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Evaluation of Qualitative Food Allergen Detection Methods and Cleaning Validation Approaches

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EVALUATION OF QUALITATIVE FOOD ALLERGEN DETECTION METHODS AND CLEANING VALIDATION APPROACHES

by

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Allergen control plans are increasingly used by the food industry to prevent allergen cross-contact and validation of these plans involves methods to detect allergen protein residues. A commonly used rapid allergen detection method is lateral flow devices, although research about their validation is lacking. The objective of this research was to investigate lateral flow devices, their specificity and sensitivity to milk proteins and milk-derived ingredients, swabbing conditions, and applications in cleaning validation.

Several lateral flow devices advertised to detect total milk did not detect whey proteins or whey-derived ingredients. The overload level of the kits was highly variable (ranging from 100-10,000 ppm milk protein), stressing the necessity of validating each kit for its intended purpose.

Milk soils were produced on stainless steel panels in order to assess swabbing conditions. There was essentially no difference in sensitivity achieved from using the swab provided by a kit as compared to a common swab, but certain swabs were better suited to scraping soils rather than absorbing liquid soils. The milk soils that were dried at high heat had a lower recovery than unheated or low heat soils.

Concentration dependent interferences with lateral flow devices and general protein tests were found with caustic cleaning solutions and oxidizing sanitizers, respectively. Four food-processing surfaces: 316 grade stainless steel, HDPE, Nylon 6/6, and Delrin, were soiled with milk and cleaned with each cleaning solution of a typical CIP system separately and then sequentially. When used separately, a commercial caustic solution was observed to outperform a commodity caustic solution. The acidic and sanitizing solutions did not contribute to milk soil removal. The stainless steel surface was most easily cleaned. The lateral flow devices were able to detect milk soils with similar frequency, while the general protein kit had a lower sensitivity. An enhanced visualization method which employed protein staining and scanning of the soils was used, but more development of this method is necessary prior to further use.

Face each new task with joy and courage

S. D. G.

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LITERATURE REVIEW

I. Introduction

Milk and dairy products are nourishing foods commonly consumed by the population and used as ingredients in the food industry. Unfortunately, milk is also a commonly allergenic food. Food allergies have been increasing in prevalence and currently there is no cure; strict avoidance of the offending food is necessary to prevent allergic reactions. In order to protect the allergic consumer and protect their company reputation from recalls, food manufacturers must ensure that cross-contact between allergenic food and non-allergenic food does not occur during processing. This is achieved through safety measures such as allergen control plans, which were originally implemented in the early 1990s and are becoming regulated through the Food Safety Modernization Act *(25, 92)*. Cleaning of equipment is a common component of allergen control plans, but the validation of cleaning is a complex, expensive and time consuming duty. Cleaning knowledge for specific processes and soils is generally held by specific manufacturers *(30)*. This review will discuss food allergy, milk allergen detection methods, cleaning methods for allergen removal, and verification and validation approaches.

II. Food Allergy

a. Overview and Prevalence

A food allergy has been defined by the National Institute of Allergy and Infectious Diseases (NIAID) as an "adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" *(65)*. The prevalence of food allergies has been estimated to affect 5% of adults and is approaching 8% of children in westernized countries *(86)*. Food allergy is also dramatically increasing in prevalence *(85)*. It is not known yet why the increase in prevalence has occurred, but risk factors such as delayed introduction, increased hygiene, genetics, gut flora, vitamin D deficiency, atopy, use of antacids, and obesity have been indicated *(86)*. There are over 160 foods that cause allergic reactions, but the "big 8" allergens, which account for over 90% of food allergies, are milk, egg, peanut, tree nuts, shellfish, fish, wheat, and soy *(39)*.

Food sensitivities can be divided into food allergies and food intolerances; individuals can tolerate a higher threshold level of the latter. Food intolerances do not involve the immune system and include anaphylactoid reactions, metabolic food disorders and idiosyncratic reactions. One of the most common food intolerances is lactose intolerance. Affected individuals are not able to metabolize lactose due to a deficiency of intestinal β-galactosidase *(93)*.

Food allergies are further differentiated into IgE-mediated reactions and cell-mediated reactions. IgE-mediated allergies are reactions to milk, eggs, peanuts, etc. while cell-mediated or non IgE-mediated reactions include celiac disease. IgE-mediated

allergies are considered immediate hypersensitivities, while cell-mediated food allergies encompass delayed hypersensitivities with symptoms developing 24-72 hours after exposure to the allergen.

b. IgE-Mediated Food Allergy

The IgE-mediated allergic reaction begins through a sensitization phase as seen in Figure 1. Allergen specific IgE antibodies are produced by plasma cells after exposure to foods containing the allergen *(93)*. This occurs through a T_H2 response producing interleukin-4 and -13 which then drive allergen-specific B cells to produce IgE *(64)*. Sensitization occurs when the allergen-specific IgE becomes attached to the surface of mast cells in the tissues and basophils in the blood *(93)*.

The second stage of the allergic response is elicitation. Upon exposure to foods containing the allergen, the antigen (allergenic protein) will cross-link the allergen-specific IgE molecules on basophils and mast cells. Through degranulation, these cells release mediators such as histamine, leukotrienes and prostaglandins *(93)*. Histamine immediately increases blood flow and causes smooth muscle contraction *(64)*. Prostaglandins and leukotrienes cause smooth muscle contraction, increase vascular permeability and stimulate mucus production *(64)*. The increase in blood flow can carry the mediators to other parts of the body and produce a systemic reaction.

The symptoms of an IgE-mediated allergic reaction are influenced by the amount of IgE present, the route of exposure, and the amount of allergen, therefore, symptoms can range from mild to severe *(64, 103)* . Mucosal mast cell activation causes diarrhea

and vomiting through transepithelial fluid loss and smooth muscle contraction *(64, 110)*. Hives and itching are produced through mast cell activation in the cutaneous tissues *(64)*. Respiratory issues such as asthma also occur. The most severe response, although rare, is the systemic reaction of anaphylaxis. This reaction lowers blood pressure, constricts the airways, and swells the epiglottis leading to suffocation *(64)*.

Food-related acute allergic reactions account for over 200,000 visits per year to emergency departments in the United States *(16)*. Children under the age of 18 accounted for 25,000 visits per year *(16)*. Of the emergency department visits, 90,000 each year are probably related to anaphylaxis. Thankfully, food-related fatalities from anaphylaxis are relatively uncommon. From 1994-1999 and 2001-2006, 32 and 31 cases, respectively, of fatal food-induced anaphylaxis were reported *(9, 10)*. The foods most frequently implicated in fatal reactions were peanuts and tree nuts; milk-related fatalities increased from 1 case to 4 cases between the two time periods *(9, 10)*. Adolescents and young adults were the most affected age category and of all the fatalities, all but one had asthma. Unfortunately, timely administration of self-injectable epinephrine was not available or used in almost all of the cases. In a study of non-fatal emergency department visits, only 11% of individuals had administered epinephrine *(15)*. Increased education about the treatment of food-related anaphylaxis, especially to the most susceptible age group, young adults, is necessary.

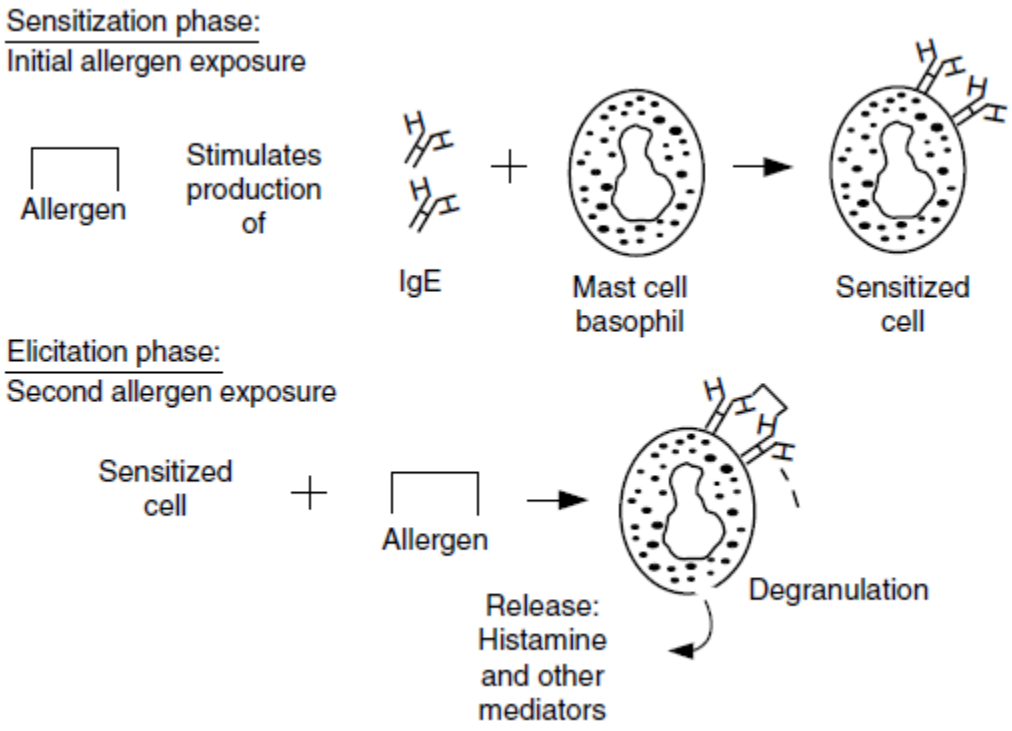

Figure 1. Mechanism of IgE-mediated allergic reaction. Adapted from *(19)*.

c. Natural History of Milk Allergy

Currently no cure exists for food allergy, so consumption of the offending food must be entirely avoided in order to prevent allergic reactions. Generally, a food allergy will develop in the first two years of life *(11)*. Resolution of food allergy is less likely for certain allergens than others. Individuals allergic to egg, milk, wheat, and soy have a higher potential for allergy resolution during childhood, but allergies to peanut, tree nuts, fish, and shellfish are more persistent throughout life *(11, 86)*. An additional predictor of persistent allergy is allergen-specific serum IgE (sIgE) levels; those with higher initial levels are less likely to experience allergy resolution *(11)*.

Milk allergy is particularly associated with childhood resolution. A consortium of observational studies published in 2013 found that over 50% of subjects had cow's milk allergy (CMA) resolution by 5 years of age (median) *(115)*. Based on examinations of milk specific-IgE levels, skin prick test (SPT)-induced wheal sizes and atopic dermatitis severity, an algorithm was produced that can predict the resolution of CMA in patients less than 15 months of age *(115)*. The resolution of milk allergy was greatly associated with lower milk-specific IgE levels, smaller SPT wheal sizes, and the absence of significant atopic dermatitis *(115)*. The resolution rates of CMA published in the late 1990s indicated 71-87% recovery in children by the age of 3 *(37, 43)*. It was noted that milk protein IgE sensitized infants had an increased risk of persistent CMA. It appears that milk allergy is becoming more persistent.

The effect of including baked milk in the diet on inducing tolerance to milk products was recently investigated. Those that included dietary baked milk in their diets were able to tolerate unheated milk at a faster rate than those that avoided all baked-milk products *(57)*. Additionally, those that were able to tolerate baked milk were also more likely to have a transient type of IgE-mediated cow's milk allergy. Individuals with transient milk allergy produce antibodies to conformational (tertiary structure) proteins. As baking destroys conformational milk protein epitopes, these individuals were able to tolerate baked milk. Conversely, those individuals who produce antibodies against sequential epitopes (which are heat-stable) were more likely to have a persistent milk allergy.

d. Diagnosis of Milk Allergy

'Self-diagnosis' of food allergy has become more commonplace as the awareness of food allergy grows, but a proper diagnosis is the best course of action. It has been generally found that the population overestimates their allergies. Through a meta-analysis of prevalence data, it was found that 35% of people reporting a reaction to food think that they have a food allergy while the prevalence of food allergy was found to be only 3.5% when considering oral food challenge results *(76)*.

The recommended components of food allergy diagnosis involve medical history, physical examination, elimination diets, skin prick tests (SPTs), allergen-specific serum IgE (sIgE) measurements, and oral food challenges (OFCs).

The medical history should investigate the types of foods and amounts that were consumed prior to symptoms to determine if any foods are more likely to be related to an IgE-mediated allergy. The timing and analysis of symptoms is of more use when diagnosing immediate hypersensitivity reactions rather than delayed reactions *(11)*. A physical exam can help to determine if visible symptoms are consistent with an allergic reaction.

The potential trigger foods identified through the medical history are used to evaluate SPTs and sIgE levels. Both of these methods can detect sensitization, but do not determine the severity of the reaction. For example, SPT wheal size is not correlated with allergic reaction severity (the coexistence of asthma is the best predictor of severe reactions) *(11)*. These methods should be used in conjunction as the results will not

always correlate (SPTs generally over-diagnose) and neither method should be used solely for diagnosis. sIgE testing measures the presence of allergen-specific antibodies in the serum while skin-prick tests measure IgE bound to cutaneous mast cells *(11)*.

The results of SPTs and sIgE levels can be further refined through an oral food challenge (OFC). Generally, allergists do not prefer conducting OFCs unless necessary because they are risky, time-consuming and expensive *(86)*. An elimination diet may be used if the risk of an OFC is particularly high *(11)*. However, double-blind, placebo-controlled food challenges are the gold standard for true food allergy diagnosis *(27)*. OFCs determine whether the food can be ingested without triggering an immediate clinical reaction *(27)*. A study of presumed food allergic children found that 89% of OFCs were negative; a proper diagnosis allows an increase in the quality of life and a more robust diet *(27)*. Finally, a food allergy diagnosis is supported when the OFC is positive and the results correlate with the medical history and laboratory tests *(11)*.

It should be noted that there are several food allergy diagnosis methods which are not recommended including: intradermal tests, total serum IgE measurements, atopy patch test, applied kinesiology, allergen-specific IgG4, and electrodermal testing *(11)*.

Component resolved diagnosis (CRD) has been recently developed as a method of food allergy diagnosis. However, CRD is not ready to replace current methods *(55)*. CRD aims to determine the specific allergenic protein source through analysis of proteins derived from rDNA technology or natural sources *(83)*. This enables identification of reactivity to allergenic proteins rather than the entire food. This approach is useful when

cross-reactivity may be a concern. A challenge associated with CRD is determining the cut-off values for diagnosis. Using CRD for diagnosis of peanut and hazelnut allergies is promising, while conflicting results have been found with other allergens, including milk *(83)*.

e. Prevention and Treatment of Milk Allergy

Prevention of Sensitization

Future methods and therapies to prevent food allergen sensitization are gaining research progress and funding recently. The Learning Early about Peanut Allergy (LEAP) study provided a monumental shift in recommendations about introduction times of allergenic foods. The study followed over 500 infants (4-11 months at introduction) with severe eczema or egg allergy and divided them into a group that consumed peanut products regularly and a group that avoided peanut products until 60 months of age. After evaluation at 5 years of age, the avoidance group had a prevalence of peanut allergy of 17.2%, while the consumption group had a peanut allergy prevalence of only 3.2% *(21)*. Later in 2015, the American Academy of Pediatrics retracted their recommendations about early food avoidance and published interim guidelines that recommended that peanut-containing products be introduced to infants with a high risk of peanut allergy at 4 to 11 months *(28)*.

Other prevention guidelines have been clarified as more information is obtained. Allergen avoidance during pregnancy and breastfeeding is not recommended *(86)*. Additionally, exclusive breast-feeding for at least four months is encouraged. A recent

study investigated the effect of early introduction of allergenic foods into the diets of breast-fed infants on development of food allergy *(70)*. The prevalence of food allergy in the early introduction group (3 months age) and the standard introduction group (6 months age) was 2.4% and 7.3%, respectively (in subjects that adhered to the protocol) *(70)*.

Another prevention tactic focuses on adjusting internal environmental exposures by influencing the gut bacteria. Probiotic supplements provide health-promoting bacteria and potentially increase synthesis of IgA and IL-10, suppress TNF-α, inhibit casein-induced T-cell activation and CD4, and Toll-like receptor 4 signaling *(66, 86)*. Consistent results have not been found to determine whether probiotics affect allergy symptoms or tolerance. A study of 119 CMA infants found that the placebo and probiotic treatment groups had no difference in tolerance to cow's milk after the 12 month treatment period *(66)*.

Building Tolerance (Desensitization)

Recently, immunotherapy (oral (OIT), sublingual (SLIT), and epicutaneous) approaches have been attempted to desensitize allergic individuals. Currently no oral, sublingual or epicutaneous immunotherapies for foods are recommended for routine treatment, but research on these methods is continuing.

A recent oral immunotherapy trial with 14 CMA children found that 13 children were desensitized at conclusion of the immunotherapy and 6 tolerated milk after one month of milk avoidance *(23)*. A comparison of OIT and SLIT with CMA children was

completed in 2011 *(56)*. The OIT group's oral food challenge threshold was 6140 or 8140 mg, while the SLIT group's was 940 mg. SLIT was less effective than OIT, and had less adverse events during treatment. A general consensus of immunotherapy has similarly concluded that OIT is more effective than SLIT *(54)*. Omalizumab, an anti-IgE monoclonal antibody, has been researched in conjunction with OIT *(69)*. Three CMA children undergoing OIT had severe reactions and subsequently underwent OIT with omalizumab. The patients reached a cow's milk dose of 40, 50, or 80 ml after treatment and no moderate or severe reactions were reported *(69)*. Omalizumab is a promising adjunct to improve efficacy and safety of immunotherapy *(54)*.

An epicutaneous immunotherapy approach for peanuts that utilizes a skin patch has received approval for a phase III clinical trial. The peanut patch delivers the peanut allergen directly to the outermost layers of the skin and is able to activate the immune system and induce desensitization without passing the antigen into the bloodstream. After the phase II study, 50% of patients that took the highest dose patch were able to tolerate 1g of peanut protein during an oral food challenge *(79)*.

Preventing Elicitation

Currently, no treatment exists for food allergy. Thus, allergen avoidance is essential. Allergic individuals must diligently read labels of packaged foods, and inquire about ingredients and cooking methods when eating at restaurants, air travel, schools, and homes. Many advocacy organizations exist and provide information to allergic individuals and the caregivers, schools, and restaurants that serve them *(53)*. Research on

threshold levels, amount of allergen to elicit an allergic reaction, has been conducted, but no thresholds have been accepted on a regulatory level *(53)*. The acceptance of thresholds could have a positive impact on necessary precautionary labeling and the variety of foods that allergic individuals can safely consume.

Treatment of Reactions

It is important to attempt to completely avoid the allergen and be prepared to treat allergic reactions or symptoms as they occur. The best way to treat an allergic reaction, especially anaphylaxis, is to administer auto-injectable epinephrine *(86)*. Unfortunately, that is not understood or applied in many cases. In a study of US food-related emergency department visits, only 16% of patients received a prescription for auto-injectable epinephrine upon discharge *(15)*. A random telephone survey in the US asked people who could have anaphylactic reactions about their plan for treatment. Only 11% would self-administer epinephrine while others would go to hospital (34%), self-administer antihistamine (27%), or call 911 (10%) *(114)*.

Education about administration of auto-injectable epinephrine and prevention of further reactions through reading of food packaging labels, and proper meal preparation or selection is needed.

III. Cow's Milk

a. Composition of Milk and Milk-Derived Ingredients

Milk is a nutritious fluid secreted by all females of the mammalian species in order to provide energy, essential fatty acids, amino acids, nitrogen, and vitamins to their neonate *(29, 68)*. The composition of milk varies by different species, animal health, and through different stages of lactation *(68)*. The average composition of milk of western bovine breeds is water, 86.6%; fat 4.1%; protein 3.6%; lactose 5.0%, and ash 0.7% *(26)*. The minor components of milk include minerals, vitamins, hormones and enzymes *(68)*. In 2013, the global milk production was estimated to be 466 million metric tons (million mt) and the main producers were the European Union, United States and India *(58)*. Skim milk powder and whole milk powder production in 2013 was 3.91 million mt and 4.5 million mt, respectively *(58)*. Approximately 1.2 million mt and 0.27 million mt of whey-derived products and casein products, respectively, were produced by the U.S. in 2013 *(58)*. Global butter production in 2015 was 9.8 million mt *(99)*.

The unique functional properties and nutritional benefits of milk have led to the development of many milk-derived ingredients which are globally desired products *(60)*. Milk powders are produced by spray-drying fresh liquid milk *(29, 58)*. Non-fat dry milk (NFDM) is a version of milk powder in which the fat has been removed. The major uses of milk powder include reconstituted dairy products, bakery mixes, confections, soups, nutritional products for children, diet beverages, processed cheeses, and animal feed *(29, 40, 58)*.

Milk protein concentrates (MPC) are produced through the partial removal of non-protein components such as lactose and minerals from skim milk (*11*). This is generally achieved through filtration or dialysis. The proteins in MPC are still in the same ratio as they would be found in liquid milk, but the total protein content may be increased up to 80%. Milk proteins as ingredients are desired because they are colorless, stable to processing, and have a bland taste *(29).*

Whey protein concentrates (WPC) are produced from ultrafiltered fluid whey in which the lactose has been removed in order to increase the protein content *(58)*. The protein content of WPCs can vary between 34-80% protein. The two-stage ultrafiltration step controls the protein content and then the product is evaporated before spray-drying *(4)*. WPC34 contains 34-36% protein, 48-52% lactose, 3-4.5% fat, 2.5-8.5% ash and 3.0-4.5% moisture while WPC80 contains 4.0-8% lactose, 80-82% protein, and 4.0-8.0% fat. Prior to 1970, whey was considered a waste product, but now WPCs are used for infant formula, sports beverages, and nutritional supplements *(4, 58)*.

Sweet whey is a co-product of cheese production containing 12.5% protein and is defined by Codex Alimentarius and U.S. Code of Federal Regulations (CFR) as a "liquid substance obtained by separating the coagulum from milk, cream or skim milk in cheesemaking…sweet whey has a maximum titratable acidity of 0.16%, calculated as lactic acid." (21 CFR 184.1979) In sweet whey production, the method of milk coagulation is through rennet treatment *(4)*. Another whey ingredient, acid whey, is produced through the production of acidified products such as cottage cheese *(4, 58)*. Milk is coagulated through the direct addition of an acid such as lactic acid *(4)*. Acid

whey has a pH of 3.9-4.5 and less lactose and whey protein. Sweet whey is produced and used more frequently than acid whey. Acid whey is harder to process through membrane filtration as the lower pH causes insolubility of some whey proteins.

Casein ingredients are prepared through "separating, washing and drying the coagulum of skim milk and/or other products derived from milk" (Codex Stan 290-1995b) *(58)*. When sodium hydroxide (raises pH to neutral) is processed with casein, sodium caseinate is produced *(58)*. Sodium caseinate is used for fat emulsification and oil/water system stabilization.

b. Cow's Milk Allergens

An IgE-mediated hypersensitivity reaction to antigens/milk proteins is responsible for cow's milk allergy (CMA) *(102, 104)*. Bovine milk contains 30-35 g/L of cow's milk proteins *(103)*. The cow's milk proteins can be divided into two sub-groups, whey and casein, based on their solubility at pH 4.6 *(105)*. The caseins precipitate under these conditions and the whey proteins stay soluble. Many of these milk proteins are potential allergens and it has been found that there is great variability in the specificity of the IgE response to different milk proteins from various milk-allergic individuals *(102, 103)*.

1) Whey Proteins

The whey proteins constitute 20% (5 g/L) of the proteins of cow's milk. The whey proteins include β-lactoglobulin (10%, BLG), α-lactalbumin (5%, ALA), immunoglobulins (3%), bovine serum albumin (BSA, 1%), and traces of lactoferrin *(102-*

105). BLG and ALA are synthesized in the mammary glands, while the other whey proteins are derived from the blood *(29)*. Lactoferrin is an iron-binding glycoprotein. The whey proteins have high levels of secondary, tertiary and occasionally quaternary structures. The protein structures are stabilized by intramolecular disulphide bonds. The proteins are not phosphorylated and are insensitive to Ca^{2+} *(29)*. The whey proteins are globular proteins.

BLG, the most abundant whey protein, is a 36 kDa dimer which contains two disulfide bridges and one free cysteine residue *(103)*. The molecule is responsible for physiochemical properties and interaction with casein during heat treatment *(103)*. BLG is thermolabile and denatured upon heating *(29)*. The sulphydryl group is exposed when denatured and forms a sulphydryl-disulphide interaction with κ-casein after heating to 75°C; this interaction influences the physiochemical properties of milk. BLG belongs to a protein family with high allergenic potential, the lipocalins. Similarities between the lipocalins exist, such as the conservation of tryptophan at position 19 in the N-terminus *(103)*.

ALA is a monomeric, 14.4 kDa globular protein *(103)*. It contains four disulfide bridges and obtains stability in its secondary structure through a high-affinity binding site for calcium *(103)*. Various experiments have determined the antigenic portions of ALA occur at hydrophobic locations and at disulphide linked portions *(102-104)*.

Jarvinen found that multiple sequential epitopes of ALA and BLG were recognized by the IgE of children who had persistent (life-long) allergy and not by those with transient CMA *(51)*.

2) Casein Proteins

The casein proteins are present in bovine milk at a concentration of 30 g/L and constitute 80% of the milk proteins. There are four proteins which make up the whole casein, α_{s1}-casein (32%), α_{s2}- casein (10%), β-casein (28%), and κ-casein (10%) *(103)*. A minor casein protein is γ-casein, which is a plasmin produced C-terminal fragment of β-casein *(29)*. All of the caseins are small and range in size from 20-25 kDa *(29)*. The casein micelle is arranged in a quaternary structure which has an average diameter of 120 nm. The micelle has a hydrophobic core and hydrophilic periphery; the micelle is suspended in whey *(103)*. The hydrophilic periphery is composed of the C-terminal of κ-casein, while the N-terminal is hydrophobic (through the presence of oligosaccharides) oriented toward the center of the micelle.

