DEVELOPMENT OF A DNA BARCODE BASED METHOD FOR FOOD TRACEABILITY AND DETECTION OF ADULTERATION OR FRAUD FOR LIQUID GOODS

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Abstract – In this paper we present the development of a DNA barcode based method to rapidly identify the source of food and detect fraud or adulteration of liquid goods. The DNA barcodes are used to apply the product identification directly on or in the food products. We applied DNA markers and traced several food items through the supply chain to demonstrate both stability of the tracers as well as ability to correctly identify source when products of different origins are commingled. We also demonstrated the feasibility of using this method for detection of adulteration of olive oil.

Keywords: traceability, source assurance, adulteration detection

1. INTRODUCTION

We live in a world of globalization, a world in which political and commercial realities are the drivers for increasing supply chain efficiencies, accountability and security. One key to achieving gains in all three areas lies in the area of traceability. For the global food industry, supply chain traceability is, today, more than ever, a high visibility issue. Traceability is the ability to trace the source of foods and their ingredients, from fork to farm.

In 2011 the CDC estimated that in the USA 3,000 deaths are caused each year due to foodborne illness. The cost to treat food poisoning comes to $14 billion a year, according to a July 2012 study published in the Journal of Food Protection, including the medical expenses of the 128,000 who are hospitalized annually. Total cost to the US economy is estimated in excess of $70 billion annually. Government regulators are increasingly concerned that due to poor traceability and increased supply chain complexity, the time it takes to respond to food-related emergencies is too long, and consumer trust in food becomes more fragile. In parallel, food adulteration and fraud are becoming major threats to the food supply. It is estimated that food fraud costs the global food industry $15 billion dollars annually, and the cost is rapidly rising.

Improved food traceability will benefit: (a) the regulators by reducing the resources required to complete an investigation; (b) public health since reduction of the duration of investigation of food related illnesses is key to the containment of an outbreak; (c) industry, by facilitating the ability to isolate the source and extent of safety and/or quality control issues, thus minimizing the delay and scope of the recall and associated liabilities; (d) producers, by allowing them to differentiate their products, which may be otherwise perceived as commodities.

Food traceability today entails a complex system of hand offs along the supply chain from producer to packer, distributor, retailer and ultimately the consumer. Investigations frequently take several weeks to complete. Advances in bio-engineering have produced a material that enables the development of a very efficient, effective and low cost food tracing system. This material referred to as SafeTracer™, is a tracer initially developed as a universal simulant for biodefense and biodetection applications. In this paper we demonstrate that SafeTracer™ can provide a method to trace food along multiple steps in the supply chain by applying the product identification directly on or in many food products, at a cost significantly lower than any other method. SafeTracer™ is a safe and versatile material already recognized by United Stated Food and Drug Administration as a Generally Recognized

As Safe (GRAS) food additive. It can be sprayed directly onto the product or mixed with a coating and will adhere to produce and other food surfaces. A practically limitless number of DNA markers are possible by using synthetic or genomic DNA, and it is possible to produce a DNA barcode containing a large amount of traceability information by combining a number of markers. Detection of SafeTracer can provide source tracing in minutes, as opposed to days or weeks.

2. THE INNOVATION

For food traceability applications, implementation is simple and does not require any significant capital investment. There already exist process steps in the supply chains of many produce and other food items where the SafeTracer™ can be safely added as a component. The DNA barcode may be added directly as a component to various coatings already in wide use such as carnauba or other wax, silicone oils, sprout inhibitors, lipid-, polysaccharide-, and protein-based edible coatings, etc. DNA encapsulated in maltodextrin, salt, starch or other material may be used for dry goods such as beans, cereals, etc. DNA may be also added directly to liquid goods such as juices, milk, oils, etc.

We have already developed approximately 800 distinct *Thermotoga maritima* sequences as tracers. We have also developed a method to utilize DNA sequences to form "barcodes." In its simplest implementation, a unique combination of sequences is used to generate a Company Code, which uniquely identifies a grower/producer. Some of our potential users are only interested in authenticating their product, and this approach can serve them well. Other users are interested in full traceability of each item through its supply chain. Figure 1 demonstrates a more advanced implementation, which provides complete traceability information.

