor optimization be recalculated: LY/G, LY/AA, LY/Gh, LY/gCT, T30/1, T70/2, PA2, P30/1 and T40/2. All samples are analyzed.
Figure 6: Optimized PCA with all samples

Figure 6 and Chart B presents the groups selected to define the “accepted” and “rejected” standards to identify the unknown samples.

<table>
<thead>
<tr>
<th>Groups</th>
<th>REJECTED standards</th>
<th>ACCEPTED standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B_R</td>
<td>G_G</td>
</tr>
<tr>
<td>2</td>
<td>B_P, O, T, K</td>
<td>G_B, D, F, J</td>
</tr>
<tr>
<td>3</td>
<td>B_L, S, Q</td>
<td>G_I, E</td>
</tr>
<tr>
<td>4</td>
<td>B_M</td>
<td>G_A, C, H</td>
</tr>
<tr>
<td>5</td>
<td>B_N</td>
<td></td>
</tr>
</tbody>
</table>

Chart B: Standard samples by Group
"Rejected Unknown Samples Identification"

By taking into account the Rejected Standard Samples and Rejected Unknowns Samples as defined in Chart A and Chart B, based on the optimized sensors: LY/G, LY/AA, LY/Gh, LY/gCT, T30/1, T70/2, PA2, P30/1, T40/2.

![Diagram of DFA on standard and unknown samples «rejected»](image)

**Figure 7: DFA on standard and unknown samples «rejected»**

**Figure 7** shows that there are no unknown samples projected near B_N sample (FF44). U_5 is also different in the quality compared to the standard samples. Another calibration Model was developed ([Figure 8](#)) without taking into account standard B_N and unknown sample U_5. This calculation was performed to better understand the discrimination and quality identification of the sample qualities.

The nine sensors are still used: LY/G, LY/AA, LY/Gh, LY/gCT, T30/1, T70/2, PA2,
Figure 8: DFA on the "rejected" standards and unknowns (without G_N and U_5)

In **Figure 8** unknown samples have been associated to the following standard groups:

<table>
<thead>
<tr>
<th>Unknown Samples</th>
<th>&quot;Rejected&quot; Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>U_4</td>
<td>3: B_L, S, Q</td>
</tr>
<tr>
<td>U_6</td>
<td>1: B_R</td>
</tr>
<tr>
<td>U_7</td>
<td>4: B_M</td>
</tr>
<tr>
<td>U_8</td>
<td>2: B_P, O, T, K</td>
</tr>
</tbody>
</table>

**Chart C: Rejected unknown sample identification**
« Accepted » unknown samples identification

Based on the ranking of the unknown samples for Rejected standard samples and Accepted unknown samples as referred into Chart A and Chart B.

Nine sensors were used: LY/G, LY/AA, LY/Gh, LY/gCT, T30/1, T70/2, PA2, P30/1, T40/2.

Figure 9: DFA on the Accepted standard and unknown samples

In figure 9 the unknown samples are projected and compare to the following standard groups:
### Chart D: Accepted unknown sample identification

#### c) Identification conclusions

The following charts present the Standard Groups to which the unknown samples have been associated. Using the **UPEI Labels**.

### REJECTED

<table>
<thead>
<tr>
<th>Groups</th>
<th>“Rejected” standard samples</th>
<th>“Rejected” unknown samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FF88R</td>
<td>SOS66</td>
</tr>
<tr>
<td>2</td>
<td>FF66 P – FF55 O – FF00 T – FF11 K</td>
<td>SOS88</td>
</tr>
<tr>
<td>3</td>
<td>FF22 L – FF99 S – FF77 Q</td>
<td>SOS44</td>
</tr>
<tr>
<td>4</td>
<td>FF33 M</td>
<td>SOS77</td>
</tr>
<tr>
<td>5</td>
<td>FF44 N</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>?</td>
<td>SOS55</td>
</tr>
</tbody>
</table>
V. CONCLUSION

By reviewing the sample data contained in this report, it can be concluded that the αFOX system is able to discriminate between the different milk qualities. The calibrations developed using the provided standards were successful at defining the qualities of the unknown samples. Further investigation of the calibration routines and the sensor optimization indicated that the quality measurement was accurate and the results supported through statistical validation.

The results are good based on the level of discrimination between the samples and the repeatability of the replicates for each sample. It is important to keep in mind that the success of this analytical technique is dependent upon a clear understanding of the analytical objective, the selection of accurate standards and validation of the calibration or predictive model with check samples.
Further efforts to define repeatable standards and sensor selection are recommended. It is further recommended that the sample selection, sample handling and method development be revalidated as part of a full validation protocol.

It is the opinion of the analyst responsible for this project that the results are good and provide sufficient data to support the suitability of the Alpha MOS technology for the routine analysis of milk samples for the identification of flavor defects.