The caseins are phosphorylated. These phosphate groups bind polyvalent cations; in milk the principal cation bound is calcium. Precipitation of α_{s1}-, α_{s2}-, and β-casein occurs when cations are bound and thus charge neutralization occurs. It should be noted that κ-casein does not bind cations strongly and thus does not precipitate. The exterior of the casein micelle is κ-casein and its calcium insensitivity protects the calcium-sensitive caseins in the center of the micelle. *(29)*

The cysteine content of the proteins affects their flexibility. Only α_{s2}- and κ-caseins contain cysteine, which occurs as disulphide bonds in these proteins. α_{s2}-casein occurs as disulphide-linked dimers, but κ-casein may connect up to ten molecules through disulphide bonds. αs_1- and β-casein are more flexible because they do not contain cysteine. *(29)*

Caseins have low levels of secondary and tertiary structures. This makes them stable to denaturing agents, susceptible to proteolysis and leads to their high surface activity and flexibility *(29)*. Secondary structures of β-casein cannot be formed because it has the highest amount of proline *(29)*.

3) Milk Proteins as Allergens

Most milk proteins are allergens. There is not a specific structure or function associated with allergenicity *(103)*. About 75% of those with CMA are sensitized to several proteins/allergens; this is referred to as polysensitization *(103)*. Additionally, there is variability in IgE sensitization, so no single allergen is responsible for all CMA; caseins, BLG and ALA are considered major milk allergens because >50% of allergic individuals are sensitized to these proteins. Sensitivity to casein has increased recently. IgE binding studies have found that both conformational and sequential epitopes are allergenic *(103)*. Further, sequential epitopes, after denaturation (perhaps through digestion) were reactive *(104)*. In a study of β-caseins, it was found that there was similar IgE reactivity with human and bovine regions and that IgE was particularly sensitive to phosphorylated seryl residues *(7, 8)*. The phosphate groups are found in β-turns and form

a hydrophilic area when brought together by folding *(104)*. IgE from individuals with persistent CMA was found to bind certain αs_1-casein segments, but younger children (likely to outgrow the allergy) were not sensitive to those segments *(14)*. Sequential IgE binding regions of α_{s2}-casein were found in the C-terminal and in the middle of the protein *(13)*.

All milk proteins are allergenic and both conformational and sequential epitopes are involved in allergy.

c. Allergen Issues with Milk-Derived Ingredients (MDIs)

When a milk-derived ingredient is selected for use; allergen issues arise. All milk proteins are potential allergens, so every MDI is potentially allergenic. Although studies have found a decrease in allergenicity to whey proteins and an increase of sensitivity to caseins, the IgE response is variable across different allergenic individuals and predictions of allergenicity cannot be made *(102, 103)*. An important consideration is the allergen load of the MDI. Allergen load is the milk-protein content of the MDI. The allergen load of the formulated food also involves the level of that ingredient that is used in the product formulation. A high protein ingredient, such as MPC80, used at a high level would have a higher allergen load than a product using sweet whey as a production aid. Additionally, hydrolyzed milk protein ingredients could also have decreased allergenicity, but their allergenicity can be challenging to predict *(103)*. Detection issues arise with MDI use because analytical methods must be able to detect milk residues both before and after processing to assure that cross contact has not occurred.

When MDIs are used, milk residues must be removed from processing equipment before manufacturing of non-milk-containing formulations. The removal of milk residues through cleaning is complex. The cleaning of equipment used to process these milk-derived ingredients would largely be based upon the nature of the product being manufactured, its physical state (wet vs dry), processing conditions, the type of equipment, the nature of the MDI, and the allergen load. Cleaning will be described fully in later sections.

IV. Detection Methods for Food Allergens and their Application to Cleaning Validation

a. Lateral Flow Device (LFD)

Lateral flow devices are a rapid and specific method used by the food industry to monitor cleaning of food processing equipment and food product cross-contact *(6, 71, 72, 75)*. The first allergen-related rapid immunoassay was developed for peanut in 1997 and the first commercialized LFD kits were released in 2004 *(62, 101)*. Since then, the market of commercialized LFD has rapidly expanded *(77, 82)*.

Lateral flow devices are a type of specific immunologic allergen detection method that relies on specific antibody-antigen recognition. The qualitative method provides a positive response when the allergen content is higher than the limit of detection. The physical structure of the test is quite similar to a home-pregnancy test; the strip can be housed in a plastic cassette and there is an area for a test line and control line to develop (Fig 2).

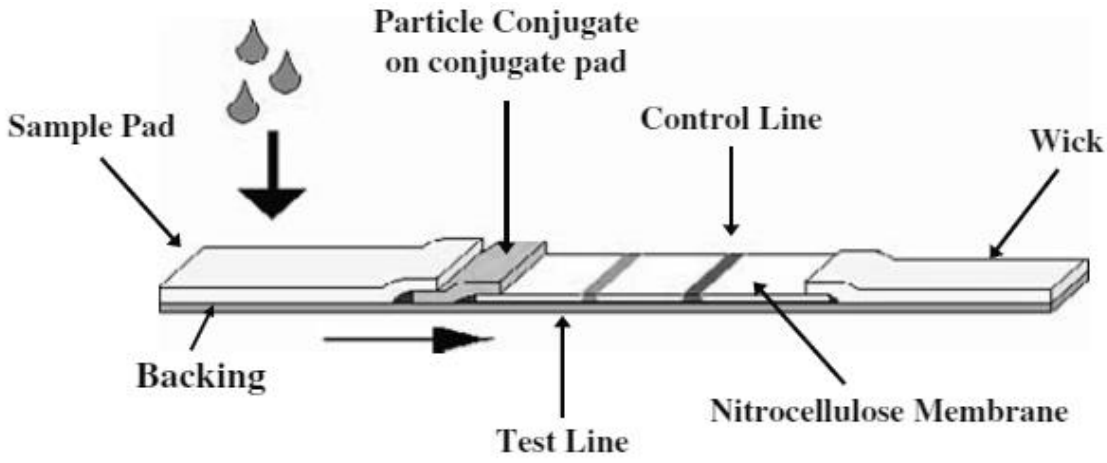

Figure 2. Configuration of lateral flow device test strip, adapted from *(67)*.

The initial step of analysis with a lateral flow device involves obtaining the sample. Equipment surfaces can be sampled with a swab; more information on swabbing will be explained later. Liquids, such as rinse water samples can also be analyzed. Many LFDs are not validated to analyze food ingredients or finished food products, especially solid foods.

The next step is extracting the sample. This step aims to extract, solubilize and make available the protein from the sample for analysis *(6)*. Most commercial kits include instructions and buffers for accomplishing this extraction. The extraction procedure can vary from hand shaking at room temperature to boiling the sample.

Subsequently, the extracted sample is introduced to the sample pad and conjugate zone (Fig 3). This zone has allergen-specific IgG antibodies which are conjugated to gold or latex. The gold and latex particles aid in development of a colored test line. The conjugated antibody has been dried in this area. If the extracted sample contains antigen

(allergen), it will bind to the conjugated allergen-specific IgG. This complex migrates to the nitrocellulose membrane. *(6, 71)*

The test zone contains a test line made of allergen-specific IgG and a control line composed of species-specific (of the antibody-producing animal) IgG *(6)*. Some kits also have an overload line which is situated between the test and control line and it is composed of antigen. The antigen-conjugated antibody complex travels and binds to the allergen-specific IgG on the test line. A colored line is formed which indicates a positive test result. The extra conjugated antibody (from the sample area) continues through the LFD and binds to the species-specific IgG on the control line. The colored control line indicates that the LFD ran properly *(6)*. As mentioned previously, the overload line is composed of antigen and reacts specifically with the conjugated antibody *(71)*. If the sample had a very high level of target antigen, the overload line would not develop because the gross amount of antigen overwhelms the conjugated antibody *(71)*. When the lateral flow device only has a control and test line, the test line may not form with high amounts of sample allergen and a false negative result is obtained; this is the hook effect or overloading.

The high dose hook effect occurs because the proportion of antibody to allergen is no longer within the working range. An excess of antibodies is required for the dose-response curve to have a positive slope and obtain semi-quantitative results *(67)*. This is why kit manufacturers may advertise that the intensity of the test line is proportional to the amount of allergen present. However, this is a statement that must be validated within a specific range of detection. When the concentration of allergen starts to exceed the

antibody amounts, the dose-response curve will level to a slope of zero. As the amount of allergen continues to increase in the sample, the slope of the dose-response curve will become negative and this correlates to a decreasing intensity of the test lines. It is important to realize that some samples may require dilution prior to testing. When selecting a kit for use it is important to understand the range of detection and know if the kit provides excess extraction buffer for dilution of high positive samples.

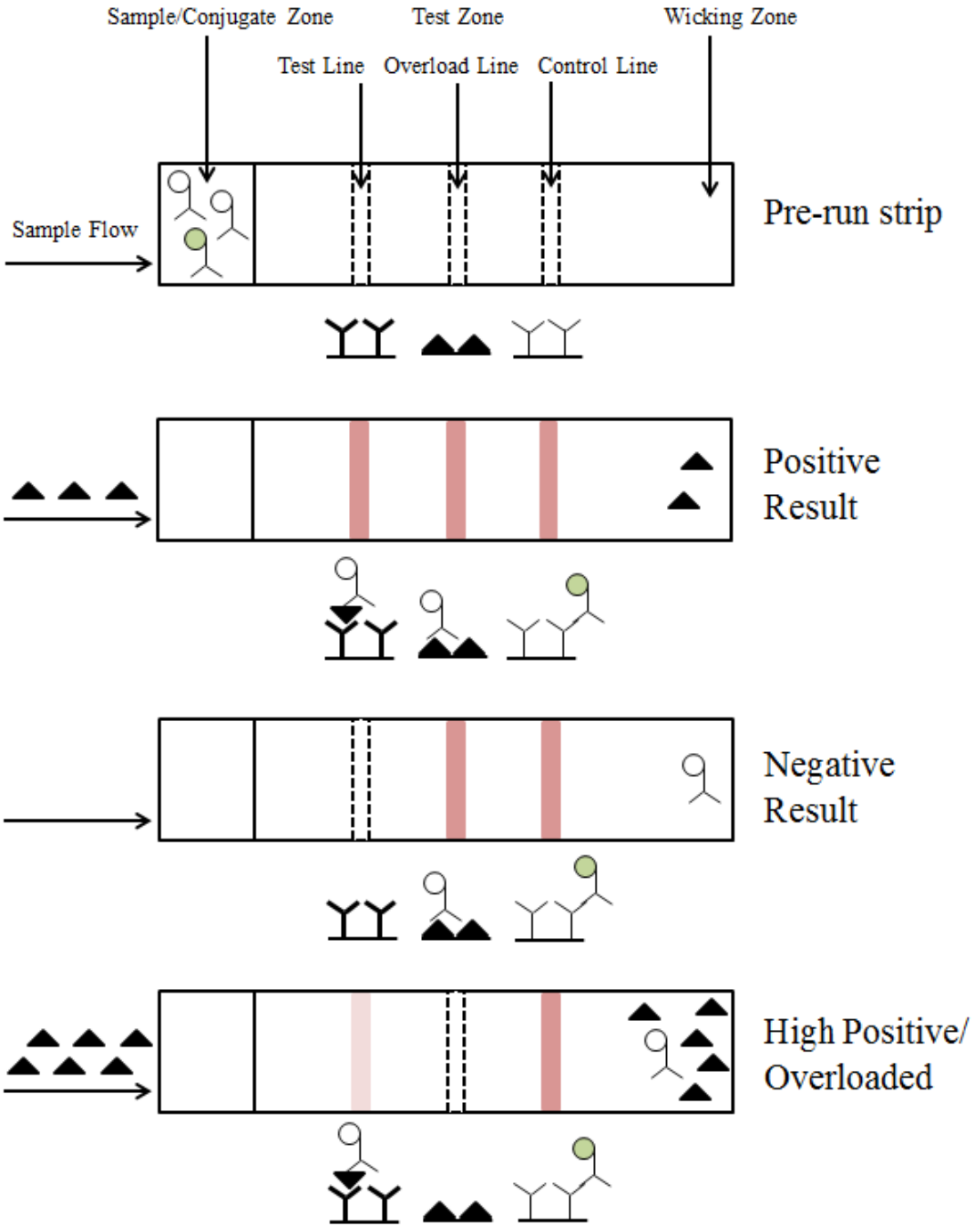

Figure 3a. Direct lateral flow device with overload line. Adapted and modified from *(67)*.

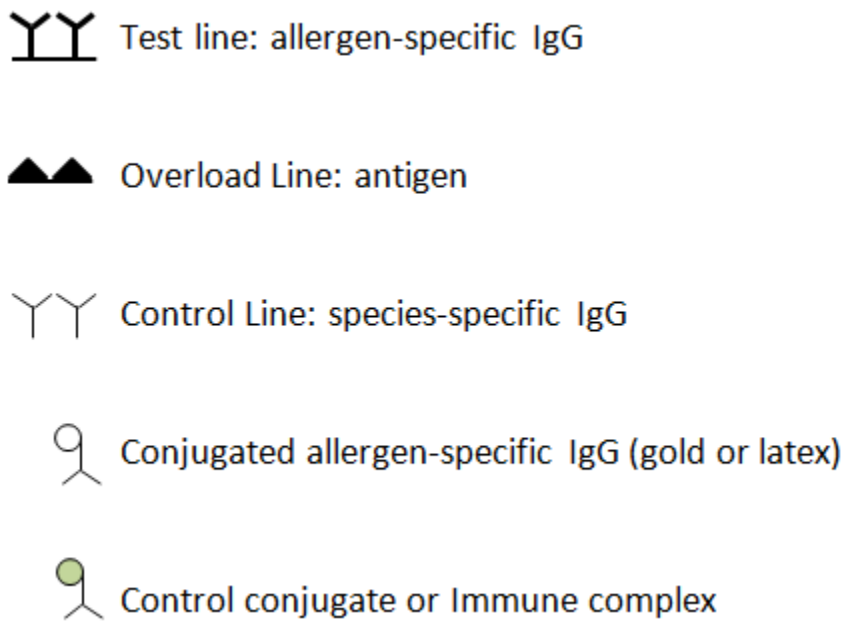

Figure 3b. Diagram key. Adapted and modified from *(67)*.

Limitations, advantages, and disadvantages

There are many factors that may affect the test results such as: manufacturing issues, operator errors, effects of the food matrix, environmental factors, sample manipulation and cross-reactivity issues *(113)*.

The use of specific monoclonal antibodies decreases the risk of false-positive results due to cross-reactivity issues *(71)*. The quality of the antibodies is very important for specific and sensitive methods *(111)*. The affinity of the antibodies for the antigen (allergen) must be quite high because the reaction time is just several seconds before the complex must enter the test zone *(12)*. A large quantity of antibodies is necessary to coat the test line of a LFD (approximately 25-100 times more than that used in an ELISA) *(12)*. Differences in antibodies may have been the reason for variability in the frequency

of false positive results between two peanut LFDs studied in an inter-laboratory investigation *(100)*.

Similarly, any changes to the antigen, such as processing, may affect detection *(71)*. When tested with ELISA, thermally processed samples containing milk had decreased recovery *(20)*. A study that tested gluten and wheat flour incurred samples in cornbread (unbaked and baked) found differing detection sensitivities between the commercial LFDs that they tested. The Morinaga and R-biopharm kits both initially detected baked and unbaked samples at the same level. The Romer kit detected gluten at the 5 ppm level in the unbaked samples, but at 20 ppm gluten in the baked samples *(84)*.

Operator error can occur during implementation of the test and interpretation of the test results. It is important to understand that strips with only a control and test line may exhibit false negative results when the allergen level is very high; this is referred to as the high dose hook effect or overloading *(6, 67)*. Differences between operators may also occur from swabbing as it is a subjective method. Some commercial kits suggest a swabbing method, such as crosshatch, but there are not suggestions for the amount and location of swabbed samples. Developing a common swabbing protocol may decrease variability between operators *(116)*.

The food and sample matrix can also affect test results. Detergents and sanitizers may interfere with the test results and it is important to ensure that equipment surfaces have been adequately rinsed prior to swabbing or testing rinse water samples *(6)*. In the validation studies of the R-biopharm milk LFD, milk, ice cream and milk powder were

applied and dried to surfaces. The milk powder sample was detected at a much higher sensitivity than the milk and ice cream samples *(72)*. A study of packaged foods not declaring peanut as an ingredient tested for peanut residues with LFD, PCR, and ELISA and found that the LFD gave false positive results when the sample was "fatty or smeary" *(117)*.

LFDs are an appealing method for cleaning validation because they are quick, specific, and cost-effective while requiring very little training or specialized equipment. Additional inter-laboratory validation studies are necessary to understand interferences, limitations, and sensitivity differences between the various commercial kits available. It is necessary for the end-user to validate the LFD for their specific ingredient and product of interest to ensure that it is detectable and understand potential effects from processing *(63, 71)*.

b. General Protein Methods

General protein methods such as the Biuret reaction and Folin-Lowry method can be used for protein determination. These methods are determining the total protein content and are not a specific method for allergenic proteins. Thus, their validity for allergen detection is unproven but commercial methods are still produced that advertise allergen detection based on general protein detection. A survey in 2006 of food manufacturers which looked into methods of equipment cleanliness verification found that general protein methods were not used frequently and only used in large companies *(94)*.

One such example is the 3M Clean-Trace Surface Protein (Allergen) swab kit which is used for equipment and surface swabbing in addition to rinse water sample testing. The kit operates based on the biuret reaction. This begins by Cu^{2+} ions being reduced by the peptide bonds in proteins to Cu^{+}. This reaction is temperature dependent and the swabs are placed in a heat block at 55°C. Subsequently, each Cu^{+} ion is bound by two bininchoninic acid molecules (BCA) and a purple colored product is formed. This colored product is proportional to the amount of protein in the sample. The 3M kit allows a qualitative protein analysis through comparison to printed colors associated with high and low protein amounts. When the assay is performed in a laboratory setting, the colored product can be quantitatively determined through absorption at 562 nm and comparison to known protein standards.

The sensitivity of the assay is lower than LFDs; the 3M kit states a detection limit of 10-20 μg milk, which relates to about 3 μg of protein. These methods may not be suitable for sole allergen detection, but may be an integral part of an overall verification method for allergen equipment cleanliness.

c. ATP

A 2006 survey of food manufacturers found that bioluminescence/ATP tests were the third most commonly used method for verification of equipment cleanliness leading to allergen removal *(94)*. However, the ATP swabs are measuring adenosine triphosphate (ATP) and are not specifically testing for protein, which is the allergenic substance. This discrepancy is not clearly distinguished as there are ATP swabs that are marketed for

allergen testing purposes, such as AllerGiene (Charm Sciences, Inc., Lawrence, MA). The test measures relative light units (RLU) and results are determined through comparison to limits. It is imperative that companies who use ATP tests must verify and establish their limits after completing validated cleaning programs and compare results with specific allergen tests.

It has been found that ATP tests do not always correlate well with allergen-specific ELISA results. A study of dry-cleaning methods for allergen removal from food processing surfaces compared the results of visual inspection, ELISA, general protein and sensitive and conventional ATP (ATP result positive when >0 RLU) *(47)*. After wiping the surfaces with sanitizing wipes, negative results were obtained by visual inspection, ELISA and general protein while conventional and sensitive ATP were positive. Another study investigated wet-cleaning methods of dairy products and compared ATP and ELISA detection methods (ATP result positive when >0 RLU) *(78)*. Very high and variable RLU backgrounds were found when stainless steel was swabbed after chlorinated alkali detergent washing. The ATP test was able to detect milk residues at similar and lower levels than the ELISAs. In a study investigating baked allergen soils on surfaces, ATP tests were able to detect at levels lower than ELISAs when the surface was determined visually clean, but not as low as general protein tests at times (ATP positive based on surface background RLUs) *(3)*.

ATP testing is not ideal for allergen detection as it does not specifically detect allergen proteins and various factors can influence the RLU readings which complicate the determination of a limit value.

d. ELISA Method Applications

The enzyme-linked immunosorbent assay (ELISA) is a specific method for allergen detection based on the specific binding of an antigen to an antibody in the wells of a microtiter plate. A color change is measured at 450 nm (exact wavelength is dependent on enzyme and substrate) and quantified in comparison to a calibration curve of known standards. The method is widely used due to its rapid, sensitive, selective and cost-effective nature that can process a large number and variety of samples *(45)*.

A survey conducted in 2006 of food manufacturers found that ELISA was the second most common allergen cleaning verification method following visual inspection *(94)*. ELISA testing was used for ingredients, equipment surfaces, rinse water, push through, and finished product *(94)*. The effectiveness of sanitation was linked to the results of equipment surface, water rinse and push through samples.

The use of an ELISA is not ideal for every situation. Decreased recoveries of milk protein have been reported with thermally processed foods *(20)*. It is important to understand the limitations of the method used. Additionally, the differences in reporting units among kits and subsequent conversion and application to a specific product must be understood. The reference materials vary by different kit manufacturers and this can complicate comparisons of kit effectiveness and matrix effects. An investigation of peanut detection from peanut butter and purified peanut proteins found that different kits had remarkably different recoveries *(52)*. It is now recommended that NIST SRM 1549 (non-fat milk powder) be used for ELISA validation of milk kits *(1)*.

V. Allergen Management in Food Industry

a. Allergens in Food Processing

As the major food allergens are also frequently used ingredients, it is common for allergens to be used in food processing. Unfortunately, cross-contact of allergenic and non-allergenic surfaces, equipment, and ingredients is possible through manufacturing and this presents a risk to food-allergic individuals.

Cross-contact, unintentional incorporation of a food allergen into a food, can occur during every portion of food processing including harvest, processing, and consumption. Some potential situations of cross-contact include: shared fields, harvesting equipment, shared off-farm storage, shared processing facilities, shared processing equipment, shared food preparation facilities, equipment, cookware, and shared serving utensils or cooking oils in restaurants *(89)*.

The actual prevalence of cross-contact is unknown, but issues of contamination have been found through adverse reactions and testing of contaminated products *(33)*. An investigation of peanut residues in packaged foods not labeled for peanut (ingredient) found 1% of products contained measurable amounts of peanut *(117)*. These positive results were generally found in products containing tree nuts; it is possible that peanuts and tree nuts were processed in the same facility and cross-contact occurred. In the EU, a study of the prevalence of milk in non-pre-packaged bakery products was completed. Levels of cow's milk protein similar to cross-contact were found in 62% of the pastry products analyzed and 35% of the savory products analyzed *(98)*. More information

about the production and storage of these items in display cases and the allergenicity of the ingredients used is needed in order for a milk-allergic consumer to understand the basis of the cross-contact.

A study that investigated methods of cleaning food processing equipment also evaluated the issue of airborne dust contamination to other processing lines. When compressed air was used to clean off equipment, a piece of hazelnut cookie dough was found in a petri dish that was placed elsewhere in the plant *(73)*. Another study of food processing methods confirmed that carry-through of allergenic materials is a cross-contact issue; peanut residues were detected in sugar cookie dough placed on food-processing surfaces that had previously been in contact with peanut butter cookie dough *(49)*.

A common effect of the potential for cross-contact in food processing is the adoption of precautionary allergen labeling (PAL) on food packages. Currently, PAL is being both under and over used by the food industry. Similar trace levels of allergens were found in products with and without PAL *(22)*. Additionally, many products with PAL had no allergen traces *(22, 117)*. PAL would be more effective if it represented an actual risk to the allergic consumer as derived from allergen threshold data.

b. Allergen Control Plans

Allergen control plans began to be implemented in the early to mid-1990s *(92)*. Resources are available about the elements and implementation of allergen control measures *(18, 38, 44, 50, 92)*. Many factors such as the type of facility, products

produced, the allergenic ingredients used and the amount of product produced may influence specific and unique allergen control measures, but the essential components of allergen control plans are described below.

An allergen control plan comprehensively addresses all aspects of food production and is updated whenever locations or processes are changed *(50, 89)*. During initial research and development, the necessity of introducing allergens into current products or new products should be discussed. Throughout the processes of receiving, storage, handling, and processing, allergens should be set apart through color coding and/or the use of symbols or icons, separate storage areas, or pallets *(46)*. Additionally, non-allergens should be stored above allergens to minimize cross-contact if leaks or spills were to happen. Suppliers must also be required to have allergen control plans and notifications of changes in the supplier's plans or ingredients should be required. During processing, runs that contain allergens can be scheduled at the end of the day or immediately prior to sanitation. In order to prevent cross-contact, rework can be used 'exact into exact', and tools and containers can be dedicated for use only with allergens. When new equipment is purchased, design elements that aid in effective sanitation should be considered. All labeling must comply with FALCPA (Food Allergen Labeling and Consumer Protection Act) of 2004 which requires that all allergenic ingredients be identified in common terms. It is important to ensure that the correct label goes on the correct package; some companies require that the previous run's packaging material be placed into storage prior to the next processing run beginning. Another approach could be to place allergen packaging films on rolls with larger cores so that non-allergen

packaging films could not fit the equipment. A validated allergen cleaning program is a vital component of allergen control plans and includes written procedures, verification methods, and acceptance criteria of results. In order to accomplish the goals of an allergen control program, all employees must be trained thoroughly in the proper protocols.