In this approach we employ, for example, 64 distinct 100 base pair DNA sequences. Each sequence represents a specific bit in a 64-bit set; presence of the sequence sets the bit to 1, and absence of the sequence sets it to 0. By employing only 64 sequences we can create 2^{64} unique combinations, which we refer to as "DNA barcodes". The major advantage of this approach is that we can analyze 2^{64} unique barcodes by employing only 64 primers and probes for detection via the Polymerase Chain Reaction process. This makes our approach highly scalable and sets us apart from other technology approaches, which, in practicality, could only be used for authentication or identification but not traceability, because of the limited number of unique sequences they could economically support.

Different sets of sequences could be combined to generate distinct sets of barcodes assigned to Growers, Packers, Re-packers, Distributors and Processor. A single piece of produce might bear many different barcodes, each identifying distinct steps in the supply chain. However multiple barcodes associated with the same step in the supply chain (for example multiple grower barcodes) would produce an inconclusive analysis, indicating supply chain mismanagement or intentional fraud.

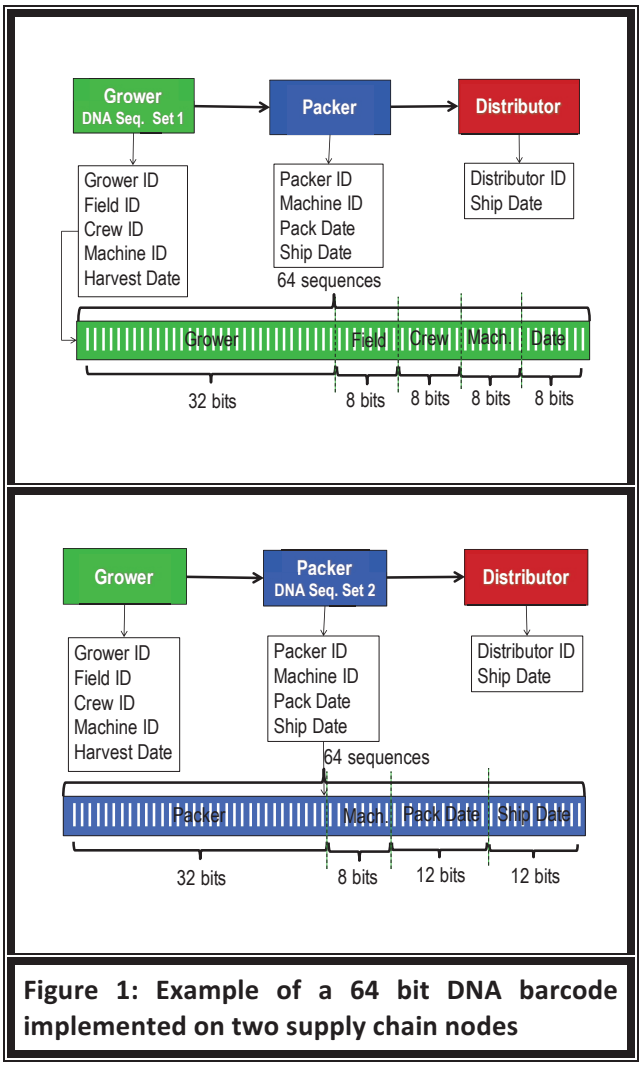

Figure 1: Example of a 64 bit DNA barcode implemented on two supply chain nodes

3. RESEARCH OBJECTIVES

The research objectives were as follows:

i. Conduct two mock food traceback projects that (a) simulate the diversity of the food supply chain and consider confounding factors, such as packaging, storage, and commingling and; (b) include olive oil and at least one product that has been the subject of significant foodborne disease outbreaks between 2005 and 2013.
ii. Evaluate carnauba wax or a similar produce coating as carrier/encapsulating agent for SafeTracer.
iii. Determine the stability of SafeTracer™ in food coatings for typical supply chain processes (such as rinsing, etc.), and environmental conditions during storage and transportation.
iv. Demonstrate ability to detect adulteration of olive oil.