The final rule of 'Current Good Manufacturing Practice, Hazard Analysis, and Risk-Based Preventive Controls for Human Food', published on September 17, 2015 as part of Food Safety Modernization Act (FSMA) now requires manufacturers to identify potential avenues of allergen cross-contact and implement preventive control measures *(25)*. Written preventive controls for food allergens are a newly required portion of overall food safety plans. The regulation includes a comprehensive approach to food production as was included in the description of current allergen control plans. The areas addressed in the rule include personnel, plants and grounds, sanitary operations, equipment and utensils, processes and controls, food safety plans, and verification methods.

A significant focus is placed on prevention of allergen cross-contact, which is defined as "the unintentional incorporation of a food allergen into a food". This includes the outer garments of employees, all equipment and utensils, food-contact surfaces, and non-food-contact surfaces. Cleaning to prevent allergen cross-contact in these situations must be done "as frequently as necessary". It is recommended that operations could be separated through "location, time, partition, air flow systems, dust control systems, enclosed systems, or other effective means" in order to decrease the potential for allergen

cross-contact. Storage conditions for ingredients after reception, packing and holding must be conducted in order to prevent allergen cross-contact. Verification methods are required to identify allergen cross-contact (hazard management); visual inspection or swabbing of equipment were acceptable examples. They are not requiring validation of food allergen controls or sanitation controls (for example, "determining if a cleaning procedure effectively removes a food allergen from equipment surfaces").

The ruling does acknowledge that the measures necessary to prevent and control allergen cross-contact will vary by the food and facility. The measures that are determined to be necessary must be written in a food safety plan. Food allergens are now included in these plans since they have been classified as a hazard. The food allergen controls "include procedures, practices, and processes employed for: ensuring protection of food from allergen cross-contact, including during storage, handling, and use; and labeling the finished food, including ensuring that the finished food in not misbranded under section 403(w) of the Federal Food, Drug, and Cosmetic Act."

The rule is flexible in how a company may prove or justify that they have complied with the regulations. This opens up a rich field of research needs in many areas such as engineering, sanitation, and materials to determine how they may supplement prevention of allergen cross-contact.

c. Usage of Allergen Control Plans

A survey in 2010 that evaluated allergen control practices during FDA inspections found that most facilities are using allergen control measures of some sort, but there is

still improvement to be made, especially in small companies *(32)*. These small companies may be most challenged and in need of the most support while developing allergen control practices to comply with the regulations of FSMA. The FDA is developing a guidance document for allergen control plans *(25)*. Additionally, small businesses and very small businesses have an additional one or two years, respectively, to comply with the FSMA regulations *(25)*.

The survey did not investigate all aspects of allergen control, but focused on receiving and storage of ingredients, equipment use and cleaning, production, and labeling *(32)*. On average, 70% of facilities segregated allergen-containing ingredients during receiving. Larger companies used this practice more frequently than smaller companies. Shared equipment during processing was used in 77% of facilities; this presents a risk of carryover of allergenic ingredients to non-allergenic products. Of the facilities that used shared equipment, 91% cleaned the equipment between allergen and non-allergen containing products and 71% had a written allergen cleaning procedure. Again, smaller companies were less likely to have a written cleaning procedure (40%).

It was found that 70% of facilities used some form of allergen control to prevent cross-contact *(32)*. Each company faces many decisions about which allergen controls to use and each decision is unique based on the products produced and facilities. For example, a company using sieving equipment for sugar and flour could purchase two sieves, schedule flour sieving last in the day, and/or could place partitions or dedicate a processing area just for flour sieving *(87)*.

VI. Cleaning in Food Industry

a. Types of Soils

In order to develop a cleaning plan, the nature of the soil to be cleaned must be understood. There are several classifications of soil systems and food soils are generally complex as they involve many food components which have very different chemical compositions *(24, 30, 41, 81)*. Overheating of carbohydrate soils is not recommended as caramelization products of the sugars and starch glues can form. Protein soils are most effectively cleaned with an alkali detergent. Mineral deposits are soluble in acid solutions, and are most commonly formed from hard water or milk *(24)*.

Additionally, the age of the soil and the temperature at which it was created influence its ability to be cleaned. Burton has described the differences in milk soils derived from pasteurization and those from ultra-high temperature *(31)*. Pasteurization milk soils are 50-60% protein (50% of that protein composed of BLG), while high temperature milk soils are 70% mineral and 15-20% protein. The soil characteristics are also vastly different: a soft soil as compared to a brittle soil. Knowledge of the components of a soil to be cleaned should be understood in order to develop the most effective cleaning protocol.

b. Food Processing Surfaces

Food processing surfaces are composed of different materials and thus present a different variable to the equation of sanitation. It is best to use surfaces which are non-absorbent, non-corrosive, non-reactive with the product produced, and cleanable *(36)*. In

addition to choosing surfaces that are easier to clean, it is also important to design and select processing equipment that is easy to clean *(41)*.

The surfaces used in a given food plant will be specific to their desired function. The following surfaces were found in food plants by the American Institute of Baking: stainless steel, plastics (polyethylene, ultra high molecular weight polyethylene, polycarbonate, PVC and vinyl), rubber, glass, wood, and cloth *(61)*. Similarly, stainless steel, plastic (polyethylene, polypropylene or polycarbonate), ceramic, rubber, sealed concrete, coated cast iron, and air filter material were listed as food environmental surfaces of interest in AOAC guidelines on the validation of microbiological methods *(5)*.

Stainless steel is generally considered one of the best food processing surfaces due to its smooth, non-porous surface that is easily cleaned. Many types of stainless steel exist, but AISI 304 and AISI 316 are relevant to the food industry. AISI 316 is used more frequently as it can tolerate higher levels of halides (such as Cl found in salty foods and chlorinated cleaning solutions) *(59)*. The surface roughness should be 0.8 μm or smoother, otherwise adaptations to the cleaning protocol may be necessary to adequately clean the rougher surface *(59)*.

Plastics have many uses in food production such as storage vessels, hoses, and covers. The main sanitation concern with plastics is that they can be porous and absorb portions of the food product; additionally, the plastic monomers may leach into the food. Plastics recommended for food use based on their ability to be cleaned include

polypropylene, polyvinylchloride, acetal copolymer, polycarbonate, and high-density polyethylene *(59)*.

Rubber's uses in food production are mainly for seals, gaskets, and joint rings. The different properties of rubber are based on the long, repetitive molecular chains, called elastomers, that are the constituents of rubber *(59)*. The recommended choices of rubber include EPDM (ethylene-propylene-terpolymers), nitrile rubber, NBR (acrylonitrile-butadiene-rubber), silicon rubber, and fluoroelastomer *(59)*. The rubber choice is dictated by its desired function, as some are compatible with high temperatures and others are not oil and fat resistant *(59)*.

Other materials such as ceramics, glass (plastic coated) and wood are used for specific and specialized uses *(59)*.

c. Cleaning Mechanism

The mechanism of cleaning is composed of four factors: time, mechanical action, concentration, and temperature *(42)*. As these four factors are adjusted, it may be possible to decrease the other factors. As cleaning time is increased, generally through soaking, the other factors may be able to decrease. When mechanical action increases, the soil will be physically removed in a shorter time. Temperature can be increased throughout certain ranges and the rate of cleaning will be increased, but knowledge of the soil is necessary as high temperatures with proteins can make the soil harder to clean *(31)*. The concentration of the cleaning chemical can be increased to an extent and this will decrease the amount of time spent cleaning. Additionally, higher cleaner chemical

concentrations will aid in removing and suspending the soils to contribute to a more efficient rinse.

Together, these factors provide the input to accomplish cleaning. More specifically, cleaning involves breaking the cohesive forces that bind a material (soil-soil) and the adhesive forces between the soil and the surface (soil-surface) *(30)*. The proportion of adhesive and cohesive forces in a product is dependent on its chemical composition and soil characteristics. For example, tomato paste can generally be removed easily by overcoming the adhesive force between the surface and the soil *(31)*. This enables pieces of soil to be removed because not as many cohesive forces have been broken *(30)*. Soils in which the cohesive bonds mainly break will still leave residue on the surface. Protein removal from surfaces is gradual because the soil is dissolved from the surface *(30)*. The adhesive forces between the soil and surface are stronger than the cohesive forces in milk soils *(31)*.

The stages of cleaning and soil removal have been investigated and described *(30, 31, 34, 35, 41)*. The following cleaning stages were described by Plett (1985) and adapted from Fryer et al. *(31)*.

1) Possible bulk reaction between components of the chemical and the bulk fluid.

2) Transport of chemical to the surface, affected by temperature, concentration and flow.

3) Transport into the deposit: penetration of chemicals into the deposit is dependent on its structure. Surface active agents can increase penetration due to wetting.

4) Reaction between the deposit and cleaning chemical include melting, mechanical break-up, wetting, swelling, desorption, emulsification, hydrolyzation, saponification and dispersion.

5) Transport to the interface: reaction products diffuse out of the deposit.

6) Transport to the bulk: concentration gradients and hydrodynamic conditions allow the transport of the reaction products into the bulk.

Cleaning for allergen removal is focused on proteins. The cleaning mechanism for proteins and allergens is similar to the general scheme of cleaning described above, but specific measures are taken in response to the soil characteristics, namely protein. The stages of protein cleaning have been described as follows: 1) swelling stage, the native protein reacts to form an open protein matrix; 2) uniform stage, the rate of cleaning is constant and the deposits are removed through surface shear and diffusion; 3) decay stage, protein matrix breaks down into a non-uniform layer and the deposits are removed through shear stress and mass transport *(34, 35)*.

The swelling initially begins as the soil is rinsed with water and the protein absorbs the water. Furthermore, alkaline solutions containing hydroxyl anions react with the protein and subsequently the protein swells, dissolves and is suspended *(17, 31)*. Sometimes chlorine is also added in the form of a chlorinated alkaline detergent which additionally helps to break down the proteins and minimize mineral deposits *(17)*. An acidic cleaner is not initially used for proteins because it will precipitate the protein and adhere it to the surface, making it much more difficult to clean and remove *(17, 81)*.

d. Detergents and Cleaning Solutions

Detergents and cleaning solutions are a minor component (5%) in the cost of cleaning, but impart a large impact on the efficacy of cleaning *(41)*. An ideal cleaning agent is able to dissolve readily in water, rinse freely, be compatible with other components, penetrate soils, emulsify fats, suspend precipitates, hydrolyze proteins, and comply with regulations *(24)*. The choice of cleaner will be based on the properties of the soil to be cleaned.

Water is of course a main component of all cleaners *(24)*. The other components of cleaning solutions can be divided into two categories, physically active ingredients and chemically active ingredients *(81)*. The physically active ingredients alter the physical characteristics of the soil such as solubility or colloidal stability, while the chemically active ingredients modify soil components to make them more soluble *(81)*.

Surfactants are a physically active ingredient. The hydrogen bonds in water are disrupted by the polar heads of surfactants. This action decreases the surface tension of the water droplet and enables it to moisten a greater surface area, thus penetrating more soils and surfaces, and increasing the cleaning action *(41)*.

The chemically active ingredients include alkaline solutions, acidic solutions, and water conditioners. The alkaline options include sodium or potassium hydroxide, and sodium, potassium or ammonium salts of phosphate, silicates or carbonates *(81)*. The alkaline detergents aid in protein dissolution. Sodium hydroxide alone is hard to rinse from surfaces, but the addition of wetting agents may help *(24)*. Acidic components aid in

the dissolution of mineral deposits and in the food industry are generally used in periodic cleans *(41)*. Water conditioners include sequestering and chelating agents which assist in the prevention of mineral deposit accumulation. This prevention occurs through the formation of soluble complexes with Ca and Mg which also helps regulate water hardness *(41)*.

e. Cleaning Methods

Cleaning methods generally fall into two categories: wet cleaning and dry cleaning. Wet cleaning includes CIP, COP, foam and gel cleaning, and physical/manual cleaning *(50)*. Dry cleaning is employed in situations such as bakeries and includes vacuuming, sweeping, scraping, wiping, and compressed air *(50)*.

When choosing a cleaning technique, it is important to consider the effectiveness, efficiency, equipment requirements, cost, verifiability, and cost of cleaning material disposal *(112)*. In general, it is best to clean equipment periodically as shown in the figure below (Fig 4) rather than allow soils to accumulate over time *(41)*. The cleaning equipment itself should also be cleaned and color-coded or labeled for specific use with or without certain allergens *(41, 87)*.

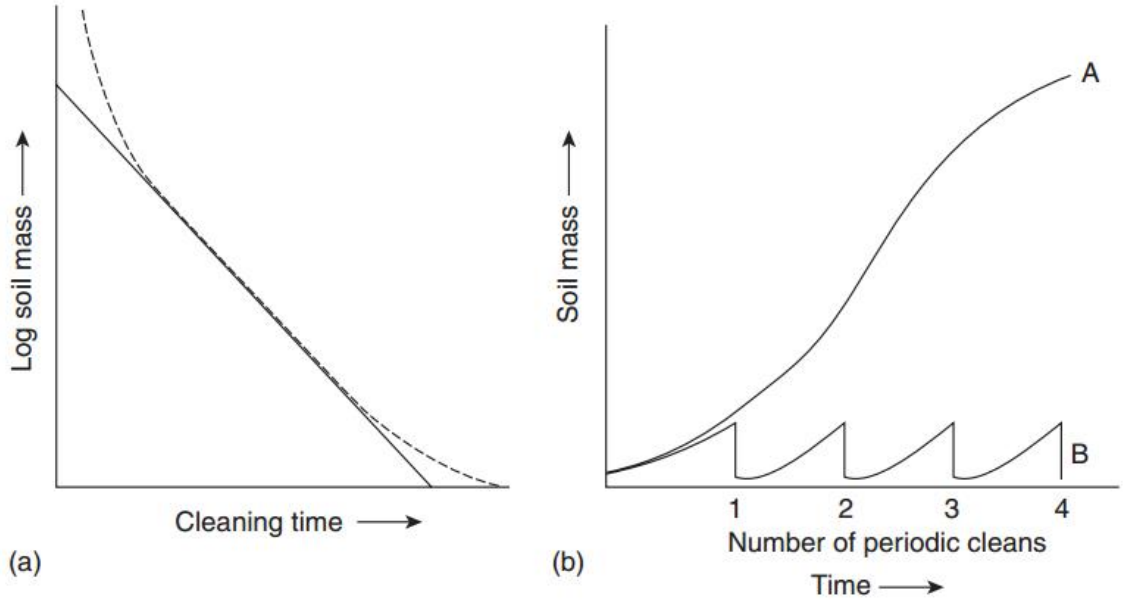

Figure 4. Soil removal and accumulation. a) Solid line is theoretical removal; dotted line is cleaning in practice. b) Build-up of soil; A, without periodic cleans and B, with periodic cleans. Adapted from *(41)*.

1) Wet cleaning methods

Wet cleaning methods include clean in place, clean out of place, foam or gel cleaning and manual or hand cleaning *(50)*. CIP allows the equipment to stay assembled while a normal stepwise cleaning process occurs including rinsing, caustic wash, rinsing, acid wash, and a final rinse. When using a COP method, the equipment is disassembled and placed into a cleaning vat capable of heating and recirculation. When using a foam or gel cleaning method, the solution is directly sprayed onto the soiled surface. Manual cleaning involves disassembling equipment and then physically brushing and cleaning the

equipment. Aspects of wet cleaning programs for allergen removal have been studied on lab-scale, pilot plant and industrial levels.

Roder, et. al studied cleaning of equipment after processing hazelnut cookie dough on a pilot and industrial scale *(73, 74)*. Their cleaning methods were based on manual cleaning with subsequent additions of hot water rinses and dish detergent. After these cleaning cycles were completed, cookie dough without hazelnut was processed on the equipment and samples at different equipment sites such as the spiral kneader, rotary molder and wire-cutting machine were sampled and tested with a hazelnut ELISA. When manual cleaning alone was used, higher amounts of hazelnut were found at the wire-cutter and thus product push-through would not be an effective method at this site. They did find that the excess hot water rinses were effective in reducing hazelnut residue in subsequent non-hazelnut cookies. The addition of detergent was not found to additionally decrease hazelnut residues. It is important to identify an appropriate cleaning procedure, although wet cleaning of commercial baking equipment is not generally recommended.

A lab scale investigation of peanut butter and milk removal from various food processing surfaces has been studied by Jackson et al. *(48, 49)*. Peanut butter was applied to stainless steel, Teflon, polyethylene, urethane and polycarbonate, washed and then swabbed for analysis with a peanut ELISA. Room temperature water was ineffective on all the surfaces while hot water was only ineffective on urethane and Teflon. Hot chlorinated alkaline detergents and acid detergents were effective on the surfaces, and room temperature chlorinated alkaline detergents were effective on some surfaces. The milk study investigated hot and cold set milk soils on stainless steel which were cleaned

at various temperatures and then swabbed for analysis with a milk ELISA. Hot water (62.8 and 73.8°C) was effective on cold milk soils, but ineffective on hot milk soils which are more resistant. The chlorinated alkaline detergent was effective on the hot milk soil at all detergent temperatures.

A study that created fouled sections of pipe with whey protein found that it is best to use alkaline-based solutions as the protein soil will swell and form an open matrix that may then be removed through shear and diffusion with a CIP system *(34, 35)*. The mineral portions of the soil are subsequently cleaned using an acidic solution.

An industrial scale study investigated cleaning of equipment after processing of wheat-battered chicken products *(109)*. The additive cleaning methods in this plant included a water rinse, foam and rinse, and lastly a sanitizer and rinse. The foam was composed of NaOH, NaOCl, and a surfactant. It was found through swabbing of equipment and testing with gliadin ELISA that the gliadin levels were reduced to approximately 1 ppm after the initial rinse step. The foam and rinse step additionally decreased the gliadin found, and the sanitize and rinse step did not additionally decrease gliadin levels.

Peanut slurry was prepared in slurry equipment that subsequently underwent rinsing, alkaline cleaning, rinsing, acidic cleaning, and rinsing. The rinsewater and final product were tested with a peanut ELISA. This wet cleaning process was effective at removing peanut residues initially after the alkaline cleaning solution *(88)*.

2) Dry cleaning methods

Dry cleaning methods include vacuuming, sweeping, scraping, wiping with cloths or brushes, and compressed air *(50)*. There is not a lot of research currently about dry cleaning methods and their implications for allergen removal. A study by Jackson et al investigated Sani-Wipes sanitizing wipes and vacuuming for allergen removal *(47)*. Slurries of peanut flour, NFDM, whole egg powder, soy flour, soy milk, and soy-based infant formula were applied to urethane, stainless steel, and Teflon and then baked for one hour at 80°C. The surfaces were then vacuumed or wiped with the sanitizing wipes and swabbed for testing with ELISA, ATP, and total protein. Positive results of allergenic resides were found after vacuuming; this method may not be effective for allergen removal. The sanitizing wipes were found to clean the surface effectively.

When using vacuuming or compressed air, it is important to consider potential cross-contact of airborne allergen particles to other processing lines during cleaning. It has been found that brushing and compressed air can move dry powders a significant distance. Holah found that particles 10-25 μm in size could be lifted by compressed air and remain airborne for greater than 16 minutes *(41)*.

f. Validation and Verification of Cleaning Methods

Validation and verification of cleaning methods for allergen removal are a complex and unique situation with many variables due to the allergen, food matrix, processing equipment, cleaning methods, and detection methods available. Validation is defined as the "process of assuring that a defined cleaning procedure is able to effectively

and reproducibly remove the allergenic food from the specific food processing line or equipment" *(42, 50).* Verification is the process of "demonstrating that validated cleaning protocols have been properly performed once the commercial manufacture of a product begins" *(50).*

1) Acceptance Limits

When validating and verifying cleaning methods, an acceptable measure of cleanliness or acceptance limits of the allergen must be used to determine when the equipment or food product is effectively cleaned. Sometimes the level of cleanliness is determined by the sensitivity level of the detection method. The detection limits of analytical methods are not necessarily practical or justifiable levels for cleaning in the context of a food allergic response. Recently, advances in determining threshold levels for various allergenic foods have been made *(90, 91, 95, 96).* The threshold values give information about the amount of food that provokes an allergic reaction; the information is gathered through double-blind challenge studies of allergic populations. These threshold values or reference doses will enable informed decisions throughout risk-assessment after cleaning and could be applied to decisions about precautionary allergen labeling as well *(22).*

Insights into applications of cleaning threshold levels can be obtained from the pharmaceutical industry, which has employed these methods for some time. It is recommended that all cleaning limits of active pharmaceutical ingredients be "practical,

achievable and justifiable" and based on toxicity data and acceptable daily intakes *(2, 106)*. Several factors are included in calculations:

1. Acceptable daily exposure (ADE) (mg/day) x Batch Size/Max Daily Dose =Maximum Safe Carryover (MSC)

2. MSC/Total Surface Area = Surface Residue ug/cm^2

3. Surface Residue/cm^2 x Area Swabbed = Residue on Swab (µg)

4. Residue on Swab (µg)/Dilution Volume (ml) = Residue level in swab sample (ppm). Adapted from *(107, 108)*.

Applying approaches such as these to the food industry will help enable safe and effective cleaning acceptance limits.

2) Sampling by Swabbing

Developing an effective sampling plan can be a statistical exercise similar to finding the needle in a haystack. It is generally best to start with "problem areas" such as gaskets, corners, and hard to reach places that may not have been fully cleaned. The physical act of swabbing these selected locations should be approached with a methodical manner to decrease subjective differences between different users *(116)*. The swab should be absorbent and have minimal particulates, while also able to release swabbed residue into the extraction solution *(97)*. It is recommended that the swab head be moist but not saturated prior to testing. The method of swabbing is not always specified, but a cross-hatch procedure that covers the area in two different directions is recommended. An

AOAC guideline for microbiological methods recommends swabbing a 1” x 1” area, while some lateral-flow device kits recommend swabbing a larger area *(5)*. When developing and validating a swabbing protocol, it is important to determine the % recovery or swabbing efficiency of the swab. A study by Schlegel et al. investigated direct sampling methods of peanut solutions from a stainless steel surface *(80)*. Using a flat zapped-head foam swab, they found that the first swab had a recovery of 68%, and by swabbing the same area with a second swab, the total recovery increased to 93% of peanut proteins applied to the surface *(80)*.

3) Detection Methods for Cleaning Assessment

Common detection methods for food allergens have been previously described. Options such as visual cleanliness, lateral flow devices, ELISA, ATP, and general protein swabs have also been used to detect allergens after cleaning by swabbing food processing surfaces or testing final product. When choosing a detection method it is important to consider if it can detect allergen residues at the level of cleanliness that is desired. Interferences of residual cleaning solution or product matrix should be considered.

VII. Summary

Current federal regulations will require higher levels of allergen control in food processing. This has created a need for validation of cleaning processes for allergen removal. Lateral flow devices provide a quick and rapid method for allergen detection, but little is known about their general usage, sensitivity, and specificity. Research of

various milk-specific lateral flow devices and cleaning methods for allergen removal will aid the food industry and kit manufacturers.

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CHAPTER TWO

EVALUATION OF COMMERCIAL MILK-SPECIFIC LATERAL FLOW DEVICES

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I. Abstract

The validation of allergen cleaning processes is becoming increasingly required. The use of lateral flow devices for this purpose has rapidly expanded, but the best practices for their use are still developing. The goal of this study was to compare commercially available milk-specific lateral flow devices (LFD) and a general protein method. Five milk proteins and seven milk-derived ingredients were tested at several concentrations with eight milk-specific LFDs and a general protein kit. Non-fat dry milk (NFDM) was prepared at concentrations between 100-10,000 ppm milk protein and analyzed by the LFDs to determine the level at which a false negative result (overload level or hook effect) was obtained. NFDM was also prepared in 0.025M PBS (pH 7.4, 0.85% NaCl) and applied to stainless steel panels (100, 30, 10, or 3 μg NFDM protein) with various drying methods and sampled with different swab conditions to determine the level of detectability. Several total milk LFD kits did not detect whey proteins or whey-derived ingredients. The overload level of the various kits ranged from 100- 10,000 ppm milk protein; a small dynamic range observed with some kits would necessitate multiple dilutions of a sample to ensure it would fall within the range of detection. When sampling stainless steel with a swab for LFD analysis, it was found that the ability to detect milk protein residues from surfaces where the milk residues were dried onto the surface with high heat was less than with low-heat. No differences in sensitivity were observed as a result of moistening the soil or the swab prior to sampling. Overall, the importance of understanding the detection capabilities of LFDs prior to use was highlighted as the performance of the milk-specific LFDs tested varied greatly.

II. Introduction

Food allergy is a growing concern for many individuals; milk allergy, one of the most common allergies, affects 2.5% of infants and children and 0.3% of adults in North America *(22)*. Milk is not only a common allergenic food for the population, but various milk-derived ingredients are among the most commonly used ingredients in the food industry *(6, 23)*. The only way to prevent an allergic reaction is to avoid the offending allergenic food. But, the implementation of a safe and effective avoidance diet is risky for the milk-allergic individual as milk is the most common undeclared allergen, has led to allergic reactions due to cross-contact, and is found in products with and without advisory labeling and foods which are associated with difficult cleaning requirements such as chocolate *(2, 7, 8)*. Undeclared milk can occur from the presence of milk in raw ingredients, processing aids, reworked product, or from carry-over from shared equipment *(23)*.

In order to protect the milk-allergic consumer, food manufacturers using milk or milk-derived ingredients should develop allergen control plans (ACP). The validation of the effectiveness of those ACPs involves the use of methods to detect milk protein residues. This can include the testing of equipment, food processing surfaces, rinse water, ingredients, or finished product. Additionally, the FDA Food Safety Modernization Act requires the development of effective ACPs and testing procedures, which, when properly implemented, can aid in the ACP validation *(5)*.

Rapid and specific methods such as lateral flow devices (LFD) are becoming increasingly used by the food industry to monitor cleaning of food processing equipment and food product contamination *(1, 16-18)*. LFDs are an immunologic allergen detection method which involves specific antibody-antigen recognition. With LFDs, the first step involves extraction of the analyte from the sample which can be a swab, rinse water, or a food or ingredient sample. With commercial milk LFDs, kit instructions vary from hand shaking at room temperature to vortexing and boiling the sample. The extracted sample is introduced onto the device and any antigens (allergens) in the sample bind to conjugated antibody (IgG conjugated to gold or colored latex, particles that aid in the development of a colored test line) in the test zone of the strip. Next, the antigen-conjugated antibody complex travels through the test zone and binds to an area of the strip where allergen-specific IgG is fixed; a positive test line is formed. Any extra conjugated antibody continues through the LFD and is bound at the control line by species-specific IgG *(1)*.

The rise in use of commercial LFD kits came rapidly *(20)*. The first allergen rapid immunoassay was developed for peanut in 1997 *(12)*. In 2004, two peanut LFDs were commercially released and joined a handful of gluten LFDs on the market *(25)*. Today, at least eight different companies produce LFDs targeting milk, casein or β-lactoglobulin (BLG, a whey protein), and LFDs are available for many other major allergens. Many factors can affect test results such as manufacturing issues, operator errors, effects of the food matrix, environmental factors, sample manipulation and cross-reactivity issues *(26)*. An additional limitation of LFDs is the high dose hook effect or overloading of the device. This occurs when the allergen level is very high in the sample and false negative

results are obtained *(1, 14)*. Matrix interferences were found to influence the detection of peanut in chocolate samples tested with LFDs as compared to sandwich ELISA while cookies had similar levels of positive results between ELISA and LFDs *(15)*. Similarly, an r-Biopharm milk LFD was validated with milk, ice cream and milk powder applied and dried on a surface; the milk powder had a lower sensitivity than the other products *(17)*. An inter-laboratory validation study of two peanut LFDs found that the sensitivity of the kits approached that of sandwich ELISA kits *(24)*. However, differences did occur in the frequency of false positives between the two commercial methods, which may be attributable to differences in antibody type and quality *(24)*. These considerations are important to take into account when selecting a suitable LFD kit. Additionally, the nature of the sample for LFD testing should be considered. Some kit inserts specify adjustments to the protocol for sorbets and thick, viscous products which may interfere with sample movement throughout the LFD by capillary action. Samples derived from swabbing can be variable as swabbing is a subjective procedure. Development of consistent swabbing procedures may reduce variability between test operators *(27)*.

While LFDs have been found to be a suitable method for qualitative screening in the food processing environment, it is still essential for allergen test kits and methods to be validated both in inter-laboratory studies and by the end-user with their actual products containing the allergenic ingredient of interest *(13, 16)*.

The purpose of this study was to investigate the milk protein and milk-derived ingredient specificity, overload level, and swab sampling characteristics of commercial milk-specific LFDs. This information will allow comparisons between the different kits

that will enable the food industry to effectively select a LFD kit for their detection purposes and to better interpret the test results.

III. Materials and Methods

a. Purified milk proteins. Purified protein fractions of the bovine milk proteins, α-, β- and κ-casein, β-lactoglobulin and α-lactalbumin (Sigma-Aldrich, St. Louis, MO), ≥ 70%, ≥ 98%, ≥ 70%, ≥ 90%, and ≥ 85% purity, respectively were used to prepare 100 ppm protein (wt/vol) solutions in 0.025 M phosphate buffered saline (PBS; pH 7.4, 0.85% saline). The solutions were prepared assuming complete purity of the proteins; SDS-PAGE images available in Appendix A. The 100 ppm protein solution was further diluted to prepare 1 and 10 ppm (vol/vol) protein solutions. The 0.025 M PBS solution was used as a negative control. The 1, 10 and 100 ppm protein samples were run in triplicate with each LFD described below except in the analysis with the Neogen Reveal Total Milk kit in which the samples were extracted in duplicate and then the extracts run in duplicate wells.

b. Milk-derived ingredients. Seven milk-derived ingredients were analyzed: low heat processed nonfat dry milk (NFDM) (Darigold, Seattle, WA), whey protein concentrate at 34% protein and 80% protein (WPC34, WPC80) (Erie Foods International, Erie, IL), sodium Caseinate (NaCas) (Erie Foods International, Erie, IL), sweet whey powder (SW) (Grande Custom Ingredients Group, Lomira, WI), acid whey powder (AW) (Saputo Dairy Foods, USA, Dallas, TX), and milk protein concentrate 80% protein (MPC80) (Idaho Milk Products, Inc., Jerome, ID). The protein content of each ingredient

was determined through analysis on a LECO FP-528 Protein/Nitrogen Determinator according to manufacturer's instructions (LECO Corporation). SDS-PAGE images and protein analysis results available in Appendix C.

Each milk-derived ingredient was used to prepare a 100 ppm protein (wt/vol) solution in 0.025 M PBS (pH 7.4, 0.85% saline). The solution was stirred for 15 minutes and then placed for 30 minutes in a 60°C shaking water bath. The solutions were directly diluted in PBS to prepare 75, 25, 10, 5 and 1 ppm protein (vol/vol) samples. Each sample was analyzed in triplicate as a liquid sample as described by the LFD kit instructions. The general protein kit was analyzed by directly pipetting 0.15 ml of the sample onto the swab head.

c. Analysis of overload level/hook effect. Samples of NFDM were prepared as previously described in 0.025 M PBS at levels between 100-10,000 ppm protein and tested in triplicate with each LFD as a liquid sample, the general protein kit was analyzed by directly pipetting 0.15 ml of the sample onto the swab head. The overload level was determined by the absence or depression of the test line and/or the absence or depression of the overload line when applicable.

d. Analysis of swab sampling conditions. A 1000 ppm NFDM protein solution was prepared in 0.025M PBS (pH 7.4, 0.85% NaCl) and directly diluted with the same PBS solution to prepare 300, 100 and 30 ppm NFDM protein solutions. A 0.1 ml aliquot of each sample was applied to a 3x5" panel made of stainless steel 316 (Ecolab, St. Paul, MN) and spread with a pipet tip to cover a 7 x 7 cm area. The soiled panel was treated in

five different ways. A) The NFDM soil was gently dried at 37°C for approximately 13 minutes and the dry soil was moistened with 0.1 ml of purified water processed through a reverse osmosis and deionizer system (ROD water) before sampling the soiled area with a dry swab provided by the kit. B) The NFDM soil was gently dried at 37°C for approximately 13 minutes and the swab head was directly moistened with 0.1 ml of moistener or water as directed by the kit insert before sampling the dry soil. C) The NFDM soil was dried at high temperature in a 350°F oven for 10 minutes; after cooling, 0.1 ml of ROD water was applied to the surface before sampling with a dry swab. D) The NFDM soil was dried at high temperature in a 350°F oven for 10 minutes; after cooling, 0.1 ml of ROD water or kit-provided moistener was applied to the swab head and the dry soil was sampled. E) The NFDM soil was sampled immediately (still moist) with a dry swab. All swabbing was conducted using a cross-hatch technique.

Initially, each kit was tested with the swab provided by the kit. The Morinaga kit does not provide swabs, so a cotton-tipped PurSwab 867-WC (Purtian Medical Products Company, Guilford, ME, USA) was used. The entire experiment was repeated as described above using the Neogen Allergen Environmental Swab (NAES) (Lansing, MI, USA) with each kit. The 3M Clean-Trace Surface Protein (Allergen) kit was excluded from the analysis using the Neogen Allergen Environmental Swab as the 3M kit comes with a specific swab for its container.

The stainless steel panels were cleaned between experiments with a warm solution of Micro-90 (International Products Corporation, Burlington, NJ, USA).

e. LFD and general protein kits. Eight commercially available total milk, casein or BLG lateral flow device kits and one total protein kit were used for analysis: Romer AgraStrip Total Milk, Romer AgraStrip Casein, and Romer AgraStrip BLG (Romer Labs, Runcorn, Cheshire, UK), Neogen Reveal 3-D Total Milk and Neogen Reveal Total Milk (Neogen Corporation, Lansing, MI, USA), bioavid Diagnostics Lateral Flow Milk (bioavid Diagnostics, Wendelsheim, Germany), Elution Technologies Bovine Total Milk (Elution Technologies, Colchester, VT), Morinaga Casein (Morinaga Institute of Biological Science, Yokohama, Japan), and 3M Clean-Trace Surface Protein Allergen (3M Health Care, St. Paul, MN, USA).

The instructions for each lateral flow device were followed with the following modifications. The Neogen Reveal 3-D extraction buffer packets were pooled and 3.75 ml of extraction buffer was placed into each sample vial. The Elution Technologies instructions described wetting the swab head for swab sampling, this was standardized to applying 0.1 ml of extraction buffer from the tube to the swab head and the instruction to swirl several times was standardized to vortexing for 10 sec. The Neogen Allergen Environmental Swabs were additionally processed by placing all swabs in a sealed plastic bag into a 60°C shaking water bath for 15 min; after cooling to room temperature, a 1 ml aliquot of the extracted solution was centrifuged for 10 minutes at 13,000 rpm (Thermo Scientific, Sorvall Legend Micro 17 Centrifuge, USA). Complete directions are available in Appendix B.

The qualitative results of each kit were read visually and interpreted according to each instruction manual. The Elution Technologies and Neogen Reveal 3-D Total Milk kits have an overload line, while the other LFD kits do not.

IV. Results and Discussion

Several total milk LFDs do not detect whey proteins. The protein specificity of the various LFDs was analyzed. The Romer, Neogen, bioavid Diagnostics and Elution Technologies total milk kits did not detect proteins from the whey fraction of milk, α-lactalbumin and β-lactoglobulin (Table 1). The discrepancy between the advertised detection capabilities and what was found is a concern for the customer using a total milk kit to detect whey ingredients. The casein and BLG kits successfully detected only their target proteins. No kit was able to detect α-lactalbumin. This is a concern as α-lactalbumin has recently garnered increased production and ingredient development and may be the sole milk-related target of interest in certain products *(3, 11)*.

LFD sensitivity to milk-derived ingredients is variable. The sensitivity of the lateral flow devices to NFDM ranged from 1 ppm protein (Romer AgraStrip Casein and bioavid Diagnostics) to 5 ppm protein (Romer AgraStrip BLG, Total Milk, Neogen Reveal, Neogen Reveal 3-D, Elution Technologies, and Morinaga Casein) (Table 2). The lowest level of NFDM that could be detected with the 3M Clean-Trace kit, a general protein method, was 25 or 75 ppm protein. While general protein swabs display lower sensitivity, they were found to be an effective method of detection when compared with

visual inspection, ELISA and ATP in a study of allergen detection after dry-cleaning methods *(9)*.

LFD specificity to milk-derived ingredients is variable. Six concentration levels of each ingredient were analyzed; the 75 ppm protein level was determined to be representative of the overall results (Table 3); complete results available in Appendix C. The test line intensity varied by kit and sometimes throughout the concentration range tested. Some kits, particularly Romer AgraStrip BLG, Neogen Reveal 3-D Total Milk, and bioavid Diagnostics had test lines which would increase and then decrease in intensity as the protein content of the sample increased. This observation may have been the beginning of the hook effect in which there is no longer an excess of antibodies due to high concentrations of analyte which inverts the dose-response curve *(1, 14)*. LFDs are generally used as a verification method of equipment cleanliness so it is important for the user to understand that a faint positive test line does not necessarily correlate to a low contamination level and that further cleaning should be initiated whenever a positive result is found *(10, 23)*.

In similarity to the milk protein specificity results, certain LFDs did not detect milk-derived ingredients made of whey. The Neogen Reveal Total Milk, Neogen Reveal 3-D Total Milk and the Elution Technologies Bovine Total Milk kits did not detect WPC34, WPC80, or sweet whey at 75 ppm protein. Soon after these results were obtained, the Elution Technologies kit underwent new development; subsequent retesting of the whey ingredients found that the kit was able to detect very, very faint positive results at the 75 ppm protein level. Acid whey was faintly detected by these kits. SDS-

PAGE analysis of the milk-derived ingredients showed that the acid whey sample was not as pure relatively and displayed traces of α-, β-, and κ-casein (data not shown). Additionally, the Romer AgraStrip Casein and Morinaga Casein kits were able to positively detect the acid whey sample.

Overall for selection of the LFD kit that is the best fit for any specific purpose, kit users should determine which kits actually detect milk residues in their products, ingredients, or environmental samples and understand the dynamic range of the LFD and the effects of overload on the LFD results.

Commercial LFDs have variable overload levels. Very high levels of allergen can overload an LFD and lead to a false negative result *(1, 14)*. This hook effect is observed in LFDs where the test line decreases in intensity and eventually does not form as the analyte (allergen) load of the sample increases. The concentration of milk protein that would result in an overload condition might vary between LFD kits. The LFDs that were evaluated had overload levels at concentrations between 100 and 10,000 (the highest concentration tested) ppm NFDM protein (Table 4); complete results available in Appendix D. Kits that have very small ranges of detection would require the user to test several dilutions of their product to ensure that it would fall within that range of detection. All test kits analyzed except Neogen Reveal 3-D come with extra extraction solution that could be used to dilute the sample prior to testing. These dilutions would also be necessary when sampling dirty equipment (positive control) to ensure that your product or ingredient of interest is detectable by the LFD kit.

In order to combat the hook effect, two companies (Neogen Reveal 3-D and Elution Technologies) produce LFDs with an additional ‘overload line’. Both of these kits became overloaded at about the same level (Table 4). But in addition to the test line not developing, the overload line does not develop which alerts the user that the sample had a high allergen load and further dilutions of the product should be tested. With the Neogen Reveal 3-D kit, the overload line decreased to intensities less than the test line in samples with higher concentrations of protein, which would lead the user to believe that the sample was at the upper detectable limit, >1000 ppm. This occurred in both the samples that were positively detected and negatively detected (the overload line decreased in intensity and test line was never positive). With the Elution Technologies kit, the overload line decreased in intensity with the higher concentrations tested of MPC80 and NFDM.

The Morinaga and 3M Clean-Trace kits are not subject to the hook effect. The Morinaga kit’s extraction solution denatures the proteins, so detection was consistent throughout all concentrations tested. The 3M Clean-Trace kit also detected strongly because the analysis relies on the Biuret method in which higher protein concentrations yield more intense color development.

Influence of swab sampling conditions on LFD detection. Experiments were conducted where the Neogen Allergen Environmental Swab was used with all of the milk LFD kits to remove any differences that might be attributed to the swab when taking samples from a stainless steel surface. In general, the swabs provided with the kits allowed slightly better sensitivity in NFDM detection than the Neogen Allergen

Environmental Swab (Table 5); complete results available in Appendix E. With all of the soil preparation methods used in this experiment, soils were easily removed from the surface. The NAES was better at scraping than absorbing soils. If a soil was crusty or extensively baked onto a food processing surface, the NAES may be a suitable swab to scrape the soil from the surface. If the soil is a liquid or still moist, a more absorbent swab would be a better choice.

The wet soil sampled with a dry swab (treatment E) was found to have the best sensitivity, followed by the low temperature oven dried samples and then the high temperature oven dried samples. The oven drying may have induced heat processing effects to the soil which have previously been found to lower detection by ELISA; additionally, the soil may have been harder to release with a swab *(4, 21).* Swabbing practices generally recommend to moisten a swab to saturation prior to swabbing, but many LFD kit inserts have variable instructions about moistening the surface or moistening the swab head *(27).* In general, no differences in sensitivity were observed between moistening the swab and moistening the surface when swabbing dry soils. Swabbing the same area with two swabs was not investigated in this research, but has previously been found to increase recovery *(13, 19, 27).* A consistent swabbing method should be developed for an allergen cleaning program *(10).*

In conclusion, commercially available milk-specific lateral flow devices vary greatly in their performance. Potential LFD users must understand the detection capabilities and limitations of the different milk LFD kits in order to make an appropriate selection. Several serious limitations were noted with LFDs. Not all total milk LFDs

detect whey proteins or whey-derived ingredients. Some kits have a very small range of detection and overload at a low level. When using LFDs to swab production surfaces, the type of soil and swab chosen may influence the sensitivity. Additionally, LFDs are a qualitative test and assumptions about the degree of contamination based on test line intensity should not be made as intensities were found to vary throughout the concentration ranges tested.

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VII. Tables

TABLE 1. *Milk protein specificity*

	Milk protein specificity[a]				
LFD kits	α-casein	β-casein	κ-casein	β-lactoglobulin	α-lactalbumin
Romer AgraStrip Total Milk	Yes	Yes	Yes	+/-[b]	No
Romer AgraStrip Casein	Yes	Yes	Yes	No	No
Romer AgraStrip BLG	No	No	No	+/-	No
Neogen Reveal Total Milk	Yes	Yes	Yes	No	No
Neogen Reveal 3-D Total Milk	Yes	Yes	Yes	No	No
Bioavid Diagnostics Milk	Yes	Yes	+/-	No	No
Elution Technologies Bovine Total Milk	+/-	No	+/-	No	No
Morinaga Casein	Yes	Yes	Yes	No	No

[a] Protein solutions were tested at 100, 10 and 1 ppm protein; this table indicates whether the kit was able to detect at one or more of the concentration levels. Each solution was tested in triplicate.

[b] +/- , a very, very faint test line that may be interpreted differently by different users.

TABLE 2. *Milk-derived ingredient sensitivity*

	Kit sensitivity of milk-derived ingredients (ppm ingredient protein, result)						
LFD kits	NFDM	MPC80	Sodium Caseinate	WPC34	WPC80	Sweet Whey	Acid Whey
Romer AgraStrip Total Milk	5, Pos	5, Pos	5, Pos	5, Pos	5, Pos	5, Pos	5, Pos
Romer AgraStrip Casein	1, f. pos[a]	1, f. pos	5, Pos	5, Pos	1, f. pos	5, Pos	5, f. pos
Romer AgraStrip BLG	5, Pos	5, Pos	Neg	5, f. pos	5, f. pos	5, Pos	5, Pos
Neogen Reveal Total Milk	5, +/-[b]	5, +/-	5, +/-	Neg	Neg	Neg	25, +/-
Neogen Reveal 3-D Total Milk	5, Pos	5, f. pos	5, f. pos	Neg	Neg	Neg	25, +/-
Bioavid Diagnostics Milk	1, f. pos	1, +/-	1, Pos	1, f. pos	1, f. pos	5, Pos	1, f. pos
Elution Technologies Bovine Total Milk[c]	5, +/-	5, +/-	10, +/-	Neg	Neg	Neg	25, +/-
Morinaga Casein	5, f. pos	5, f. pos	5, f. pos	75, +/-	100, +/-	Neg	25, +/-
3M Clean-Trace Surface Protein (Allergen)	75, Pos	75, pos	75, pos	25, +/-	25, +/-	25, +/-	25, Pos

[a] f. pos, faint positive

[b] +/-, a very, very faint test line that may be interpreted differently by different users.

[c]Elution Technologies Bovine Total Milk kit underwent development during this time and thus some samples were retested with a newly developed kit. No change in detection was determined for NFDM, but WPC34, WPC80, and SW were initially detected at a +/- level at 75 ppm protein with the newer version of the kit.

TABLE 3. *Milk-derived ingredient specificity at 75 ppm protein*

	Detection of milk-derived ingredients prepared at 75 ppm protein, (no. positive/ no. tested)[a]						
LFD kits	NFDM	MPC80	Sodium Caseinate	WPC34	WPC80	Sweet Whey	Acid Whey
Romer AgraStrip Total Milk	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
Romer AgraStrip Casein	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)[c]	Pos (3/3)	Pos (3/3)	Pos (3/3)
Romer AgraStrip BLG	Pos (3/3)	Pos (3/3)	Neg (0/3)	Neg (0/3)	f. pos (2/3)	f. pos (3/3)	+/-[d] (3/3)
Neogen Reveal Total Milk	Pos (3/3)	Pos (3/3)	Pos (3/3)	Neg (0/3)	Neg (0/3)	Neg (1/3)	+/- (3/3)
Neogen Reveal 3-D Total Milk	Pos (3/3) O<T[b]	Pos (3/3) O<<T	Pos (3/3) O<T	Neg (0/3) O: +/-	Neg (0/3) O: +/-	Neg (0/3)	f. pos (3/3)
Bioavid Diagnostics Milk	f. pos (3/3)	f. pos (3/3)	Pos (3/3)	f. pos (3/3)	f. pos (3/3)	+/- (2/3)	f. pos (2/3)
Elution Technologies[e] Bovine Total Milk	f. pos (3/3)	f. pos (3/3)	+/- (2/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (2/3)
Morinaga Casein	Pos (3/3)	Pos (3/3)	Pos (3/3)	+/- (2/3)	Neg (1/3)	Neg (0/3)	Pos (3/3)
3M Clean-Trace Surface Protein (Allergen)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

[a] The protein content of each ingredient was determined through LECO Dumas protein analysis. Solutions were tested at 100, 75, 25, 10, 5, and 1 ppm protein. Analysis was performed in triplicate and the result denotes the majority result of the measurements.

[b]O, overload line; T, test line; Neogen states that when O intensity is less than T intensity, the LFD is overloaded

[c]f. pos, faint positive

[d] +/-, a very, very faint test line that may be interpreted differently by different users.

[e]Elution Technologies Bovine Total Milk kit underwent development during this time and thus some samples were retested with a newly developed kit. WPC34, WPC80, and SW were detected as +/- at 75 ppm protein.

TABLE 4. *Overload level of LFDs with NFDM*

LFD kits	Overload level (ppm NFDM protein)[a]
Romer AgraStrip Total Milk	2 500
Romer AgraStrip Casein	~10 000[b]
Romer AgraStrip BLG	1 000
Neogen Reveal Total Milk	~10 000[b]
Neogen Reveal 3-D Total Milk	1 000
Bioavid Diagnostics Milk	100
Elution Technologies Bovine Total Milk	750
Morinaga Casein	>10 000[b]
3M Clean-Trace Surface Protein (Allergen)	>10 000[b]

[a] The overload level was defined as the absence or depression of the test line and/or the absence or depression of the overload line when applicable.

[b]Overload not observed at 10,000 ppm NFDM protein.

TABLE 5. *Influence of various swabbing conditions on sensitivity of LFD kits*

LFD kits	Swab	Method[a]	Sensitivity (µg NFDM protein, result)
Romer AgraStrip Total Milk	Kit provided swab	A	3, delayed +/-
		B	3, +/-
		C	10, Pos
		D	10, f. pos
		E	3, f. pos
	NAES[b]	A	3, +/-
		B	10, Pos
		C	10, Pos
		D	10, +/-
		E	3, +/-
Romer AgraStrip Casein	Kit provided swab	A	3, f. pos
		B	3, f. pos
		C	3, delayed f. pos
		D	3, delayed f. pos
		E	3, Pos
	NAES	A	3, f. pos
		B	3, f. pos
		C	3, delayed f. pos
		D	3, delayed f. pos
		E	3, f. pos
Romer AgraStrip BLG	Kit provided swab	A	3, +/-
		B	10, f. pos

		C	10, +/-
		D	10, +/-
		E	3, f. pos
	NAES	A	10, f. pos
		B	10, f. pos
		C	Neg
		D	Neg
		E	3, +/-
Neogen Reveal Total Milk	Kit provided swab	A	30, pos
		B	10, +/-
		C	30, +/-
		D	30, +/-
		E	30, +/-
	NAES (not applicable)		
Neogen Reveal 3-D Total	Kit provided swab	A	10, f. pos
Milk		B	10, f. pos
		C	10, f. pos
		D	10, +/-
		E	3, +/-
	NAES	A	10, f. pos
		B	3, +/-
		C	10, +/-
		D	10, +/-
		E	10, f. pos
bioavid Diagnostics Milk	Kit provided swab	A	3, f. pos
		B	3, f. pos

		C	3, Pos
		D	3, f. pos
		E	3, Pos
	NAES	A	3, f. pos
		B	3, f. pos
		C	3, f. pos
		D	3, f. pos
		E	3, f. pos
Elution Technologies Milk	Kit provided swab	A	3, f. pos
		B	3, +/-
		C	3, +/-
		D	3, +/-
		E	3, f. pos
	NAES	A	3, +/-
		B	3, +/-
		C	10, Pos
		D	10, +/-
		E	3, f. pos
Morinaga Casein	Kit provided swab	A	3, f. pos
		B	3, f. pos
		C	3, f. pos
		D	3, f. pos
		E	3, f. pos
	NAES	A	3, f. pos
		B	3, f. pos
		C	3, f. pos

		D	3, +/-
		E	3, f. pos
3M Clean-Trace Surface	Kit provided swab	A	10, +/-
Allergen		B	10, f. pos
		C	10, f. pos
		D	10, +/-
		E	10, f. pos
	NAES (not applicable)		

[a]Methods:

A) The NFDM soil was gently dried at 37°C for approximately 13 minutes and the dry soil was moistened with 0.1 ml of ROD water before sampling the soiled area with a dry swab provided by the kit.

B) The NFDM soil was gently dried at 37°C for approximately 13 minutes and the swab head was directly moistened with 0.1 ml of moistener or water as directed by the kit insert before sampling the dry soil.