We collaborated with Borton & Sons, a large apple, pear and cherry producer in Yakima, WA. Recently, prepackaged caramel apples were identified as the cause of a listeria outbreak in 2014-2015, although no Borton fruit was implicated. Borton expressed high interest in our method, and we executed a pilot program, which was partially funded by the grant and partially self-funded. Borton processes 350 million tons of apples every season. Borton is a leading, vertically integrated fruit company that combines experience and cutting edge techniques to get the highest volume yields of the highest quality product in the industry. Borton also sources their produce from many fields, orchards and local farmers, and package and distribute to a wide range of vendors domestically and internationally, through multiple shippers.

The specific objectives of this pilot were to: (a) integrate our method into the apple packing process (which is very similar to those of most pomme fruit, stone fruit, citrus, etc.); (b) perform lot tracking on the "as-is" packing process – changing the barcode applied to the product without significant incremental steps, and with low risk of "cross-contamination" from lot to lot; and (c) conduct a preliminary evaluation of the propagation of the barcodes in the processing plant.

The apple pilot was conducted on December 1, 2015 at the Borton apple packing plant in Yakima, WA. The plant operated at a throughput of approximately 30,000 lbs/hr.

The plant waxing system applied a mix of carnauba, shellac and other waxes at a rate of 1-gallon wax solution per 10,000 lbs of apples. The DNA marker was simply added to wax in the facility's 5-gallon wax tank at a concentration of 180 ng/gal, demonstrating the ease of implementation. Apples were sampled in sets of three every five minutes after the wax application and curing (Figure 2). Control apple samples were collected at 3 time points: time zero, the half way point, and at the end of the 8 hour experiment. The control apple samples were collected as the apples rolled on the brushes before moving into the furnace for wax curing (referred to as "-heat"). At the beginning of the experiment one set of apple samples was collected after rolling out of the furnace onto the conveyor belt (referred to as "+heat"). Control environmental samples were also collected at the end of the experiment by swabbing the wax brushes and the conveyor.

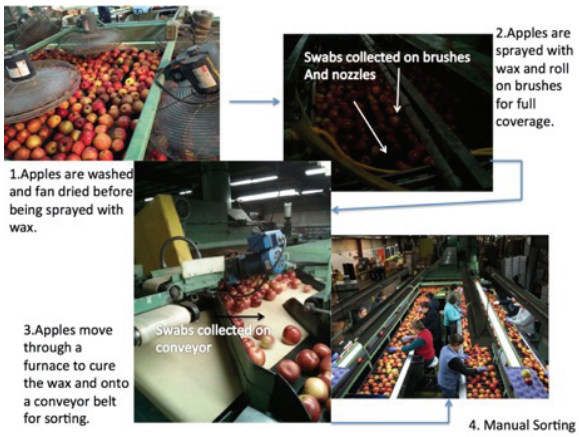

Figure 2: Apple Packing Process

At the end of the day the plant personnel followed standard operating procedures to clean the wax nozzles and brushes. After the restart of the line the following day, plant personnel removed two boxes of apples at the end of the packing line, one around the beginning of the day at 6:00 am and one around the end of the day at 4:00 pm containing approximately a total of 250 apples. These samples were used to determine if there was residual marker left in the system after the cleaning of the wax nozzles and brushes, and evaluate the feasibility of changing the barcode from lot to lot without significantly increasing the plant downtime, and without significant risk of cross-contamination from lot to lot. The plant also held back tagged apples that were stored under room conditions and

were tested one, two and three months after the application of the barcode to evaluate stability over time.

Samples were processed by swabbing the surface with a sterile cotton swab, TE buffer was added to the sample, vortexed for approximately 30 seconds and tested in triplicate on a Cepheid ThermoCycler. Table 1 contains the experimental results. Although numerical values are presented for the average number of cycles it took for fluorescence to be detected and the standard deviation for the three tests run for each sample, we believe the results are mostly qualitative. The amount of material collected by swabbing can vary for a number of reasons even if the marker had been applied uniformly. We consider a cycle count below or equal to 37 as a positive result and a cycle count above 37 as negative.