C) The NFDM soil was dried at high temperature in a 350°F oven for 10 minutes; after cooling, 0.1 ml of ROD water was applied to the surface before sampling with a dry swab.

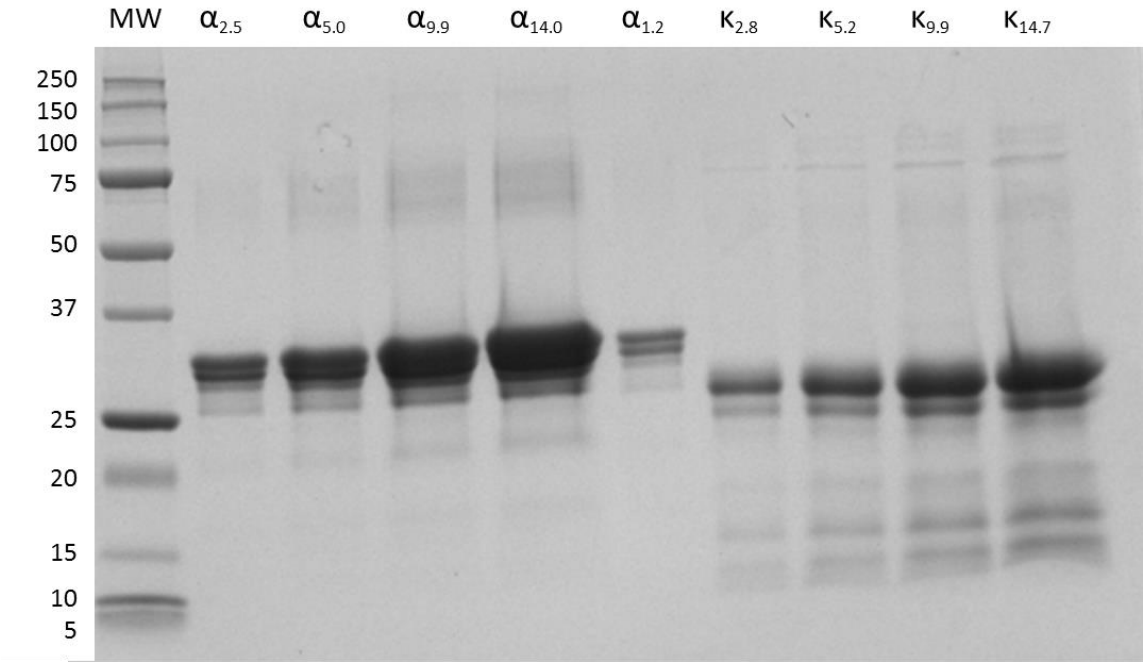
D) The NFDM soil was dried at high temperature in a 350°F oven for 10 minutes; after cooling, 0.1 ml of ROD water or kit-provided moistener was applied to the swab head and the dry soil was sampled.

E) The NFDM soil was sampled immediately (still moist) with a dry swab. All swabbing was conducted using a crosshatch technique.

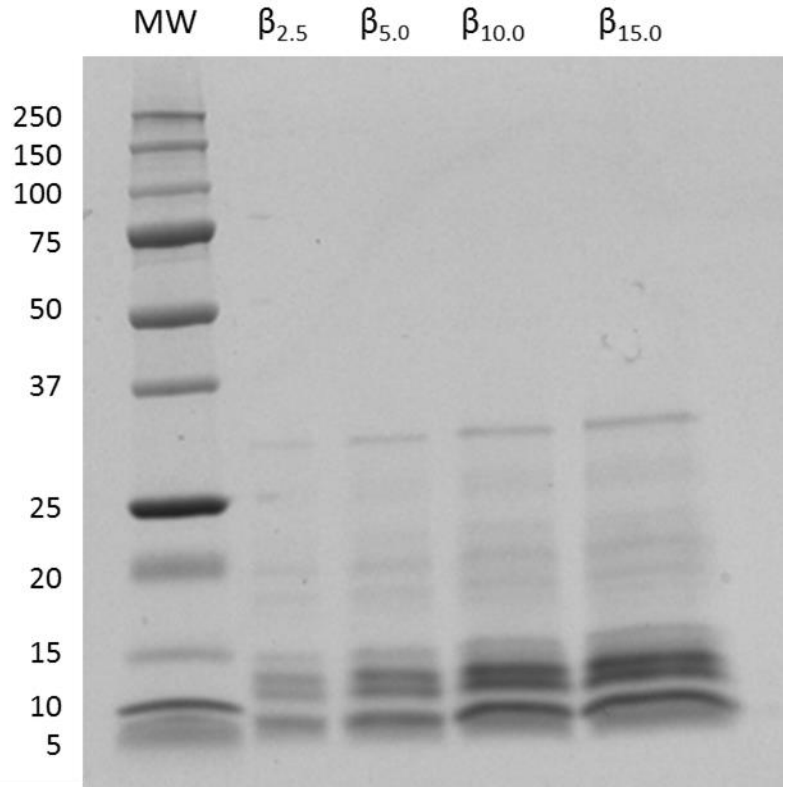
[b]NAES= Neogen Allergen Environmental Swab

APPENDIX A: Purified Milk Proteins

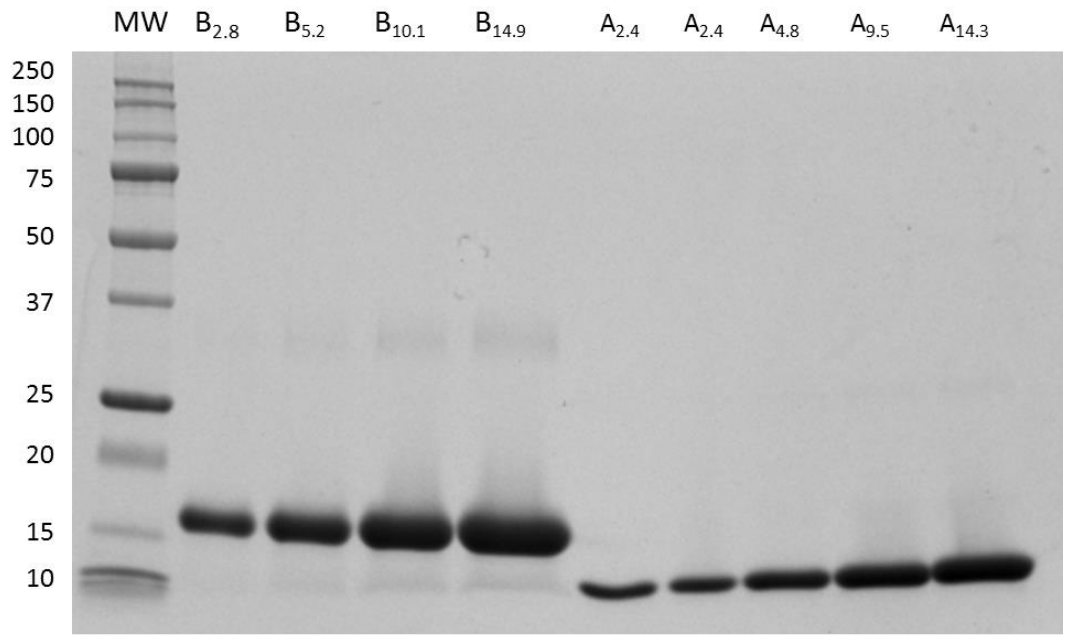
The purified milk proteins were analyzed by SDS-PAGE in order to determine their purity. The samples were initially analyzed with a Lowry protein assay to determine the amount of protein loaded in each well of the gel.



Supplementary Figure A1. SDS-PAGE Image of Purified Milk Proteins. The milk proteins were prepared under reducing conditions and the gel was run at 200V, fixed, stained and imaged. The lanes are as follows: MW: molecular weight marker (kDa); α: α-casein; κ: κ-casein. The amount of protein loaded on the gel (μg) is designated by the subscript.



Supplementary Figure A2. SDS-PAGE Image of Purified Milk Proteins. The milk proteins were prepared under reducing conditions and the gel was run at 200V, fixed, stained and imaged. The lanes are as follows: MW: molecular weight marker (kDa); β: β-casein. The amount of protein loaded on the gel (μg) is designated by the subscript.



Supplementary Figure A3. SDS-PAGE Image of Purified Milk Proteins. The milk proteins were prepared under reducing conditions and the gel was run at 200V, fixed, stained and imaged. The lanes are as follows: MW: molecular weight marker (kDa); B: β-lactoglobulin; A: α-lactoglobulin. The amount of protein loaded on the gel (μg) is designated by the subscript.

The molecular weight of the α-casein subunits are 23.6 (α_s1) and 25.2 kDa (α_s2) *(2)*. In Figure 1, it can been seen that α-casein has two distinct bands in this region, with the stronger band at a higher molecular weight. K-casein has a strong band around 25 kDa, which is close to its molecular weight of 19.0 kDa *(2)*. The SDS-PAGE gel of β-casein (Figure A2) has a faint band near its molecular weight of 24 kDa, but the more prominent bands are above and below 10 kDa. This may be γ-casein, a hydrolysis product of β-casein, or it may be an effect of the surface hydrophobicity of β-casein which may bind more SDS and thus travel a disproportionate distance *(1)*.

B-lactoglobulin and α-lactalbumin are shown in Figure A3. They have strong bands near their molecular weights, 18.3 and 14.2 kDa, respectively.

APPENDIX B: Lateral Flow Device Directions for Liquid Samples

A. Romer AgraStrip Total Milk, Casein and BLG Lateral Flow

The Romer AgraStrip lateral flow kit requires extraction of 0.2 ml of the sample in extraction buffer, standardization of the volume 3 ml (1:16 dilution), and then shaking by hand for 1 minute. A portion of the extracted sample (0.4 ml) is added to an incubation vial, shaken for 15 seconds and allowed to stand for 5 minutes. After this time, the lateral flow test strip is inserted into this solution and read after 5 minutes.

µg specific protein = (µg/ml level of protein solution x 1:16 dilution) x 0.4 ml sample in vial

stated LOD: 1 ppm= 0.025 µg

B. Neogen Reveal for Total Milk Allergen Lateral Flow

A 10 ml sample is extracted in 90 ml of extraction solution (1:10 dilution) and shaken in a 60°C water bath for 15 minutes. Then 0.1 ml of the extracted sample supernatant is transferred to a well in which the test strip is placed and read after five minutes.

µg specific protein = (µg/ml level of protein solution x 1:10 dilution) x 0.1 ml sample in well

stated LOD: 5 ppm= 0.05 µg

C. Neogen Reveal 3-D for Total Milk Allergen Lateral Flow

The Neogen Reveal 3-D lateral flow procedure was standardized: 1 ml of sample was added to 3.75 ml of extraction buffer (1:4.75 dilution) and shaken in the provided tube for one minute. Then, 0.1 ml of the extracted sample was directly added by pipet to the cavity in the head of the device. The device was read after 5 minutes of development.

µg specific protein = (µg/ml level of protein solution x 1:4.75 dilution) x 0.1 ml sample absorbed

stated LOD: 5-10 ppm= 0.1- 0.2 µg

D. bioavid Diagnostics Lateral Flow Milk

The bioavid Diagnostics lateral flow requires 0.2 ml of sample to be added to 0.2 ml of running buffer (1:2 dilution). The vial is tapped on a counter to mix the contents and allowed to stand for 5 minutes. The test strip is inserted into this solution and read after 3 minutes.

µg specific protein = µg/ml level of protein solution x 1:2 dilution

stated LOD: 1 ppm= 0.5 µg

E. Elution Technologies Milk Rapid Kit Lateral Flow

The Elution Technologies lateral flow kits required 0.1 ml of sample to be added to 0.9 ml of extraction/running buffer (1:10 dilution) followed by mixing well. Of this solution, 0.1 ml is directly added to the LFD cartridge and the results are read at 5 min and confirmed at 11 min.

µg specific protein = (µg/ml level of protein solution x 1:10 dilution) x 0.1 ml sample
stated LOD: 1-2 ppm= 0.01-0.02 µg

F. Morinaga Casein Lateral Flow IIR

The Morinaga lateral flow requires 1.0g of sample to be added to 19 ml of sample extraction solution (1:20 dilution). The diluted sample is heated in boiling water for 10 minutes, followed by centrifugation for 20 minutes. Then, 0.1 ml of the supernatant is diluted with 0.9 ml of diluting solution (1:10 dilution) to prepare the test solution. A 0.2 ml portion of the test solution is applied directly to the lateral flow test stick and the result is read after 15 minutes.

µg specific protein = (µg/ml level of protein solution x 1:200 dilution) x 0.2 ml sample
stated LOD: 5 ppm= 0.005 µg

G. 3M Clean-Trace Surface Protein (Allergen)

The Biotrace International heating block was allowed to reach 55°C before analysis began. A 0.150 ml sample was applied directly to the swab head of the 3M protein swab. The device was activated, shaken for a minimum of 5 seconds and then placed into the heating block for 15 minutes. The resulting color change of the solution and swab was interpreted in comparison to the 3M label and a negative control. A purple color was deemed positive, gray +/- and green negative.

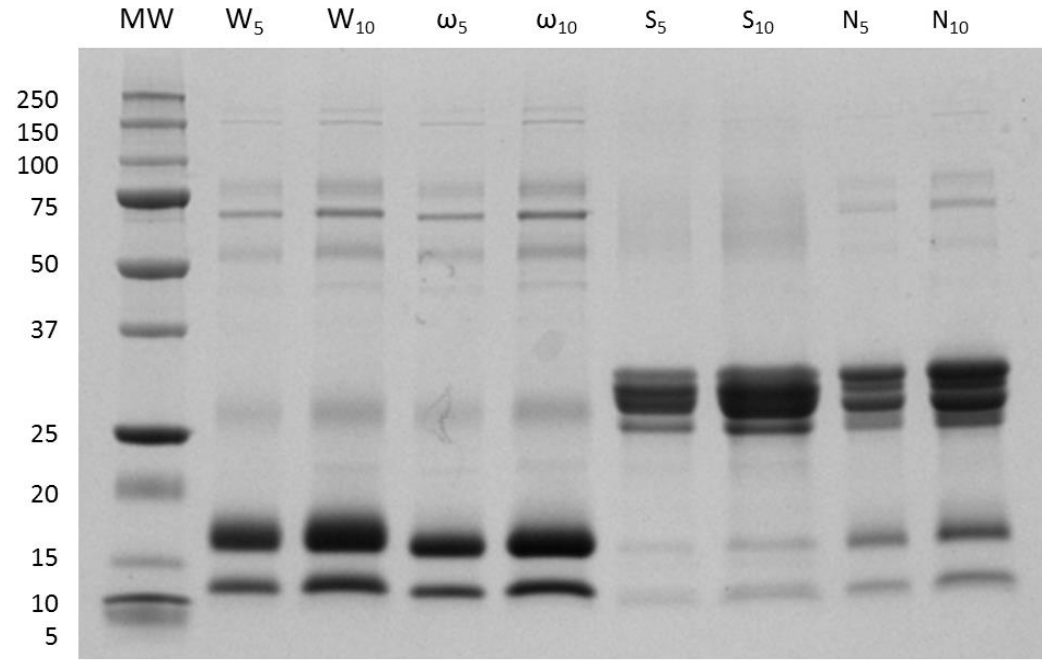
µg specific protein = (µg/ml level of protein solution x 0.150 ml)
stated LOD: 2.59-5.18 µg whole milk protein
25 ppm= 3.75 µg

APPENDIX C: Lateral Flow Device Analysis of Milk-Derived Ingredients

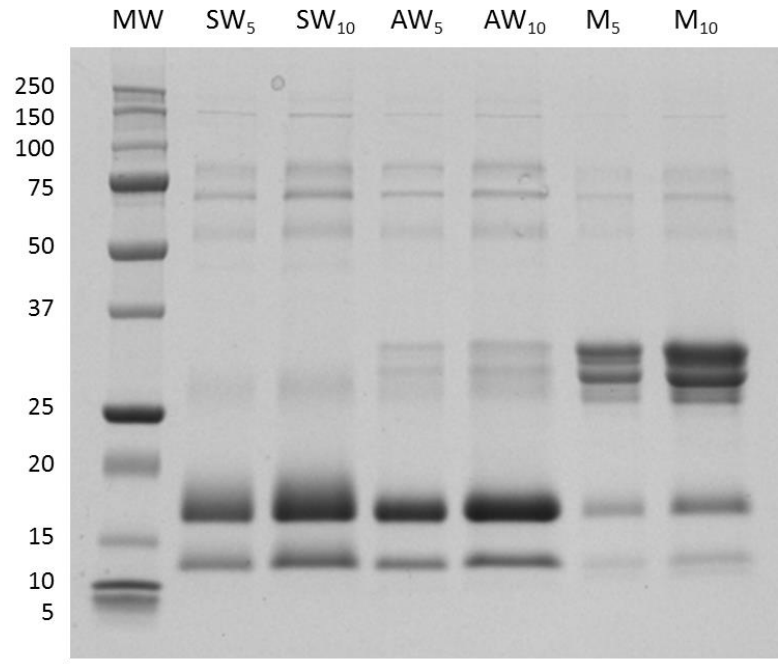
Each milk-derived ingredient was analyzed by LECO Dumas to determine the protein content (Table C1).

SUPPLEMENTARY TABLE C1. *LECO Dumas Protein Analysis of Milk-Derived Ingredients*

Ingredient	LECO % protein (g/100g)
NFDM	37.52
MPC80	79.24
NaCas	89.88
WPC34	32.55
WPC80	78.47
Acid Whey	11.31
Sweet Whey	12.28



Supplementary Figure C1. SDS-PAGE Image of Milk-Derived Ingredients. The milk-derived ingredients were prepared under reducing conditions and the gel was run at 200V, fixed, stained and imaged. The lanes are as follows: MW: molecular weight marker (kDa); W: WPC34; ω: WPC80; S: NaCas; N:NFDM. The amount of protein loaded on the gel (μg) is designated by the subscript.



Supplementary Figure C2. SDS-PAGE Image of Milk-Derived Ingredients. The milk-derived ingredients were prepared under reducing conditions and the gel was run at 200V, fixed, stained and imaged. The lanes are as follows: MW: molecular weight marker (kDa); SW: Sweet Whey; AW: Acid Whey; M: MPC80. The amount of protein loaded on the gel (μg) is designated by the subscript.

The SDS-PAGE images of the milk-derived ingredients are displayed in Supplementary Figures C1 and C2. The prominent bands of the WPC34, WPC80, SW and AW samples are seen around 17 kDa and 12 kDa. These bands correspond to the whey proteins BLG and ALA, respectively. The WPC samples and sweet whey also have shading above 25 kDa. This could be contributed to trace amounts of casein, most likely κ-casein. The acid whey sample is not as pure relatively and displays traces of α-, β- and κ-casein (Fig. C2).

The NFDM and MPC80 SDS-PAGE images (Figs C1 and C2) display bands characteristic of both casein and whey proteins. The casein banding above 25 kDa is more intense than the BLG and ALA bands (17 and 12 kDa), representing the higher abundance of caseins (80%) to whey proteins (20%) in milk.

The SDS-PAGE image of NaCas (Fig C1) shows the most intense bands for the casein proteins. Traces of BLG and ALA are also visible. In comparison to the lanes with NFDM and MPC, the whey protein content is reduced in the NaCas sample.

Bands are also observed in some of the samples at 150 kDa; this can be attributed to traces of immunoglobulins which make up about 3% of milk proteins. The bands in the 60-75 kDa range may be traces of BSA and lactoferrin which have molecular weights of 66.3 and 80 kDa, respectively *(2)*.

Complete Results of Lateral Flow Analysis of Milk-Derived Ingredients

Chapter 2 contains a discussion of kit sensitivity to milk-derived ingredients (Table 2) and milk-derived ingredient specificity at 75 ppm protein (Table 3). The supplementary tables presented below show the results of all tested concentrations with each lateral flow device. Further discussion is not provided with these supplementary tables because the 75 ppm protein level discussed in Chapter 2 is sufficiently representative.

Abbreviations:
Neg, negative.
Pos, positive.
f. pos, faint positive.
+/-, a very, very faint test line that may be interpreted differently by different users.
WPC34, whey protein concentrate, 34% protein.
WPC80, whey protein concentrate, 80% protein.
NaCas, sodium caseinate.
NFDM-LH, non-fat dry milk, low-heat processed.
MPC80, milk protein concentrate, 80% protein.
O, Overload line (of Neogen Reveal 3-D kit)
T, Test line (of Neogen Reveal 3-D kit)

SUPPLEMENTARY TABLE C2. *Romer AgraStrip Total Milk kit*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
WPC80	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
Sweet Whey	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
Acid Whey	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NaCas	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NFDM-LH	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
MPC80	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE C3. *Romer AgraStrip Casein kit*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (1/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)
WPC80	f. pos (2/3)	Pos (2/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
Sweet Whey	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
Acid Whey	Neg (0/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NaCas	Neg (1/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NFDM-LH	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
MPC80	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE C4. *Romer AgraStrip BLG kit*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (0/3)	f. pos (3/3)	f. pos (3/3)	f. pos (2/3)	Neg (0/3)	Neg (0/3)
WPC80	Neg (0/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (2/3)	Neg (0/3)
Sweet Whey	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	+/- (3/3)
Acid Whey	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	+/- (3/3)	+/- (3/3)
NaCas	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
NFDM-LH	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
MPC80	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE C5. *Neogen Reveal Total Milk kit*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
WPC80	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
Sweet Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (1/3)	Neg (1/3)
Acid Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (2/3)	+/- (3/3)	+/- (3/3)
NaCas	Neg (0/3)	+/- (2/3)	+/- (3/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)
NFDM-LH	Neg (0/3)	+/- (3/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
MPC80	Neg (0/3)	+/- (3/3)	f. pos (2/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE C6. *Neogen Reveal 3-D Total Milk*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3) O: +/-	Neg (0/3) O: +/-
WPC80	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3) O: +/-	Neg (0/3) O: +/-
Sweet Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3) O: +/-
Acid Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (3/3)	f. pos (3/3) O faint but >T	f. pos (3/3) O faint but >T
NaCas	Neg (0/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3) O≈T	Pos (3/3) O<T	Pos (3/3) O<T
NFDM-LH	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3) O≈T	Pos (3/3) O<T	Pos (3/3) O<<T
MPC80	Neg (0/3)	f. pos (3/3)	Pos (3/3) O >T	Pos (3/3) O≈T	Pos (3/3) O<<T	Pos (3/3) O<<T

SUPPLEMENTARY TABLE C7. *Bioavid diagnostics lateral flow milk*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)
WPC80	f. pos (2/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)
Sweet Whey	Neg (0/3)	Pos (3/3)	f. pos (3/3)	f. pos (2/3)	+/- (2/3)	Neg (1/3)
Acid Whey	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (2/3)	f. pos (3/3)
NaCas	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NFDM-LH	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	+/- (3/3)
MPC80	+/- (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	+/- (3/3)

SUPPLEMENTARY TABLE C8. *Elution Technologies Total Milk*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
WPC80	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
Sweet Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (1/3)	Neg (0/3)	Neg (0/3)
Acid Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (2/3)	+/- (2/3)	+/- (3/3)
NaCas	Neg (0/3)	Neg (0/3)	+/- (3/3)	+/- (2/3)	+/- (2/3)	f. pos (3/3)
NFDM-LH	Neg (0/3)	+/- (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)
MPC80	Neg (0/3)	+/- (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)

SUPPLEMENTARY TABLE C9. *Morinaga Casein Lateral Flow IIR*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein	5 ppm	10 ppm	25 ppm	75 ppm	100 ppm
WPC34	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (2/3)	+/- (3/3)
WPC80	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (1/3)	+/- (3/3)
Sweet Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
Acid Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (3/3)	Pos (3/3)	Pos (3/3)
NaCas	Neg (0/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NFDM-LH	Neg (0/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
MPC80	Neg (0/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE C10. *3M Clean-Trace Surface Protein (Allergen)*

	Protein content of sample, result, (no. positive/no. tested)					
Sample	1 ppm protein (0.15 µg)	5 ppm (0.75 µg)	10 ppm (1.50 µg)	25 ppm (3.75 µg)	75 ppm (11.25 µg)	100 ppm (15 µg)
WPC34	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (2/3)	Pos (3/3)	Pos (3/3)
WPC80	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (2/3)	Pos (3/3)	Pos (3/3)
Sweet Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (3/3)	Pos (3/3)	Pos (3/3)
Acid Whey	Neg (0/3)	Neg (0/3)	Neg (0/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
NaCas	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Pos (3/3)	Pos (3/3)
NFDM-LH	Neg (0/3)	Neg (0/3)	Neg (1/3)	Neg (1/3)	Pos (3/3)	Pos (3/3)
MPC80	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (1/3)	Pos (3/3)	Pos (3/3)

*Pos: purple; +/-: gray (counted as positive); Neg: green.

APPENDIX D: Overload Levels of Lateral Flow Devices Tested with NFDM

SUPPLEMENTARY TABLE D1. *Overload level of LFDs with NFDM, complete data*

	Level of NFDM protein tested, Result, (no. positive/no. tested)							
LFD Kit	100 ppm	250 ppm	500 ppm	750 ppm	1,000 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Romer AgraStrip Total Milk	Pos (3/3)	X	Pos (3/3)	X	f. pos (3/3)	Neg (1/3)	Neg (0/3)	Neg (0/3)
Romer AgraStrip Casein	Pos (3/3)	X	Pos (3/3)	X	Pos (3/3)	X	f. Pos (3/3)	+/- (3/3)
Romer AgraStrip BLG	f. pos (3/3)	X	+/- (3/3)	X	+/- (3/3)	Neg (0/3)	X	X
Neogen Reveal TM	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	f. pos (3/3)	X	f. pos (3/3)	f. pos (3/3)
Neogen Reveal 3-D TM	Pos (3/3) O<T	Pos (3/3) O<T	Pos (3/3) O<<T	Pos (3/3) O<<T	Pos (3/3) O<<<T	X	Over T +/-	Over T +/-
bioavid Diagnostics Milk	+/- (2/3)	Neg (1/3)	Neg (0/3)	X	X	X	X	X
Elution Technologies Milk	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	+/- (GC) (3/3)	+/- (GC) (3/3)	GC (3/3)	GC (3/3)	X
Morinaga Casein	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	X	Pos (3/3)	Pos (3/3)
3M Clean-Trace (Allergen)	Pos (3/3)	X	Pos (3/3)	X	Pos (3/3)	X	Pos (3/3)	Pos (3/3)

Note: 'Over', Overloaded; the overload line was absent, and the T (test) line was very faint.

GC, Grossly Contaminated (test and overload lines absent or very, very faint).

X, sample concentration not analyzed

O, Overload line

T, Test line

Discussion:

It is important to understand the usable range of detection of lateral flow devices as very high levels of allergen can overload the LFD and lead to a false negative result. This hook effect is observed in some kits where the test line decreases in intensity and eventually does not form as the sample concentration increases. It is important for users to take action when a positive result is received regardless of the intensity of the line. Two kits (Neogen Reveal 3-D and Elution Technologies) have additional overload lines in their test zones that do not develop when the sample has high levels of allergens.

The Neogen Reveal 3-D and Elution Technologies kits, which both have overload lines, behaved similarly. The overload line of the Reveal 3-D kit was less intense than the test line at 100 ppm NFDM protein, but it became much fainter at 1,000 ppm. At 5,000 ppm the overload line did not develop and the LFD was overloaded. These results were mirrored in the Elution Technologies kit in which the LFD overload line was less intense than the test line at 750 and 1,000 ppm and the LFD was overloaded at 2,500 ppm (absence of test and overload lines). At 100 ppm both kits had overload lines that were less intense than the test lines. This would erroneously lead the user to believe that the sample was >1000 ppm NFDM.

The LFDs without an overload line that detected milk were quite variable. The bioavid Diagnostics kit was overloaded at 250 ppm NFDM protein. A range of dilutions of the sample extract would be necessary to ensure that a sample potentially fell into bioavid's range of detection. The Romer AgraStrip Total Milk kit had a very intense positive result at 100 ppm, but the test line intensity decreased significantly at 500 ppm and at 2,500 ppm the test result was negative. The Neogen Reveal Total Milk kit was able to detect NFDM protein up to 10,000 ppm, but after 1,000 ppm the test line was very faint and lingering at this intensity through 10,000 ppm.

The casein specific kits both detected NFDM in the entire range analyzed. The positive test lines of the Morinaga Casein kit were all similarly intense over the concentration range tested. The Romer AgraStrip Casein kit detected the highest level (10,000 ppm) with a very, very faint intensity (pos/neg) and the intensity of the test lines generally decreased as the protein concentration of the test sample increased. The intensity of the AgraStrip lines did increase and the results were easier to interpret within four minutes of removal of the strip from the test vial.

The Romer AgraStrip BLG kit states that it can detect 1-100 ppm BLG; this would correspond to an upper limit of 1000 ppm milk protein. It was found that the kit only positively detected at 500 and 1000 ppm with a very, very faint result.

The 3M Clean-Trace (Allergen) kit would not be susceptible to a hook effect. It was able to detect NFDM through the highest level tested, 10,000 ppm NFDM protein. A color change of the solution to purple denotes a positive result and the intensity of the purple color increased as the protein concentration of the sample increased.

APPENDIX E: Evaluation of Swabbed Samples with Lateral Flow Devices

SUPPLEMENTARY TABLE E1. *Preliminary swab moistening conditions study*

	3M Clean Trace Surface Protein (Allergen)			**Neogen Reveal 3-D Total Milk (3D swab)**			**Neogen Reveal Total Milk (NAES)[a]**		
	(no. positive/ no. tested)			**(no. positive/no. tested)**			**(no. positive/no. tested)**		
µg protein NFDM	Wet -->Dry Swab	Wet--> Wet Swab	Dry --> Wet Swab	Wet-->Dry Swab	Wet--> Wet Swab	Dry --> Wet Swab	Wet-->Dry Swab	Wet--> Wet Swab	Dry --> Wet Swab
PBS	Neg (0/1)			Neg (0/3)	Neg (0/3)	Neg (0/2)	Neg (0/2)	Neg (0/2)	Neg (0/2)
1 µg	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
3 µg	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
10 µg	Neg (1/3)	f. pos (3/3)	Pos (3/3)	f. pos[c] (3/3)	f. pos (3/3)	f. pos (3/3)	+/- [d](3/3)	Neg (0/3)	+/- (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	+/- (3/3)	f. pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3) (O<T)	Pos (3/3) (O<T)	Pos (3/3) (O<T)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)

	Neogen Veratox Total Milk (NAES)										
			Wet Soil → Dry Swab			Wet Soil → Wet Swab			Dry Soil → Wet Swab		
µg protein NFDM	**ppm NFDM[b]**	% CV	ppm NFDM	%CV	% Recovery	ppm NFDM	%CV	% Recovery	ppm NFDM	%CV	% Recovery
PBS			BLQ								
1 µg	**2.9**	7%	BLQ			BLQ			BLQ		
3 µg	**20.7**	13%	3.18	12%	15%	BLQ			4.05	12%	20%
10 µg	**90.5**	13%	19.4	16%	21%	5.9	20%	7%	23.8	4%	26%
30 µg	**300**	7%	181	17%	60%	59.2	11%	20%	92.6	16%	31%
100 µg	**995**	8%	642.5	9%	65%	300.5	16%	30%	370.8	10%	37%

[a]NAES, Neogen Allergen Environmental Swab

[b]The ppm NFDM of the Veratox samples was determined by directly pipetting 0.1 ml of the sample onto the NAES swab head. The % recovery was calculated from the sample ppm NFDM as determined by the Neogen Veratox Total Milk kit.

[c]f. pos, faint positive test result

[d]+/-, a very, very faint test line that may be interpreted differently by different users.

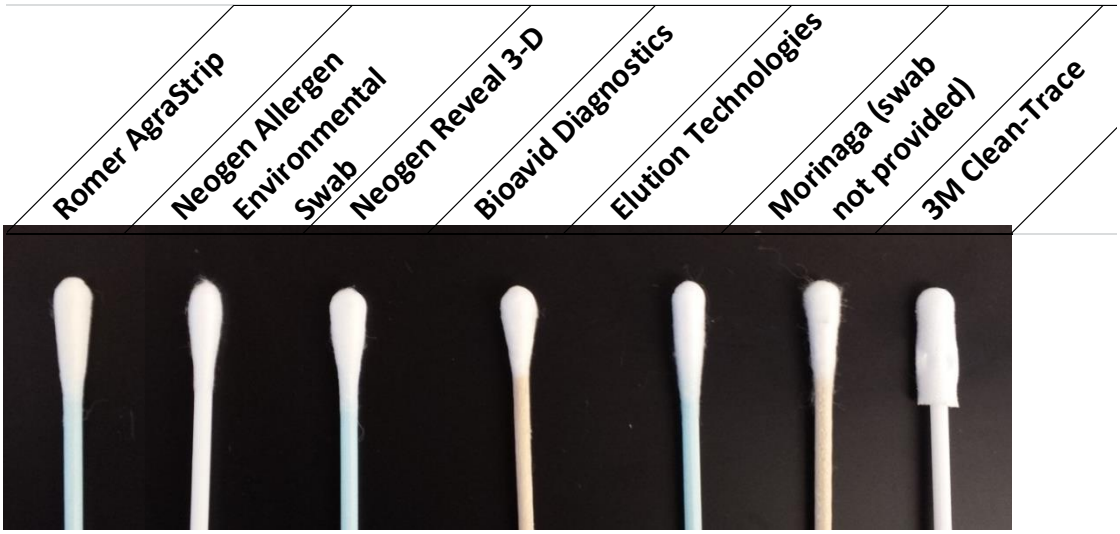
SUPPLEMENTARY TABLE E2. *Summary of preliminary swab moistening study*

	LFD or ELISA kit		
Soil and swab condition	Neogen Reveal 3D Total Milk (initial protein level detected)	Neogen Reveal Total Milk (initial protein level detected)	Neogen Veratox Total Milk (ave. % recovery)
Wet soil → dry swab	10 µg	30 µg	40 %
Wet soil → wet swab	10 µg	100 µg	19 %
Dry soil → wet swab	10 µg	30 µg	29 %

Each LFD kit provides different recommendations about swab sampling and under which sampling conditions to moisten swabs. For example, some kits recommend moistening swabs and do not differentiate between wet and dry samples. Theoretically, moistening a swab to sample a wet soil would further dilute the sample and lower recovery. In order to analyze this situation, we compared three combinations of soil and swab conditions as outlined above (Table E2). Complete results are available in Table E1.

No differences in detection were encountered that could be attributed to pre-moistening of the swab within the sensitivity range of the Neogen Reveal 3-D kit. The Neogen Reveal kit was able to detect the wet soil-dry swab and dry soil-wet swab with no differences, but the wet soil-wet swab was only detected at higher levels. The average recovery using the Veratox kit was 40% wet soil-dry swab, 29% dry soil-wet swab and 19% wet soil-wet swab.

The LFD kits, Neogen excluded, recommend moistening the swab head in every situation, but adjusting the protocol to use an unmoistened swab when testing wet surfaces has the potential to increase recovery of the soils. It is also important to develop a consistent swabbing approach to ensure that each soil or surface is tested similarly.



Supplementary Figure E1. Kit provided swabs.

SUPPLEMENTARY TABLE E3. *Kit Provided Swab Materials and Specifications*

Kit	**Stated LOD-swabs**	**Moistener**	**Swab**
Romer AgraStrip Total Milk	1 µg/25 cm^2 milk protein	Extraction buffer (provided)	provided
Neogen Reveal Total Milk	5 ppm	Extraction solution (provided) for dry samples	Neogen Allergen Environmental Swab -polyester
Neogen Reveal 3-D Total Milk	20 µg milk/100 cm^2	Extraction Buffer (provided) for dry samples	Sterile Rayon swab (provided)
Bioavid Diagnostics Milk	1-10 µg material with allergenic potential	PBS-Tween (provided)	Cotton swab (provided)
Elution Technologies Bovine Total Milk	1 ppm	Extraction buffer (provided)	Pur-Wraps® Polyester tipped sterile swab
Morinaga Casein	250 µg/ml (in swabbing solution)	water	Not provided Use Puritan 867-WC (cotton swab)
Romer AgraStrip Casein	1 µg/25 cm^2 casein	Extraction buffer (provided)	provided
Romer AgraStrip	0.5 µg/25 cm^2	Extraction buffer	provided

β-lactoglobulin	BLG	(provided)	
3M Clean-Trace Surface Protein (Allergen)	3 µg total protein or 10-20 µg whole milk powder	Provided (to swab or surface to be tested for dry samples)	provided

Complete Swabbing Results

A) The NFDM soil was gently dried at 37°C for approximately 13 minutes and the dry soil was moistened with 0.1 ml of ROD water before sampling the soiled area with a dry swab provided by the kit.

B) The NFDM soil was gently dried at 37°C for approximately 13 minutes and the swab head was directly moistened with 0.1 ml of moistener or water as directed by the kit insert before sampling the dry soil.

C) The NFDM soil was dried at high temperature in a 350°F oven for 10 minutes; after cooling, 0.1 ml of ROD water was applied to the surface before sampling with a dry swab.

D) The NFDM soil was dried at high temperature in a 350°F oven for 10 minutes; after cooling, 0.1 ml of ROD water or kit-provided moistener was applied to the swab head and the dry soil was sampled.

E) The NFDM soil was sampled immediately (still moist) with a dry swab. All swabbing was conducted using a crosshatch technique.

NAES= Neogen Allergen Environmental Swab

SUPPLEMENTARY TABLE E4. *Romer AgraStrip Total Milk tested with Romer swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	+/- (3/3) delay	+/- (3/3)	Neg (1/3)	Neg (0/3)	f. pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE E5. *Romer AgraStrip Casein tested with Romer swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	f. pos (3/3)	Delayed f. pos (3/3)	Delayed f. pos (3/3)	Pos (2/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE E6. *Romer AgraStrip BLG tested with Romer swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	+/- (2/3)	Neg (0/3)	Neg (1/3)	Neg (0/3)	f. pos (3/3)
10 µg	f. pos (3/3)	f. pos (3/3)	+/- (3/3)	+/- (3/3)	f. pos (3/3)
30 µg	f. pos (3/3)	f. pos (3/3)	+/- (3/3)	+/- (3/3)	f. pos (3/3)
100 µg	+/- (3/3)	+/- (3/3)	Neg (1/3); delayed +/-	Neg (1/3); delayed +/-	f. pos (3/3)

SUPPLEMENTARY TABLE E7. *3M Clean-Trace Surface Protein (Allergen)*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (1/3)	Neg (0/3)
10 µg	+/- (2/3) *	f. pos (3/3)	f. pos (2/3)	+/- (3/3)	f. pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE E8. *Bioavid Lateral Flow Milk with bioavid cotton swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	f. pos (3/3)	Pos (3/3)	f. pos (3/3)	Pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	f. pos (3/3)	Pos (3/3) O	Pos (3/3)

SUPPLEMENTARY TABLE E9. *Neogen Reveal Total Milk with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
10 µg	Neg (0/3)	+/- (2/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
30 µg	Pos (3/3)	Pos (3/3)	+/- (3/3)	+/- (3/3)	+/- (3/3)
100 µg	Pos (3/3)	Pos (3/3)	f. pos (3/3)	+/- (3/3)	f. pos (3/3)

SUPPLEMENTARY TABLE E10. *Neogen Reveal 3-D Total Milk with provided swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	Neg (1/3)	Neg (0/3)	Neg (1/3)	Neg (0/3)	+/- (3/3)
10 µg	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	+/- (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	Pos (3/3)
100 µg	Pos, O<T (3/3)	Pos, O<T (3/3)	Pos (3/3) O<T	Pos (3/3)	Pos, O<T (3/3)

SUPPLEMENTARY TABLE E11. *Morinaga Casein Lateral Flow with cotton swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE E12. *Elution Technologies Bovine Total Milk with provided swab*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	+/- (3/3)	+/- (3/3)	+/- (3/3)	f. pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	f. pos (3/3)	f. pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)[a]	Pos (3/3)[a]

[a] overloading beginning to develop

SUPPLEMENTARY TABLE E13. *Romer AgraStrip Total Milk with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	+/- (3/3)	Neg (1/3)	Neg (0/3)	Neg (0/3)	+/- (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	+/- (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)-lighter	Pos (3/3)	Pos (3/3)	Pos (3/3)	+/- (3/3)

SUPPLEMENTARY TABLE E14. *Romer AgraStrip Casein with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	f. pos (3/3)	Delayed f. pos (3/3)	Delayed f. pos (3/3)	f. pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE E15. *Romer AgraStrip BLG with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	Neg (0/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)	+/- (3/3)
10 µg	f. pos (3/3)	f. pos (3/3)	Neg (1/3) Delayed +/-	Neg (1/3) Delayed +/-	+/- (3/3)
30 µg	f. pos (3/3)	f. pos (3/3)	Neg (1/3) Delayed +/-	Neg (1/3) Delayed +/-	+/- (3/3)
100 µg	f. pos (3/3)	+/- (3/3)	Neg (0/3) Delayed +/-	Neg (0/3) Delayed +/-	+/- (3/3)

SUPPLEMENTARY TABLE E16. *Bioavid diagnostics milk lateral flow with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	+/- (3/3)	f. pos (3/3)	f. pos (3/3)	Pos (3/3)	f. pos (3/3)*

SUPPLEMENTARY TABLE E17. *Neogen Reveal 3-D Total Milk with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	Neg (1/3)	+/- (2/3)	Neg (0/3)	Neg (0/3)	Neg (0/3)
10 µg	f. pos (3/3)	f. pos (3/3)	+/- (3/3)	+/- (3/3)	f. pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	f. pos (3/3)	Pos, O<T (3/3)
100 µg	Pos, O<T (3/3)	O, O<T (0/3)	O, O<T (1/3)	O<T (1/3)	O, O<T (0/3)

SUPPLEMENTARY TABLE E18. *Morinaga Casein Lateral Flow with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	f. pos (3/3)	f. pos (3/3)	f. pos (3/3)	+/- (3/3)	f. pos (3/3)
10 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
30 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

SUPPLEMENTARY TABLE E19. *Elution Technologies Milk with NAES*

NFDM protein	A RT → HOH surface	B RT → moist swab	C Oven → HOH surface	D Oven → moist swab	E Wet→ dry swab
3 µg	+/- (3/3)	+/- (3/3)	Neg (1/3)	Neg (1/3)	f. pos (3/3)
10 µg	Pos (3/3)	f. pos (3/3)	Pos (3/3)	+/- (3/3)	Pos (3/3)
30 µg	Pos (3/3)	f. pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)
100 µg	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)	Pos (3/3)

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CHAPTER THREE

EFFECTS OF CLEANING ON REMOVAL OF MILK SOILS FROM VARIOUS FOOD PROCESSING SURFACES AS DETECTED BY COMMERCIAL MILK-SPECIFIC LATERAL FLOW DEVICES AND GENERAL PROTEIN TESTS

I. Abstract

The contributions of cleaning detergents to allergen removal (non-fat dry milk) from several food processing surfaces and the applicability of lateral flow devices (LFDs) as a detection method for cleaning validation were investigated in this study. Each cleaning solution was tested for interferences with the detection methods; the caustic solutions gave false negative results with LFDs, while the sanitizer caused false positive results with a general protein kit. Each detergent was then used separately to clean milk-soiled surfaces. The caustic solutions easily removed the milk soil while the acid and sanitizing solutions left a soiled surface. The full CIP process was tested sequentially and found to remove the milk soil. Greater soil suspendibility was observed when cleaning with a commercial caustic solution as compared to a commodity caustic solution. The plastic surfaces developed various amounts of surface roughening throughout the experiment which could harbor milk protein soils while the stainless steel surface was consistently cleaned. This study provides important points to consider when designing and executing cleaning validation studies in food processing facilities.

II. Introduction

Food allergy affects approximately 5% of the population and the prevalence is reportedly increasing *(26)*. A food-allergic individual must avoid foods they are allergic to in order to prevent allergic reactions. This can be a challenge as many packaged foods are produced on shared equipment and cross-contact could occur on occasion *(28)*. A survey conducted during FDA food processing facility inspections found that 77% of facilities do share processing equipment and that of those, 91% clean the equipment between allergen and non-allergen containing products and that 71% had a written allergen cleaning procedure *(11)*. The implementation of the Food Safety Modernization Act now requires manufacturers to develop and implement effective allergen control plans to mitigate the risk associated with allergen cross-contact and mispackaging *(8)*.

Despite these control measures, shared equipment practices could lead to carryover of food allergen residue to other ingredients or finished products after changeover. A large study of packaged foods consumed by allergic individuals found measurable peanut traces in 1% of the products *(30)*. Milk protein was found in 67% of non-pre-packaged pastry products not stated to contain milk *(29)*. Hidden allergen residue that is present in packaged foods that do not declare the allergen in the ingredient statement or through precautionary allergen labels are especially troublesome as allergic consumers do not know if the product is safe to consume. Allergic consumers are also becoming increasingly aware that food products bearing precautionary allergen labels have a low probability of containing detectable allergen residue based on published reports of retail products that have been analyzed *(2)*. While a small proportion of these

products contained allergen residue, consumers cannot rely on precautionary allergen labeling for guidance either, as products with and without precautionary allergen labeling contained similar levels of trace allergens *(5)*.

Allergen cleaning programs can be implemented to decrease the risk of cross-contact. Several studies have investigated cleaning of peanut butter, milk soils, and hazelnut cookie dough from food processing surfaces *(16, 17, 23, 24)*. The efficacy of cleaning was dependent on the soil, surface, detergent, and cleaning temperature; therefore, it is important for manufacturers to understand the cleaning process and validate their specific cleaning programs. It is generally acknowledged that proteins, especially when heated, are one of the hardest soils to remove *(4, 6, 9, 10, 12-15, 18, 25)*. A general washing protocol is as follows: caustic wash, rinse, acid wash, rinse, sanitizer, drip dry *(7)*. The caustic and acid washes are generally used at a 1% concentration at 60-80°C for 15-30 minutes, while sanitizers are used at 0.13-0.26% concentrations at 20-60°C for 0.5-10 minutes *(7)*. Caustic washes aid in protein dissolution while acidic washes aid in the dissolution of mineral deposits *(14, 25)*. The quality of a detergent can greatly impact cleaning efficiency and many commercial detergent solutions contain functional additives such as surfactants, water conditioners, and catalyzing agents that improve the cleaning effectiveness.

The use of lateral flow devices (LFDs) to test rinse water and equipment surfaces as a means of allergen cleaning validation has gained increased use in recent years. LFDs provide a quick, allergen-specific, and cost effective detection method. The method must be verified to detect the ingredient of interest in the product matrix with the cleaning

program, as these factors may affect the reliability and sensitivity of detection of the allergenic residue of interest *(20, 22)*. While many food companies are relying upon the accuracy of these LFDs for allergen detection and validating cleaning, officially recognized validation protocols of qualitative methods have not been released and little has been published *(19)*. As a result, manufacturers of LFDs often conduct internal cross-reactivity and validation studies on a number of matrices to determine the relative sensitivity of the LFDs and it is recommended that food manufacturers conduct a positive control analysis of the residue they are attempting to detect to ensure that the LFD is fit for their intended purpose of cleaning validation.

The objectives of this study are to evaluate the contribution of each CIP step on the removal of milk soils from various food processing surfaces, determine the efficacy of milk-specific LFDs as a detection method for cleaning validation, and evaluate any potential interferences of cleaning chemicals with LFD detection of milk.

III. Materials and Methods:

a) Cleaning chemical interferences. Low heat processed nonfat dry milk (NFDM) (Darigold, Seattle, WA) was used to prepare a NFDM solution in 0.025 M phosphate buffered saline (PBS; pH 7.4, 0.85% saline). Cleaning solutions were prepared in purified water processed through a reverse osmosis and deionizer system (ROD water): commodity caustic (NaOH, Fisher Chemical, Fair Lawn, NJ), commercial caustic (Exelerate CIP, Ecolab, MN), commercial acid (Envirocid Plus, Ecolab, MN), and a commercial sanitizer (Vortexx, Ecolab, MN). The solutions were combined in such a way

to prepare a 200 ppm NFDM protein (wt/vol) solution in a caustic or acid cleaning solution at 0.03, 0.3 and 1% (vol/vol) cleaner levels. The sanitizer solution was prepared as a 200 ppm NFDM protein (wt/vol) solution in a 0.003, 0.03 and 0.3% (vol/vol) sanitizer solution. Each cleaner was tested without NFDM as a negative control and the 200 ppm NFDM protein (wt/vol) solution was tested as a positive control. Each test was completed in triplicate.

b) Surfaces for CIP. Four food-processing surfaces were provided by Ecolab (St. Paul, MN); 316 grade stainless steel, high density polyethylene (HDPE), Nylon 6/6, and Delrin that were cut to approximately 7.7 cm x 12.7 cm, referred to herein as 'panels'. The panels were renewed between each experiment by scrubbing with dish soap, boiling (or highest temperature tolerable) in 1% NaOH for 30 min, scrubbing with dish soap, and boiling (or highest temperature tolerable) in 1% nitric acid for 30 min followed by a thorough ROD water rinse.

c) Cleaning chemicals and milk solution for CIP. Four cleaning solutions were used; two caustic solutions: 1) a commercial caustic Exelerate CIP (Ecolab, MN) and 2) a commodity caustic prepared from sodium hydroxide (Fisher Chemical, Fair Lawn, NJ), 3) an acid cleaner: Envirocid Plus (Ecolab, MN), and 4) an oxidizing sanitizer: Vortexx (Ecolab, MN). When the contribution of each CIP solution was being analyzed, the caustic and acid solutions were prepared in ROD water at 1, 0.3 and 0.03% (vol/vol). The sanitizer was prepared at 0.3, 0.03 and 0.003% (vol/vol) levels. When the complete CIP process was being analyzed, the mid-level concentration for each cleaner was selected, 0.3% for caustic and acid solutions and 0.03% for the sanitizer, as these are

approximately the manufacturer's recommended cleaning and sanitizer concentrations. A low-heat processed non-fat dry milk solution was prepared in 0.025 M PBS (pH 7.4, 0.85% NaCl) at a concentration similar to liquid milk (1/3 cup NFDM and 1 cup PBS). The solution was stirred and then extracted in a 60°C shaking water bath for 30 minutes.

d) Simulated CIP cleaning process. The NFDM solution was applied to the lower three-quarters of the panel (food-processing surface material) with a 2 inch foam brush (Loew-Cornell A1308-1043) and dried with a heat gun (Wagner, Plymouth, MN). The milk application and drying was repeated until 0.3-0.35g of milk soil was applied to the surface. 900 ml of cleaning solution was heated in a 1L beaker on a stirring hot plate (Corning PC-420D, Corning, NY). Two panels were suspended in the solution by binder clips, supported by a dowel resting on the top of the 1L beaker (Appendix A). The caustic and acid solutions were brought to 60°C and cleaning took place for 15 min at 90 rpm to simulate time and turbulent flow of a CIP system. The panels were removed from the cleaning solution and set vertically for 2 minutes to drip dry before rinsing by dipping into a 1 L beaker filled with cold distilled tap water, then dried with compressed air. The sanitizer solution was brought to 25°C and cleaning took place for 2 min at 90 rpm to also simulate CIP processing. The panels were removed to drip dry and were not rinsed. Initially, each cleaning solution was tested separately to determine their soil removal efficacy (ex) panel soil-Envirocid Plus-rinse-qualitative residue analysis or panel soil-Vortexx-drip dry-qualitative residue analysis). Subsequently, the complete CIP analysis tested the cleaners sequentially using each caustic solution as diagramed below.