Sample	Average CT	St. Deviation	Barcode Result
-HEAT CONTROL 1	36.43	0.57	Positive
+HEAT CONTROL 1	36.7	0.26	Positive
BRUSHES 1	37.50	0.7	Negative
CONVEYOR 1	36.87	0.7	Positive
1	36.50	2.26	Positive
2	37.00	0.85	Positive
3	34.30	0.43	Positive
4	32.43	0.49	Positive
5	38.43	0.68	Negative
6	34.90	0.53	Positive
7	35.70	1.48	Positive
-HEAT CONTROL 2	36.13	0.38	Positive
BRUSHES 2	31.00	0.62	Positive
CONVEYOR 2	36.03	0.32	Positive
8	32.33	1.29	Positive
9	33.60	0.17	Positive
10	34.33	1.64	Positive
11	33.93	0.25	Positive
12	33.63	1.76	Positive
13	33.60	0.46	Positive
14	33.80	6.5	Positive
15	33.30	0.26	Positive
16	36.50	0.95	Positive
-HEAT CONTROL 3	35.47	0.7	Positive
BRUSHES 3	33.30	0.3	Positive
CONVEYOR 3	34.47	0.2	Positive
Dec 2, 6:00AM 17	37.50	0.56	Negative
Dec 2, 4:00PM 18	39.50	22.8	Negative
Sample stored for 30 days	30.47	1.38	Positive
Stored Sample 60 days	39.92	0.54	Positive
Stored Sample 120 days	32.43	0.08	Positive
Stored Sample 150 days	32.33	0.01	Positive

Table 1: Results from Apple Tagging Exepriments

The first observation from the experimental results is that the wax curing (heat) process does not appear to degrade the barcode, as shown in the first two rows of the table. We generally observed a build up of the barcode in the wax system, with the counts gradually decreasing during the first half of the experiment and stabilizing around 33 for the second half. All but one samples were positive; we believe that the negative sample was caused by variation in the swabbing force and area. We also notice that after the cleaning of the wax system, the following day the results were negative. Finally, the apples that were stored for thirty, sixty, ninety, one hundred and twenty and one hundred and fifty days all produced positive results, demonstrating the long term stability of the barcodes.

The olive oil experiments were performed in collaboration with the Olive Center at Unverisity of California, Davis. The UC Davis Olive Center is a self-funded university and industry coalition that brings together nearly 100 UC faculty members, research specialists and farm advisors who address the research and education needs of California olive growers and processors. The center also collaborates with institutions worldwide.

The experiments were performed with several different olive oil samples, to make the most efficient use of time, considering that the olive harvest was late during the project period. Olive oil produced by olives harvested at the UC Davis campus were used for the maturity experiments. Commercially obtained olive oil was used for the chemical composition, taste and adulteration tests.

a) Effect of olive maturity on stability of the barcode

Each level of olive fruit maturity (green, medium and mature) has characteristics that could play a role in how stable the DNA barcode is in the oil. Green olives are high in polyphenols (anti-oxidants) and green/grass flavor components, and the chlorophyll content is high. Medium olives are medium in polyphenols and start to develop some ripe-fruity characteristics. They have close to a maximum amount of oil per dry weight. In mature fruit the skin turns from purple to black (although some varieties never turn completely black); the polyphenol and the chlorophyll contents decline, the carotenoid content increases, and the oil yield is high.

The UC Davis Olive Center harvested the olives at the three maturity levels in October/November 2015, milled the olives and provided them for the experiments. We created a “stock barcode” formulation by dissolving 100ul of the barcode in 5 ml of acetone, which was then added to 10 ml of olive oil. We then prepared the samples by mixing 900uL of each of the three oils (green, medium and mature) with 100 uL of the stock barcode, resulting in an initial marker concentration of 0.28 ng/uL. We performed a 1:10 dilution series and samples were tested in triplicate. Original samples were run

without purification steps, resulting in negative results for all samples. We believe enzymes present in the olive oil were inhibiting the reaction. As a result, two purification kits were used to clean up the sample for testing; a QIAmp DNA Stool Kit made by Qiagen and the more simplified PCR purification kit made by DNALand Scientific. Samples were diluted prior to purification and no further dilutions were made after purification.

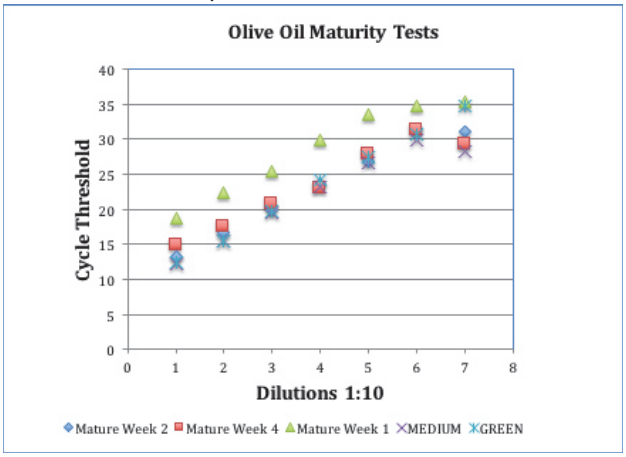

Figure 3: Olive Oil Sample Analysis Results

Figure 3 shows the results of the series, i.e. the number of PCR counts before fluorescence was detected for green, medium and mature oil one week after it was added and for mature oil 2 and 4 weeks after it was added. Only Mature Week 1 series was done using the Qiagen kit and the rest were done using the DNALand kit. The figure shows that the Qiagen kit was less effective at cleaning up the samples in comparison to the simple PCR purification kit. It also shows that the barcode remains stable in olive oil over a period of one month. However, olive oil shelf life can be two years or longer, and therefore we intend to conduct a longer stability study, including the green and medium oils in the future.

b) Effect of the barcode on chemical composition and taste

Commercially obtained extra virgin olive oil (California Olive Ranch, 2014 harvest) was used for the chemical composition and sensory tests. Each chemical test compared two samples: a "blank" and a "barcoded". 100ul of the barcode was initially dissolved in 5 ml of acetone, which was then added to 10 ml of olive oil. That was then heated to 80^0 C overnight to evaporate the acetone, and subsequently added to the sample that is referred to as "barcoded." The resulting marker concentration was 1.13 ng/ml of olive oil. The blank sample was drawn directly from the commercial product. The chemical analysis was performed at the UC Davis Olive Center Laboratory. The following tests were performed.

Free Fatty Acidity (FFA)

Free fatty acids (FFA) are the breakdown compounds from triacylglycerols via a chemical reaction called hydrolysis or lipolysis. A high level of FFA in recently extracted oil shortens the shelf life of oil. Factors such as fruit fly, fruit diseases, delays between harvesting and milling, improper extraction and prolonged contact between the oil and vegetation water may increase FFA.

Peroxide Value (PV)

Peroxides are the primary oxidation products in olive oil. When oil is exposed to oxygen, light, or heat, fatty acids are oxidized, which results in off-flavors, and diminishes nutritional value. The more oxidized an olive oil is, the higher the PV would be. However, it is important to note that as oxidation advances and secondary oxidation products are formed, peroxides are degraded and the level of PV decreases.

UV Absorbance

The ultraviolet light absorbance is measured by applying UV light through the oil at several specific wavelengths on spectrophotometer. Absorbance at 232 nm (K_{232}) indicates the primary oxidation level, while absorbance at 268 nm (K_{268}) indicates the secondary oxidation level. The absorption curve in the UV of oil is influenced by the oxidation products, some of which provoke an increase of the absorption at 232 nm and others at 270 nm. This curve is called Delta K (ΔK). ΔK detects oil treatments with color removing substances, and the presence of refined or pomace oil, by measuring the difference between absorbance at 270 nm and 266 nm – 274 nm using spectrophotometer

Fatty Acid Profile

Fatty acid profile is a measure of the proportion of individual fatty acids in the oil and is an important part of the oil purity and quality. In terms of purity, the USDA has set limits for each fatty acid in olive oil to prevent adulteration from other seed/nut oils. This is due to the fact that each seed/nut oil has a different characteristic fatty acid profile. In terms of quality, the proportion of the different fatty acids can influence the stability of the oil and the nutritional value of the oil. The fatty acid profile of olive oil is influence by cultivar and environment.