Panel soil → Exelerate CIP → rinse → Envirocid Plus → rinse → Vortexx → drip dry →qualitative residue analysis

or

Panel soil → NaOH → rinse → Envirocid Plus → rinse → Vortexx → drip dry → qualitative residue analysis.

For each concentration and cleaning solution, four panels were prepared and cleaned in two beakers.

e) LFD and general protein kits. Two commercially available LFD kits and one total protein kit were used for analysis. Romer AgraStrip Casein (Romer Labs, Runcorn, Cheshire, UK), Neogen Reveal 3-D Total Milk (Neogen Corporation, Lansing, MI, USA), and 3M Clean-Trace Surface Protein Allergen (3M Health Care, St. Paul, MN, USA) were evaluated.

All the samples were evaluated by applying the sample to the swab head (interferences study) or swabbing the surface directly (CIP studies). The test kit instructions were followed with the following modifications. The contents of the Neogen Reveal 3-D extraction buffer packets were pooled and 3.75 ml of extraction buffer was used in each sample tube. The swab heads were moistened with 0.1 ml of provided extraction solution or moisturizer prior to swabbing the surfaces.

f) Scanning method. The soil level on the panels after cleaning was also analyzed through a staining and computerized scanning method. After cleaning, Coomassie

Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories, Hercules, CA) was applied to the panel surface for 30 seconds, rinsed with RO water and allowed to dry. The stained panels were scanned using a HP Officejet Pro 8600 flatbed scanner (600 dpi, brightness adjusted to 100 for stainless steel only). The scanned images were analyzed with Image J (http://rsb.info.nih.gov/ij/download.html). The same size area was selected and analyzed for each coupon surface. The mean value analysis used the histogram function to determine the mean gray value and standard deviation. The data were analyzed for significant differences at the 5% level ($p<0.05$) with Fisher's LSD using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). The % clean analysis compares the pixels at each gray value of a washed panel to a control clean panel. The principle being that any pixel not within the control clean spectrum is dirty. Calculations were made to determine what percentage of the washed panel is clean.

IV. Results and Discussion

Cleaning chemical interferences. It was found that caustic cleaning solutions can induce concentration dependent interferences with immunochemical-based LFDs, with increasing concentrations of cleaning solutions resulting in faint or no qualitative detection of the solution containing 200 ppm NFDM protein (Table 1). The commodity caustic NaOH solution decreased the intensity of positive test lines. If low amounts of cross-contact samples or surfaces were tested, the result would likely be interpreted incorrectly as a false-negative response. The Exelerate CIP cleaning solution produced false negative results at the 1 and 0.3% cleaner level for Reveal 3-D and at the 1% cleaner level for Romer AgraStrip Casein. The false negative results derived from testing

with the Exelerate CIP solution could be a result of oxidation by sodium hypochlorite. The 3M Clean-Trace Surface Protein (Allergen) kit was not affected by the caustic cleaning solutions at any of the concentrations that were evaluated. When selecting equipment swabs and rinse water samples, the sampling should be completed after thorough rinsing to remove any cleaning chemical residues or after checking and potentially adjusting the pH of the sample. If detection of allergenic residues in caustic solutions is necessary, general protein methods may be a suitable surrogate method.

The acidic cleaning solution did not interfere with LFD or general protein detection methods of NFDM (Table 1).

The oxidizing sanitizer solutions gave false positive results at 0.3% and 0.03% when tested with the 3M general protein swab. The chemistry of the general protein swab is based on the biuret reaction in which color development in proportion to the protein content is based on a complex containing bicinchoninic acid (BCA). It is known that BCA is not compatible with hydrogen peroxide, one of the ingredients of Ecolab's Vortexx sanitizer *(3)*. General protein testing of equipment surfaces should not be attempted after sanitization with oxidizing sanitizers, as false positive results may occur. LFD immunochemical-based methods can be used, but the Reveal 3-D Total Milk test may exhibit depression of the test line intensity rendering it unable to detect lower levels of milk protein residue.

Contribution of each CIP step to allergenic soil removal from common food contact surfaces. Cleaners: In order to determine which portions of a CIP process contribute to allergen removal from the representative food contact surfaces utilized in the food industry (316 grade stainless steel, Nylon 6/6, HDPE and Delrin), each cleaning solution was tested individually. The commercial caustic solution from Ecolab was generally found to produce visually clean surfaces after cleaning. The commodity caustic solution (NaOH) produced surfaces that were mostly clean with a few visible pieces of soil stuck to the surface. These observations were consistent with the knowledge that proteins are best removed by alkaline cleaning solutions. The protein reacts, swells, and dissolves upon contact with hydroxyl anions *(4, 12, 13, 25)*. It was observed that while cleaning in the NaOH solution, the soil would thin and peel off as large pieces of film or break into smaller pieces at higher concentrations of NaOH. It is possible that a protein film was created (new disulfide bonds are formed from exposed sulfhydryl groups due to heat and alkaline conditions). The commercial caustic solution was generally observed to dissolve and disperse the soils in the cleaning solution much more readily than the commodity solution. This is most likely an effect of the formulation of the commercial caustic solution which contains water conditioners, which lower surface tension and help suspend the soil in solution *(4, 21)*.

After cleaning with the acid detergent, Envirocid Plus, the NFDM soil was essentially intact on the surface of the panel. At the lowest acid cleaner concentration, some soil came off which looked stringy in solution and the soil was generally softer than the more brittle soils produced after cleaning with higher acid concentrations. These

results are consistent with known effects of cleaning solely with acid; when used first, it will fix the protein soil to the surface or precipitate the protein and thus make it harder to clean *(4, 25)*. It would be of interest to determine if milk in an acidified product would result in increased protein buildup.

Vortexx is an oxidizing sanitizer and it is not expected to contribute to allergen removal. After cleaning with the sanitizer, a highly soiled surface was produced.

Detection: General protein and allergen-specific LFDs were used for milk residue detection on common food contact surfaces found in the food industry. The general protein method had a lower sensitivity and was unable to detect low soil amounts that the LFDs were able to detect as shown in the caustic wash results (Tables 2, 3, 4, 5). For cleaning validation purposes, the use of milk-specific LFDs should be considered by food companies. The use of a general protein detection method may be suitable for routine analysis during the cleaning process but the food company should determine if the general protein test provides suitable sensitivity to detect the residue to meet any established corporate cleaning target limits.

Detection of NFDM protein residue by swab and qualitative analysis was dependent on the characteristics of the soil. For example, the 0.3 and 1% concentrations of Envirocid Plus produced visually greater soil (protein aggregates) than the 0.03% solution, but only the 0.03% solution soil required dilution prior to testing because the other higher concentration cleaners produced soils that did not release well from the panels (Tables 2, 3, 4, 5). This would not be a major safety concern because the soil was

very obvious and the cleaning process would have been initiated again prior to testing to remove the visible soils.

It should be noted that a major difference between the LFDs used is the presence of an overload line. Of the two LFDs evaluated in this study, only the Neogen Reveal 3-D test has an overload line. When there is a high allergen load, the kit without an overload line could display a false negative result due to the so-called hook effect commonly observed at high concentrations of allergen residue while a kit with an overload line will show that a high positive result was obtained. There were several times when a visually clean surface yielded a positive result and care should be taken to test several dilutions of a sample when using a LFD without an overload line to ensure that a faint or no line in the LFD does not correspond to a false negative response.

The cleaning solution interference experiments found that the caustic cleaning solutions gave false negative results at the higher cleaning solution concentrations with the LFD kits (Table 1). Some discrepancies between kit results may be a result of these concentration dependent interferences, such as stainless steel at 1% NaOH level (Table 2). Other discrepancies may be due to variation in soil formation, cleaning, and the ability of the swab to remove the soil from the surface and the extraction buffer from the kit to recover the residue from the swab.

A visual measure of residual protein on the panels after cleaning was analyzed through staining the protein soil with Coomassie Blue, then scanning the panels and performing an image analysis with the ImageJ software. Using the mean value analysis, it

was generally observed that with higher concentrations of the acid wash and sanitizer, the surface became ‘dirtier’ (Figures 1, 3, 5, 7). Higher concentrations of the commercial caustic solution (Exelerate) produced a ‘cleaner’ surface. The commodity caustic solution (NaOH) did not follow a clear trend with the cleaner concentration, but was always below the commercial caustic ‘cleanliness’ level. Not every surface would be suitable to scanning analysis. The clean Delrin and HDPE surfaces were naturally whiter and approached the limit of the gray scale while the Nylon 6/6 surface which has a slight yellow tint was closer to the gray scale range observed with the stainless steel range. It should also be noted that the mean value of clean for stainless steel is lower than the mean value for dirty, while this trend is reversed for the plastic surfaces.

The percent clean analysis (Figures 2, 4, 6, 8) was found to be quite variable with high standard deviations. This could be a result of variances in the control clean panels that were pooled and used for comparison against the washed panels. Comparing each washed panel to its own control clean panel may have given less variable results, but was not feasible in this study. Due to this variability, this scanning method for analysis of residual soil that may remain after cleaning is not recommended for further use.

Surfaces: Based on the number of positive results (LFD and general protein) after cleaning with the caustic wash solutions, the 316 grade stainless steel had the easiest soil removal with only 25 positive results. Nylon 6/6 and HDPE were similarly well-cleaned with 30 and 32 positive results, respectively. HDPE (high density polyethylene) and Nylon 6/6 are both strong plastics and Nylon 6/6 in particular is a thermoplastic which contribute to the stability of the surface and ability to remove soils even after several

cleaning cycles. Delrin had 50 positive results, but it was observed that the surface deteriorated throughout the cleaning renewal process resulting in a roughened surface. The Delrin deteriorated to an extent that it would no longer be used in a food-processing environment and replaced with new Delrin material. This result may reflect changes to the surface of the panel that allowed harborage sites for soils.

Delrin is an acetal polymer (polyoxymethylene) used in conveyor belt materials. It is stiff, has high wear resistance, but less resistance to alkaline solutions than similar polymers. Two studies that investigated the removal of bacterial biofilms or food-borne pathogens from various food-processing surfaces found that Delrin had greater biofilm development or lower amounts of bacterial reduction as compared to other plastics and stainless steel *(1, 27).*

Contribution of a full CIP process to allergenic soil removal from common food contact surfaces. The ability of a milk soil to be removed during the cleaning process from various food processing surface materials using a complete CIP program was investigated. Two different caustic cleaners, a commercial caustic solution (Exelerate CIP) and a commodity caustic solution (NaOH) were used. After cleaning with Exelerate CIP, the surfaces were visually clean. After cleaning with NaOH, there were a few small, thin milk soils remaining on the panel surfaces that were not removed during the subsequent rinses, acid wash, and sanitizing. It was observed during the cleaning process, that the milk soils removed with NaOH would not dissolve into the solution; rather they would form an aggregated mass at the surface of the cleaning solution, or precipitate and

fall to the bottom of the beaker. A cleaner that suspends the soil and is easily rinsed away is preferred *(21)*.

The qualitative LFDs and a scanning method were again used to evaluate the simulated CIP process. The Romer AgraStrip Casein kit and Neogen Reveal 3-D Total Milk LFDs had comparable results that were predominately negative after the simulated CIP process (Table 6). An exception occurred with the Nylon 6/6 surface that was cleaned with Exelerate CIP and tested with the Romer AgraStrip Casein kit where positive results were observed upon multiple replications. This could be a result of differences in the detection limit of the two kits; previous studies have found the Romer kit to detect swabbed samples at 3 μg milk protein, while the Neogen kit was able to initially detect samples at 10 μg milk protein (thesis Ch 2).

The mean value analysis correlated with the visual observations and lateral flow device results (Figure 9). The mean gray values of the stainless steel, Nylon 6/6 and HDPE surfaces (after the cleaning process and staining with Coomassie Blue), were similar to the mean gray value of their control clean surfaces. It was observed with the plastic surfaces that the mean value after cleaning with Exelerate CIP was slightly closer to the mean value of the clean control coupon than after cleaning with NaOH. The soil particles left on the surfaces after cleaning with NaOH were detectable through this method. The lack of difference in the LFD results is probably a result of low swab recovery of dry, thin soils. It would be of interest to determine if a small, thin soil of this type would be likely to dislodge in further food processing.

The Delrin surface deteriorated through the cleaning renewal process which resulted in a rough surface with areas to harbor soil. This was observed in the mean value analysis, in which the coupons that were stained after cleaning had mean values that were similar to a completely dirty control (Figure 9). It is of concern that the LFD did not have more positive results with this surface. This is most likely due to a lack of swab recovery and an uneven, rough surface. The % Clean analysis again yielded results which were variable and did not correlate with the other detection methods (Figure 10).

V. Conclusions

This study evaluated several aspects of cleaning validation studies. Caustic cleaning solutions displayed concentration dependent negative interferences with LFDs while sanitizing solutions yielded false positive results with general protein tests. Caustic cleaning solutions effectively removed milk protein soils in a simulated CIP system, while acidic and sanitizing solutions did not contribute to protein removal. Stainless steel was most easily cleaned, followed by HDPE and Nylon 6/6. The Delrin surfaces deteriorated throughout the study. In addition to qualitative tests, two scanning image methods were used to determine cleanliness; further development of these methods is necessary.

VI. Acknowledgements

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VII. References

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VIII. Tables and Figures

TABLE 1. *Interferences of cleaning chemicals with LFD and general protein detection of NFDM*

	3M Clean-Trace Surface Protein (Allergen)[a]	Neogen Reveal 3-D Total Milk	Romer AgraStrip Casein
Pos control: 200 ppm NFDM protein	Pos-purple	Pos	Pos
COMMODITY CAUSTIC			
1% NaOH	neg-colorless/light blue	neg	neg
1% NaOH +200 ppm NFDM protein	Pos-purple	+/-[b]	faint pos
0.3% NaOH	neg-light green	neg	neg
0.3% NaOH + 200 ppm NFDM protein	Pos-purple	faint pos[b]	pos
0.03% NaOH	neg-light green	neg	neg
0.03% NaOH + 200 ppm NFDM protein	Pos-purple	faint pos	pos
COMMERCIAL CAUSTIC-ECOLAB			
1% Exelerate CIP	neg-light green	neg	neg
1% Exelerate CIP +200 ppm NFDM protein	gray-lavender	neg	neg
0.3% Exelerate CIP	neg-light green	neg	neg
0.3% Exelerate CIP + 200 ppm NFDM protein	Pos-purple	neg	+/-
0.03% Exelerate CIP	neg-light green	neg	neg
0.03% Exelerate CIP + 200 ppm NFDM protein	Pos-purple	pos	pos
COMMERCIAL ACID-ECOLAB			
1% Envirocid Plus	neg-light green	neg	neg
1% Envirocid Plus +200 ppm NFDM protein	Pos-purple	pos	pos
0.3% Envirocid Plus	neg-light green	neg	neg
0.3% Envirocid Plus + 200 ppm NFDM protein	Pos-purple	pos	pos
0.03% Envirocid Plus	neg-light green	neg	neg
0.03% Envirocid Plus + 200 ppm NFDM protein	Pos-purple	pos	pos
COMMERCIAL SANITIZER-ECOLAB			
0.3% Vortexx	pos-intense purple	neg	neg
0.3% Vortexx +200 ppm NFDM protein	pos-intense purple	faint pos	pos
0.03% Vortexx	pos-purple	neg	neg
0.03% Vortexx + 200 ppm NFDM protein	pos-dark purple	pos	pos
0.003% Vortexx	neg-colorless/light blue	neg	neg
0.003% Vortexx + 200 ppm NFDM protein	pos-purple	pos	pos

[a]The results are analyzed by color development. Green is negative while purple is positive.

[b]+/-, very faint positive result; f.pos, faint positive result.

Table cell coloring at right denotes false negative and false positive results. false negative false positive

TABLE 2. *Cleaning of 316 grade stainless steel soiled with 0.3-0.35g of NFDM*

Cleaner	Conc (%)[a]	Kit (no. positive/ no. tested) 3M Clean-Trace	Romer AgraStrip Casein	Neogen Reveal 3D Total Milk	Visual Observation
Commodity Caustic	0.03	Neg (0/4)	Pos (4/4)	Neg (0/4)	
	0.3	Neg (1/4)	f. pos[b] (2/4)	Neg (1/4)	Soil peeled off as film
	1	Pos (4/4)	Neg (0/4)	Neg (0/4)	Soil peeled off as film
Exelerate CIP	0.03	f. pos (4/4)	Pos (4/4)	+/-[d] (4/4)	Visually clean
	0.3	Neg (0/4)	Neg (0/4)	Neg (0/4)	Visually clean
	1	Neg (1/3)	Neg (0/4)	Neg (0/4)	Visually clean
Envirocid Plus	0.03	Pos (4/4)	f. pos x100[c] (4/4)	Overloaded (4/4)	Soil in solution stringy
	0.3	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Soiled
	1	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Soiled
Vortexx	0.003	n/a[e]	f. pos (4/4)	Pos (4/4)	Majority of soil removed
	0.03	n/a	f.pos (4/4)	Overloaded (4/4)	Majority of soil removed
	0.3	n/a	Pos x100[c] (4/4)	Overloaded (4/4)	Majority of soil removed

[a]Each concentration level was tested in quadruplicate.

[b]f.pos, faint positive result.

[c]This extract was diluted 100 fold prior to testing.

[d]+/-, very faint positive result

[e]n/a, not analyzed because of false positive result between cleaner and kit.

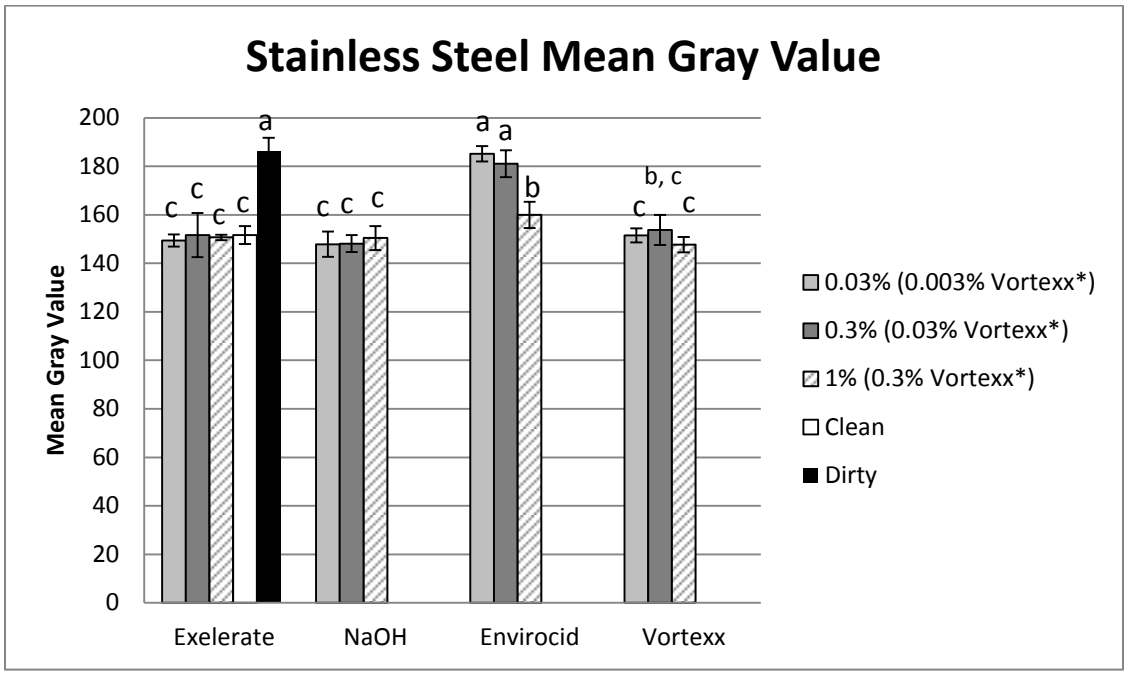


Figure 1. Mean gray value analysis of 316 grade stainless steel surfaces soiled with 0.3-0.35g NFDM. Means followed by same letter are not significantly different ($p>0.05$). *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

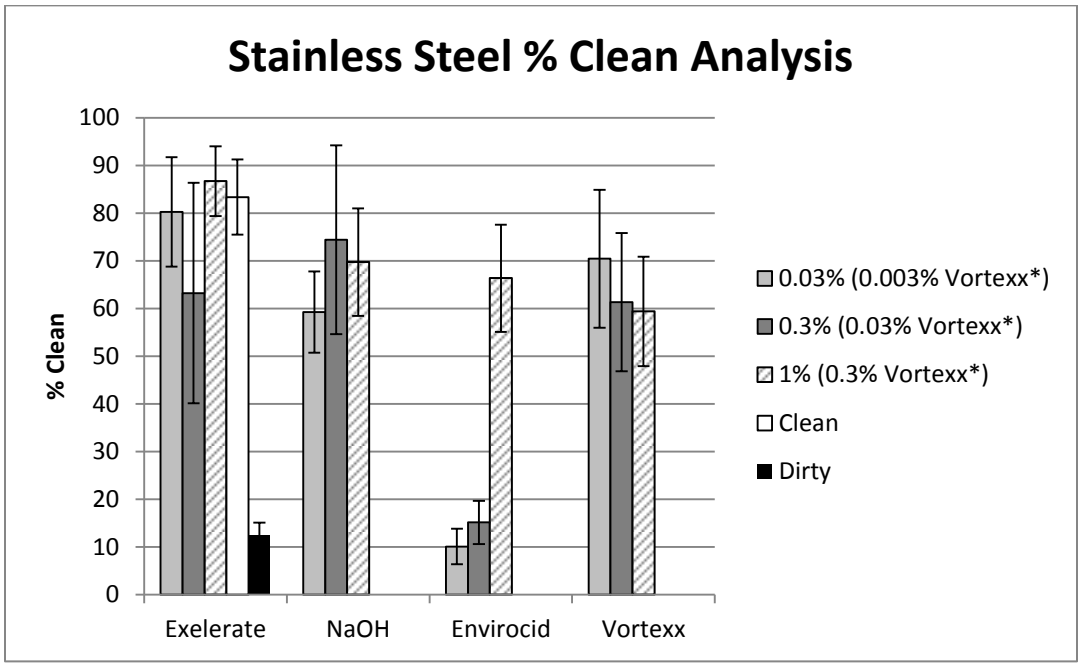


Figure 2. % Clean analysis of 316 grade stainless steel surfaces soiled with 0.3-0.35g NFDM. *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

TABLE 3. *Cleaning of Nylon 6/6 soiled with 0.3-0.35g of NFDM*

Cleaner	Conc (%)[a]	Kit (no. positive/no. tested) 3M Clean-Trace	Romer AgraStrip Casein	Neogen Reveal 3D Total Milk	Visual Observation
Commodity Caustic	0.03	+/-[b] (2/4)	Pos (4/4)	Neg (0/4)	Visually clean
	0.3	Neg (0/4)	f. pos[b] (4/4)	Neg (0/4)	Visually clean, a few pieces of soil
	1	Neg (0/4)	Neg (1/4)	Neg (0/4)	Visually clean, a few pieces of soil
Exelerate CIP	0.03	Pos (3/4)	Pos (4/4)	+/- (4/4)	Visually clean
	0.3	+/- (2/4)	f. pos (4/4)	Neg (0/4)	Visually clean
	1	+/- (2/4)	Neg (0/4)	Neg (0/4)	Visually clean, sol'n yellow
Envirocid Plus	0.03	Pos (4/4)	Pos x50[c] (4/4)	Overloaded (4/4)	Soiled, some came off in sol'n a bit stringy
	0.3	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Completely Soiled
	1	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Completely soiled
Vortexx	0.003	n/a[d]	Pos (4/4)	Overloaded (4/4)	Lower soil
	0.03	n/a	Pos (4/4)	Overloaded (4/4)	Mid-soil
	0.3	n/a	f. pos (4/4)	Overloaded (4/4)	Completely soiled, some stringy bits of soil came off

[a]Each concentration level was tested in quadruplicate.

[b]+/-, very faint result; f.pos, faint positive result.

[c]This extract was diluted 50 fold prior to testing.

[d]n/a, not analyzed because of false positive result between cleaner and kit.