The results for all tests are shown in Table 2. The results for "Blank" and "Barcoded" samples are very similar and well within the USDA limits for extra virgin olive oils. Based on the chemical analysis of the "Blank" and "Barcoded" samples, we determined that adding the barcodes to the olive oil does not change the quality or purity of the product.

Sample Name		Blank	Barcoded
Test Date		12/16/15	12/16/15
	USDA Limits		
FFA	≤0.8	0.21	0.21
PV	≤20	7.3	7.4
K232	≤2.50	1.62	1.65
K268	≤0.22	0.09	0.08
Delta K	≤0.01	0.001	0.001
Fatty Acid Profile			
Palmitic Acid (C16:0)	7.5 – 20.0	17.1	17.5
Palmitoleic Acid (C16:1)	0.3 – 3.5	1.5	1.5
Heptadecanoic Acid (C17:0)	≤0.3	0.1	0.1
Heptadecenoic Acid (C17:1)	≤0.3	0.2	0.2
Stearic Acid (C18:0)	0.5 – 5.0	1.6	1.7
Oleic Acid (C18:1)	55.0 – 83.0	66.7	66.4
Linoleic Acid (C18:2)	3.5 – 21.0	12.0	11.9
Linolenic Acid (C18:3)	≤1.5	0.6	0.5
Arachidic Acid (C20:0)	≤0.6	0.1	0.1
Gadoleic Acid (Eicosenoic) (C20:1)	≤0.4	0.1	0.1
Behenic Acid (C22:0)	≤0.2	0.0	0.0
Lignoceric Acid (C24:0)	≤0.2	0.0	0.0

Table 2: Olive Oil Chemical Analysis Results

A triangle test was used for the sensory (taste) test. The triangle test is a discriminative method used in sensory science to gauge whether an overall difference is present between two products, or to determine whether a treatment has significantly changed a product. Experienced judges evaluate products in a controlled environment to determine whether perceptible and statistically significant differences exist between them. Thirteen olive oil panelists were used as judges. Eleven judges evaluated two sets of triangle tests and two judges evaluated single sets for a total (N) of 24.

For each of the three oils, approximately 12-13 mL was poured into glasses specified by the International Olive Oil Council, according to method COI_T20_Doc 5 Rev_1 2007 Glass for oil tasting. The glasses were covered with plastic lids, labeled with 3-digit random number codes and placed on metal serving trays corresponding to the master triangle test sheet, which indicated whether there were two control oils or two adulterated oils in the triangle test.

The tests were conducted in accordance with sensory protocol COI_T20_Doc 15 Rev 7 Organoleptic Assessment of Virgin Olive Oil. Each judge completed a "Triangle Test" form for each set of olive oils, and tried to describe how the sample they chose differed from the other two oils. Paper ballots were used to collect the data. Data were analyzed using statistical tables.

The null hypothesis was that there is no sensory difference between the "blank" olive oil and the "barcoded" olive oil. There were 12 "correct" answers (panelist chose the olive oil that was different from the other two) out of 24 – 6.8% of the time these results could have been obtained by chance alone. Thus at the 5% level of significance, the null hypothesis is not rejected; the two olive oils were not perceived to be significantly different. Panelist comments for correct triangles were evaluated and sorted according to "blank" and "barcoded."