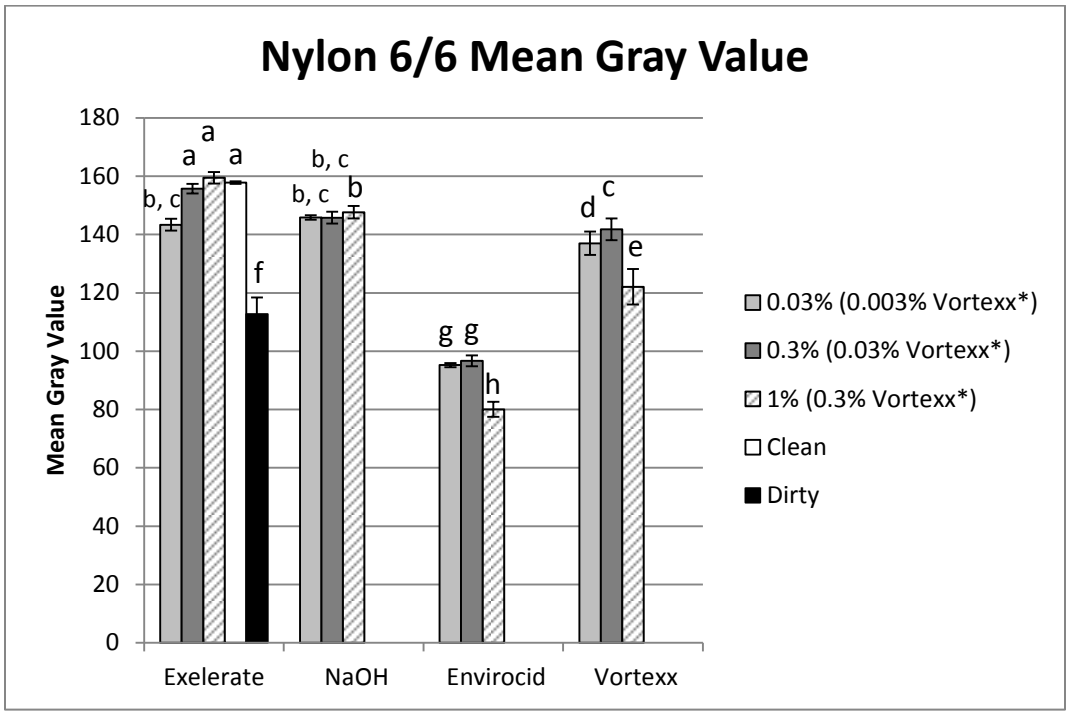


Figure 3. Mean gray value analysis of Nylon 6/6 surfaces soiled with 0.3-0.35g NFDM. Means followed by same letter are not significantly different ($p>0.05$). *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

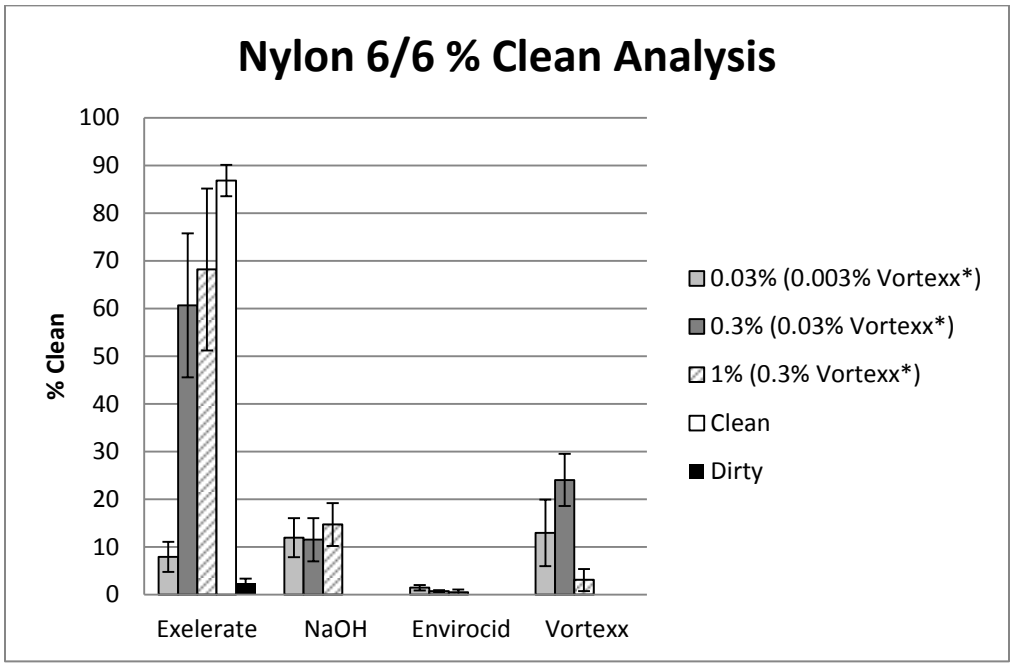


Figure 4. % Clean analysis of Nylon 6/6 surfaces soiled with 0.3-0.35g NFDM. *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

TABLE 4. *Cleaning of HDPE soiled with 0.3-0.35g of NFDM*

Cleaner	Conc (%)[a]	Kit(no. positive/no. tested) 3M Clean-Trace	Romer AgraStrip Casein	Neogen Reveal 3D Total Milk	Visual Observation
Commodity Caustic	0.03	Neg (0/4)	Pos (4/4)	+/-[b] (4/4)	Clean
	0.3	Pos (3/4)	f. pos[b] (4/4)	+/- (3/4)	Films released
	1	Neg (1/4)	f. pos (2/4)	Neg (0/4)	Films released, sol'n yellow
Exelerate CIP	0.03	Neg (0/4)	Pos (4/4)	f. pos (2/4)	Clean
	0.3	Neg (0/4)	f. pos (4/4)	Neg (1/4)	Clean
	1	Neg (0/4)	Neg (0/4)	Neg (0/4)	Clean, slight yellow sol'n
Envirocid Plus	0.03	Pos (4/4)	Pos x50[c] (4/4)	Overloaded (4/4)	Soiled, some stringy soil released
	0.3	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Soiled
	1	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Soiled-more than lower conc.
Vortexx	0.003	n/a[d]	f. pos (4/4)	Overloaded (4/4)	Soiled
	0.03	n/a	Varied (3/4)	Overloaded (4/4)	Soiled
	0.3	n/a	f. pos x50[c] (4/4)	Overloaded (4/4)	Soiled

[a]Each concentration level was tested in quadruplicate.

[b]+/-, very faint result; f.pos, faint positive result.

[c]This extract was diluted 50 fold prior to testing.

[d]n/a, not analyzed because of false positive result between cleaner and kit.

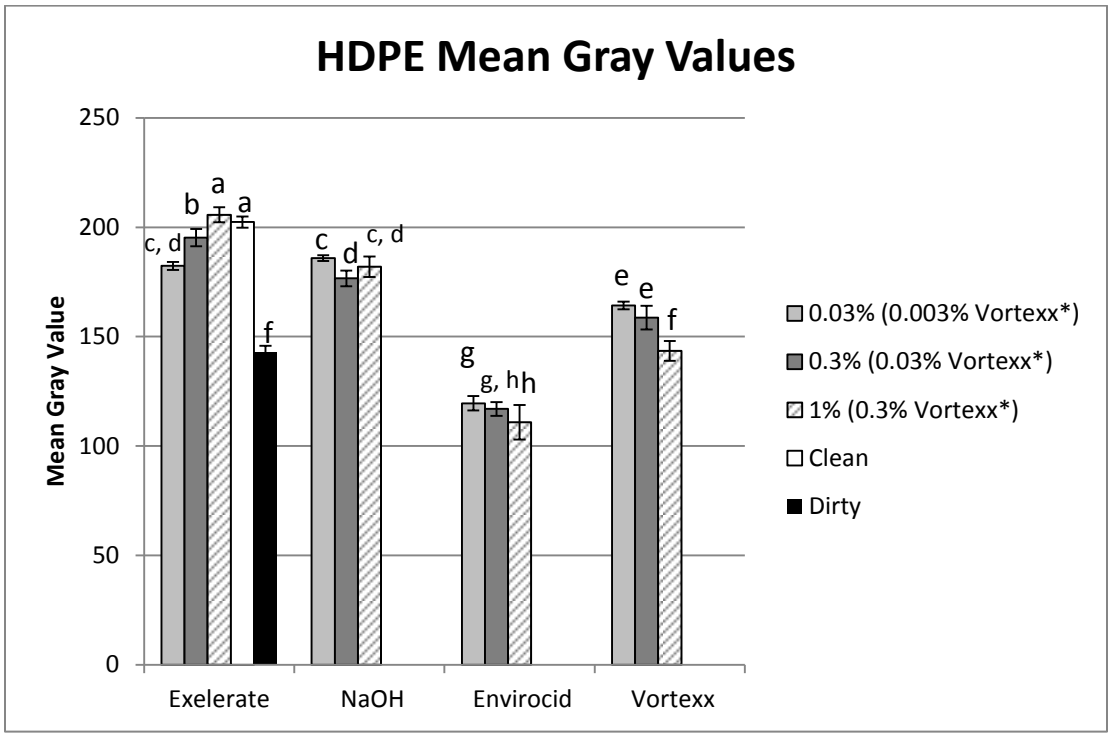


Figure 5. Mean gray value analysis of HDPE surfaces soiled with 0.3-0.35g NFDM. Means followed by same letter are not significantly different ($p>0.05$). *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

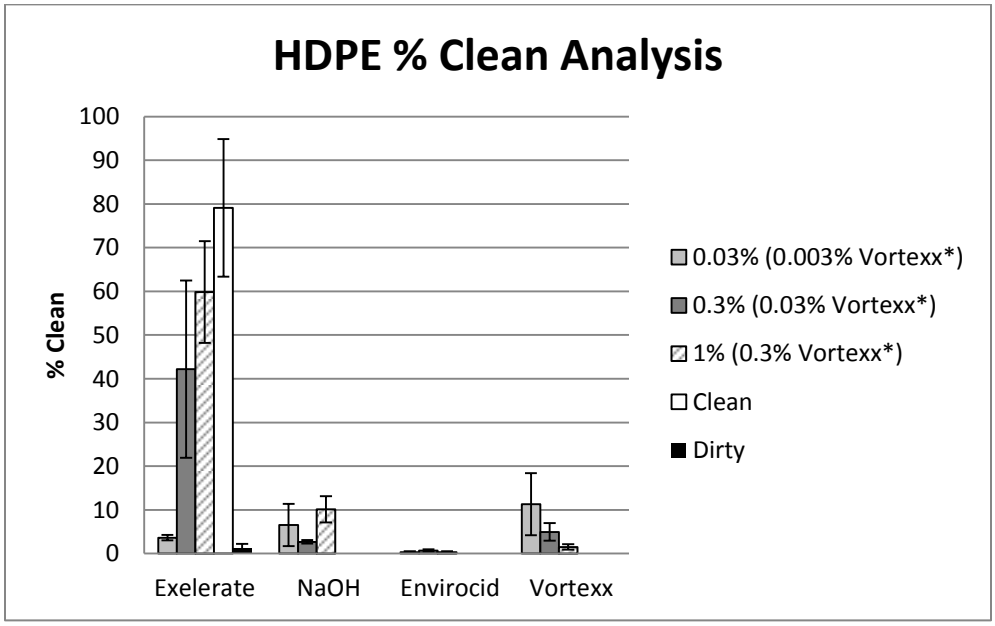


Figure 6. % Clean analysis of HDPE surfaces soiled with 0.3-0.35g NFDM. *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

TABLE 5. *Cleaning of Delrin soiled with 0.3-0.35g of NFDM*

		Kit			
Cleaner	Conc (%)[a]	3M Clean-Trace	Romer AgraStrip Casein	Neogen Reveal 3D Total Milk	Visual Observation
Commodity Caustic	0.03	f. pos[b] (2/4)	Pos (4/4)	+/-[b] (2/4)	Clean
	0.3	Pos (4/4)	Pos (4/4)	Overloaded (4/4)	Soil films coming off in sol'n, sol'n yellow
	1	f. pos (2/4)	f. pos (4/4)[d]	Overloaded (4/4)	Films coming off in sol'n; Sol'n yellow
Exelerate CIP	0.03	f. pos (2/4)	Pos (4/4)	f. pos (4/4)	Clean
	0.3	Neg (1/4)	f. pos (4/4)	Neg (0/4)	Clean
	1	Neg (1/4)	Neg (0/4)	Overloaded (4/4)	Clean, sol'n yellow
Envirocid Plus	0.03	Pos (4/4)	Pos x50[c] (4/4)	Overloaded (4/4) O faint	Soiled
	0.3	Pos (4/4)	Pos (4/4)	Overloaded (4/4) O<T	Soiled
	1	Pos (4/4)	Pos (4/4)	Overloaded (4/4) O faint	Soiled
Vortexx	0.003	n/a[e]	Pos (4/4)	Overloaded (4/4) O<<<T	Soiled, some removed when lifting out of sol'n
	0.03	n/a	f. pos (4/4)	Overloaded (4/4) O not visible	soiled
	0.3	n/a	Pos x50[c] (3/3)	Overloaded (4/4) T faint, O not visible	Soiled, some soil came off in stringy/clumpy bits

[a]Each concentration level was tested in quadruplicate.

[b]+/-, very faint result; f.pos, faint positive result.

[c]This extract was diluted 50 fold prior to testing.

[d]Nearing overload.

[e]n/a, not analyzed because of false positive result between cleaner and kit.

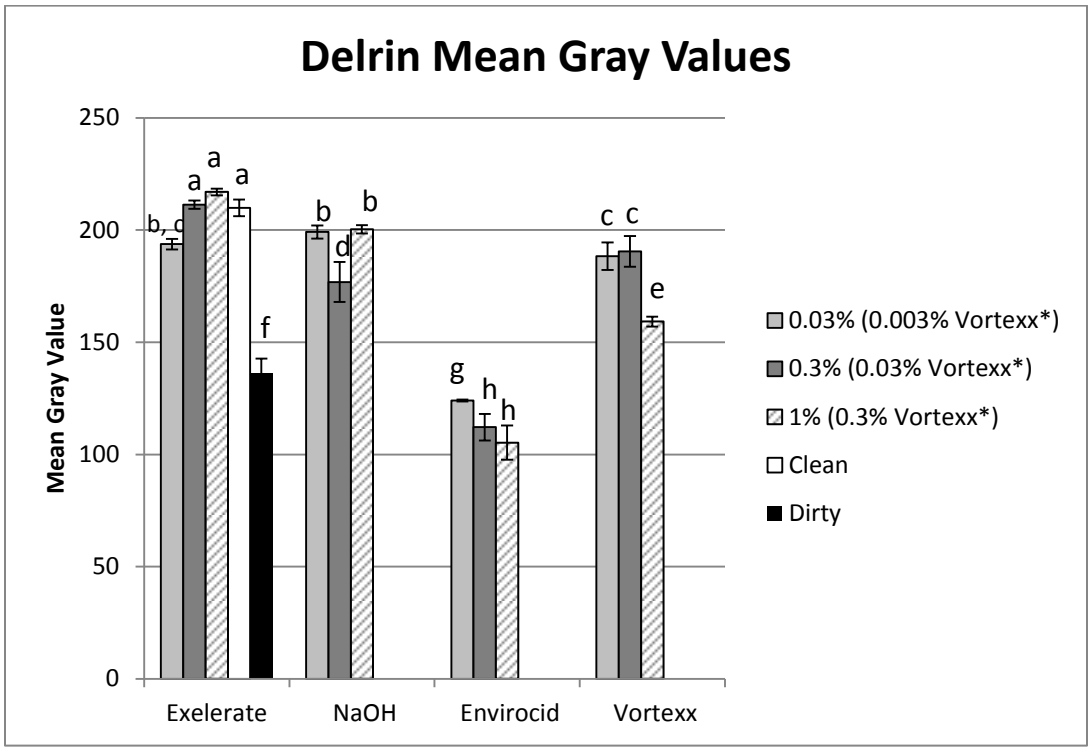


Figure 7. Mean gray value analysis of Delrin surfaces soiled with 0.3-0.35g NFDM. Means followed by same letter are not significantly different ($p>0.05$). *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

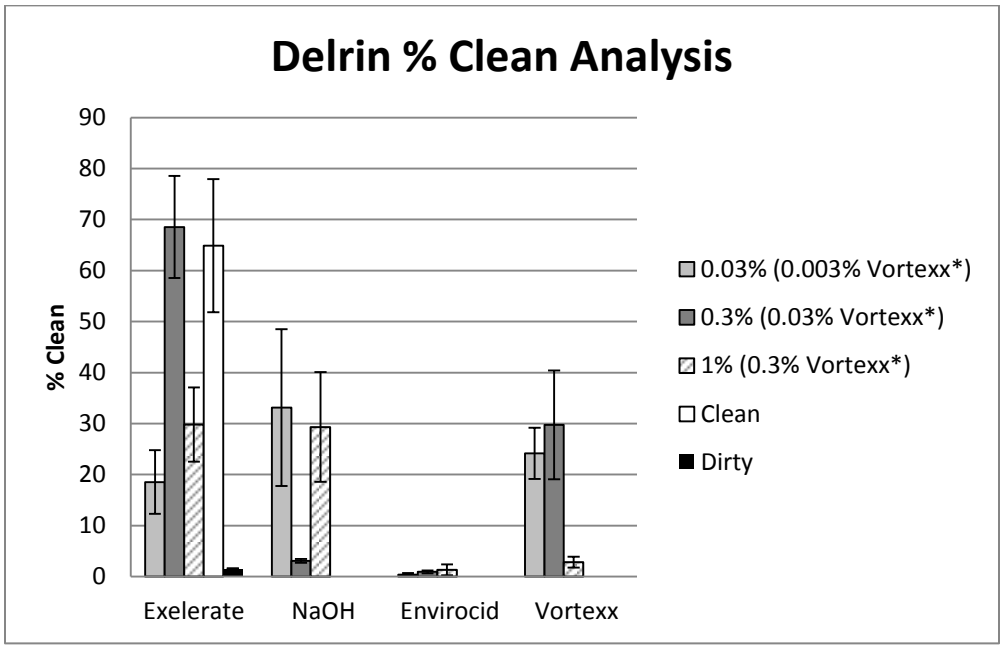


Figure 8. % Clean analysis of Delrin surfaces soiled with 0.3-0.35g NFDM. *Vortexx was applied at a different concentration than Exelerate, NaOH, and Envirocid.

TABLE 6. *Effect of caustic cleaner as part of CIP process on cleaning of 0.3-0.35g of NFDM from various food processing surfaces*

		LFD Kit result (no. positive/no. tested)	
Surface	Caustic Cleaner	Romer AgraStrip Casein	Neogen Reveal 3-D Total Milk
Stainless Steel 316	Exelerate CIP	Neg (0/4)	Neg (1/4)
	Commodity Caustic	Neg (0/4)	Neg (0/4)
Nylon 6/6	Exelerate CIP	Pos (4/4)	Neg (0/4)
	Commodity Caustic	Neg (0/4)	Neg (0/4)
HDPE	Exelerate CIP	Neg (1/4)	Neg (1/4)
	Commodity Caustic	Neg (0/4)	Neg (0/4)
Delrin	Exelerate CIP	Neg (1/4)	Neg (1/4)
	Commodity Caustic	Neg (1/4)	faint pos (2/4)

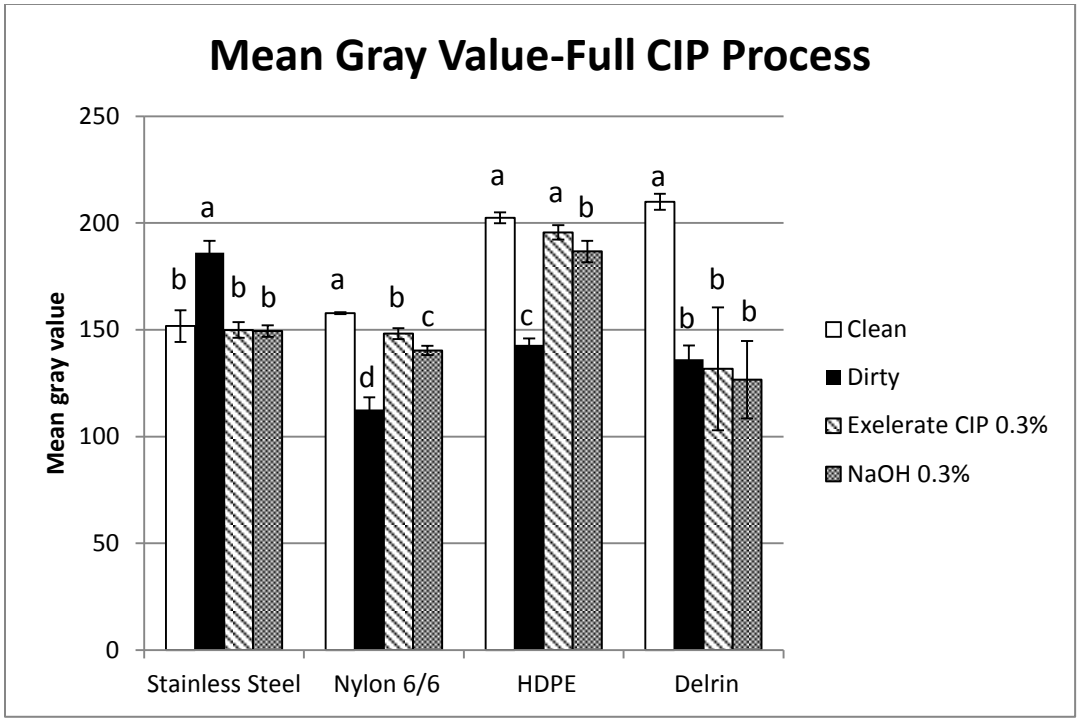


Figure 9. Mean gray value analysis of various food processing surfaces after full CIP. Means followed by same letter are not significantly different ($p>0.05$); each surface was analyzed separately.

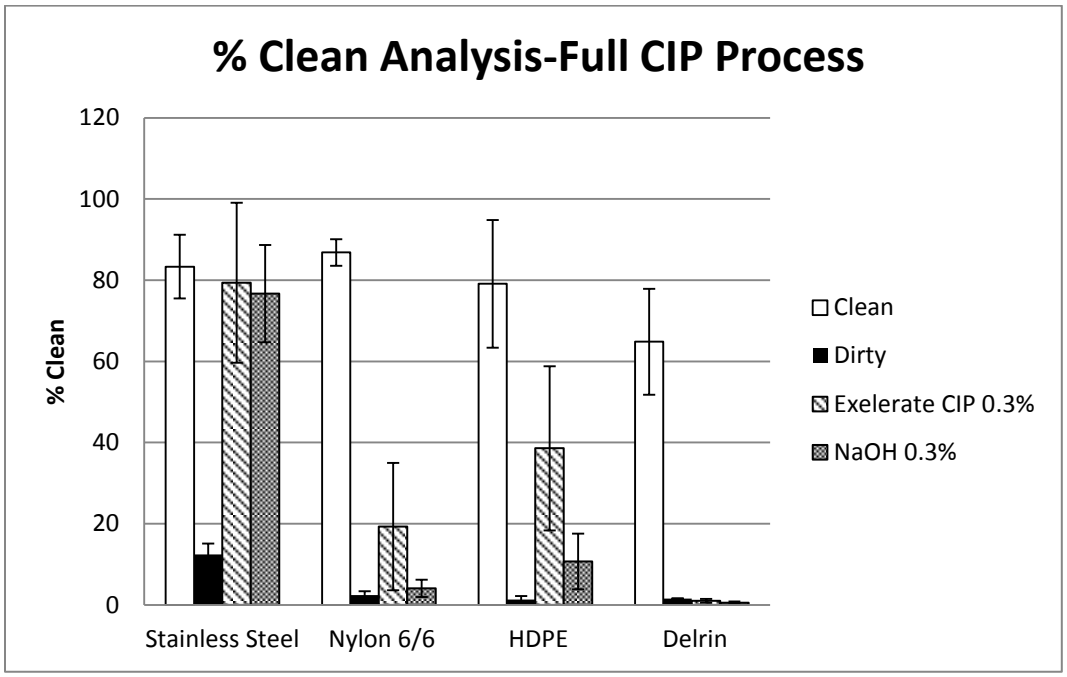
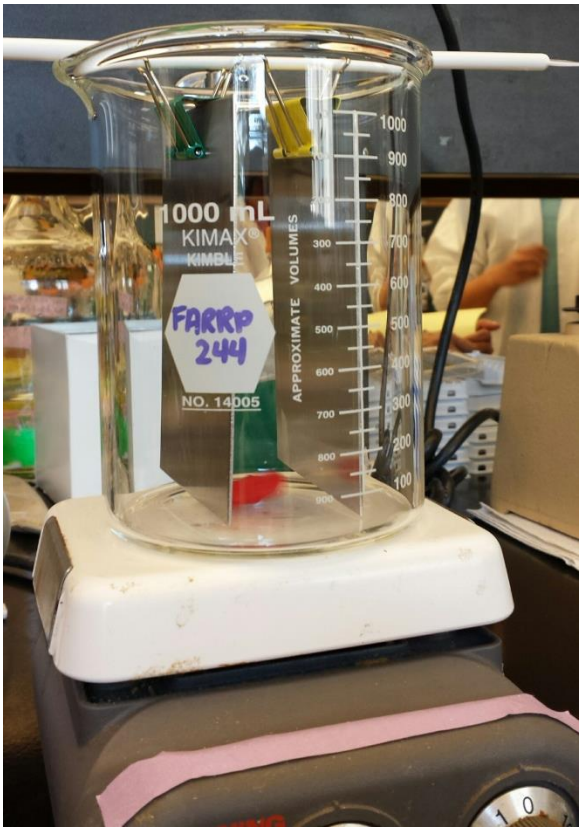


Figure 10. % Clean analysis of various food processing surfaces after full CIP.

APPENDIX A



Supplementary Figure A1. Panels suspended in beaker. Two panels suspended by binder clips threaded on dowel. Stir bar in beaker and 900 ml of cleaning solution added when cleaning.