The summary of correct panelist comments indicates that no clear pattern could be observed. For example, three panelists described the control as more pungent than the adulterated oil, but one found the control less pungent. In conclusion, at the 6.8% level of significance, there was a statistically significant difference between the "barcoded" olive oil and the "blank" olive oil. However, at the 5% level the oils were not significantly different. Since this result is borderline, we intend to repeat the test in the future with a larger sample size to increase the power of the test.

c) Olive Oil Adulteration Detection

The adulteration study was done at the request of our Italian Olive Oil partners. The collaborators in Italy used one locally produced oil and followed the sample protocol that we provided (Figure 4) to produce four samples:

Sample 1: a 250 mL "pure" olive oil sample that had not been marked with our barcodes

Sample 2: a 250 mL "pure" olive oil sample that had been marked with our barcode at 1.13 ng/uL

Sample 3: a second 125 mL "pure" olive oil sample identical to sample 3, which had been further diluted by an equal amount of unmarked olive oil, to simulate a 250mL sample that had been adulterated by 50%.

Sample 4: a 125 mL "pure" olive oil sample that had been marked with our barcode at the same rate as above.

The samples were analyzed in triplicate using both Qiagen and DNALand kits, approximately 60 days after they were tagged. The results are shown in Figure 4. For sample 1, no fluorescence was observed after 37 PCR cycles, and it is therefore considered negative. Samples 2 and 4 were expected to be positive and identical, and were

found to be as expected. For sample 3 ("adulterated") fluorescence was observed at significantly higher PCR cycles, which indicates lower presence of the barcode, suggesting adulteration. Although this experiment was very simple, it demonstrated the feasibility of using quantitative PCR methods to detect adulteration or olive oil, assuming that application of the barcodes takes place at a known rate.

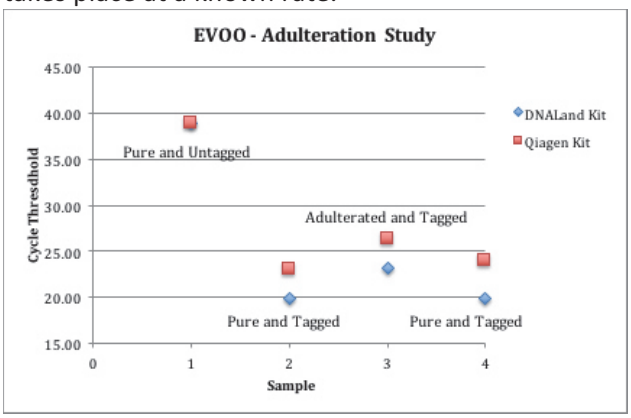

Figure 4: Oilve Oil Adulteration Experiment Results

4. CONTINUING RESEARCH

Since the completion of this work we have continued the evaluation of the DNA barcodes. Specifically in our present work, which we will describe in a subsequent paper:

- We are demonstrating our ability to correctly identify commingled produce with different origin barcodes.
- We are demonstrating our ability to extract multiple barcodes from a single item.
- We are demonstrating stability of the barcodes over a nine month period.

5. CONCLUSIONS

- We demonstrated the feasibility of using DNA barcodes for traceability in the food supply chain.
- We demonstrated stability of the DNA barcodes over supply chain conditions and times associated with a single commodity.
- We demonstrated that no cross contamination occurs between cases of produce from different origins that move together through the supply chain.
- We demonstrated our ability to correctly identify commingled produce with different origin barcodes.
- We demonstrated simplicity of integration into the apple packing process (which is very similar to those of most pomme fruit, stone fruit, citrus, etc.).
- Preliminary results suggest that it is feasible to use DNA barcodes for lot-tracking on the "as-is" packing process – changing the barcode applied to the product "on the fly" without significant incremental steps and with low risk of "cross-contamination" from lot to lot.
- We demonstrated stability of the barcodes in olive oil over a one-month period.
- We demonstrated stability of the barcodes on apples over a three-month period.
- We demonstrated that the presence of the tracers does not alter the chemical composition of the olive oil
- We performed sensory tests to evaluate the effect on the tracers on the olive oil taste and preliminary results are encouraging.
- We demonstrated that quantitative PCR can be employed to detect a 50% adulteration of olive oil.