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Differences between qualitative and quantitative determination of alkaline phosphatase measurement methods

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Abstract:
Background*: This study has been carried out to clarify the differences between the qualitative and quantitative methods for detecting the presence of alkaline phosphatase (ALP) in pasteurized milk. After a historical overview on milk pasteurization, the individual methods are described, mentioning the positive and negative points of each method.*
Materials and Methods*: A comparison was conducted between a series of different commercially available kits for proper milk pasteurization process (both quantitative and qualitative) and an experimental test kit, named "AP test".*
Results: *Several commercial kits, e.g. Lactognost Heyl III are obsolete and the accepted limits from the regulatory Authorities for proper milk pasteurization must be revised. Until today, the quantitative methods were more sensitive in comparison to qualitative ones. The application of a new experimental qualitative test, named "AP test", has made the qualitative methods 5 times more sensitive in comparison to quantitative methods (Charm F-AP).*
The new method could detect proper pasteurization at 3.5 mU/L of ALP concentration in pasteurized milk with color change from white (proper pasteurization) to yellow-green (non-proper pasteurization). The method uses no instruments and provides immediate results in 5 seconds and works at environmental temperatures that range from 2 ^{0}C up to 42 ^{0}C. Moreover, the results were stable, repeatable and not affected by the origin of the milk, the geographical area of the milk examined, the environmental conditions and the different pasteurization pattern or pasteurization time.
Conclusion*: "AP test", a novel enzymatic biochemical method, shows great potential as a screening test for a correct pasteurization process, making it the most sensitive test with a detection limit of 3.5 mU/L. It is fully compliant with the requirements and guidelines of W.H.O. which clearly state that proper pasteurization of milk means total inactivation of ALP. Convenience, speed and user-friendly orientation make the AP test an ideal tool for the dairy industry.*
Key Words*: Alkaline phosphatase , AP test, Milk Pasteurization, Quantitative Method, Qualitative Method.*

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I. Introduction

With very few exceptions, pasteurization is a crucial step in the processing of milk. Despite the long history of milk pasteurization as a well-measured public health strategy, the effectiveness of the process in terms of food safety outcomes remains a major concern. Especially as new pathogens appear, or as old pathogens reemerge. In order to confirm that conventional heat pasteurization is still valid, an effective food safety strategy must be defined to determine whether milk and milk products are properly pasteurized to reduce pathogens[1].

Milk pasteurization is now considered a common practice. To conduct a scientific examination of the procedure, it is necessary to have a thorough look into the history of milk pasteurization. Forty years before Pasteur's original studies, in 1824, recommendations were made for boiling milk at home before giving it to newborns. The National Milk Standards Committee was the first professional organization in the United States to establish a minimum time-temperature combination for the pasteurization of milk in 1911: 62.8 °C for 30 minutes (now known as the batch or holder method). Many people at the time believed that this heat treatment was slightly more than the recommended amount of heat exposure to kill the Mycobacterium tuberculosis[2], one of the most dangerous milk-borne infections at the time. After thorough analysis and research into the possibilities of the 'holding method' of milk pasteurization, the time-temperature combination introduced in 1911, became the first official and legally recognized method. The original Pasteurized Milk Ordinance was

published in 1924, using commercially available equipment as an effective pasteurization technique in the United States. The Ordinance stated that "a heating process of at least 61.1 °C for 30 minutes" could describe pasteurization.

A High Temperature Short Time pasteurization (HTST) requirement of 71.7°C for 15 seconds was added to the 1933 edition of the U.S. Public Health Service Milk Ordinance and Code, as a result of additional research on the thermal destruction of M. tuberculosis and other infections. In establishing the standard, attention was also paid to how the HTST treatment affected the creaming ability of the milk.

It was discovered in the late 1930s that M. tuberculosis/bovis was not as heat resistant as Coxiella burnetii, the cause of Q Fever[3]. According to studies published in 1956, some C. burnetii cells could survive at 61.7°C for 30 minutes, if they were present in large quantities in raw milk. The U.S. Public Health Service recommended raising the limit for the "holding method" of pasteurization to 62.8°C for 30 minutes, as a result of these investigations. It was also proposed to add at least an additional 2.8°C to the holding temperature for products with a fat content higher than whole milk or with added sugar.

The aforementioned pasteurization requirements have not changed since their introduction, with the exception of slight rounding of numbers to account for Fahrenheit-Celsius conversions. The International Dairy Federation states that 63°C for 30 minutes is the minimum time-temperature combination now accepted worldwide or 72°C for 15 seconds.

As a measurement of the effectiveness of the pasteurization process for milk, the phosphatase test is frequently used in quality control and food safety programs. Unexpectedly, the enzyme alkaline phosphatase, which is naturally present in raw milk, becomes inactive at 71.7°C after 15 seconds exposure.

The aim of the present study was to compare a series of different commercially available kits for proper milk pasteurization process (both quantitative and qualitative) with a newly developed test kit, named "AP test".

II. Material And Methods

The different commercial or experimental methods for alkaline phosphatase detection used in the milk industry can be divided into qualitative and quantitative. The activity of ALP is expressed as mU/L, where one unit equals the amount of enzyme that catalyzes the conversion of 1 umol of substrate per minute. The values for the measurement of ALP vary according to the origin of the milk. The accepted value for a successful milk pasteurization permitted by the regulatory authorities of the USA and the European countries is 350 mU/L and the ALP test is considered negative when the measured activity in cow's milk does not exceed this value[4].

II.1.Method Classification

The methods used to detect the activity of ALP can be classified into 4 categories:

A. Photometric
B. Fluorimetric
C. Chemiluminescent
D. Immunochemical

These methods have been applied for many years, however only photometric, fluorimetric and chemiluminescence methods have been recognized as valid methods to verify pasteurization of dairy products.

II.2. Photometric

Photometric methods are based on reactions using chromogenic substrates. The usual substrates are phenylphosphate, p-nitrophenyl phosphate and phenolphthalein phosphate, which are hydrolyzed to inorganic phosphate and phenol, p-nitrophenol or phenolphthalein, respectively[5]. In particular, in the first photometric methods applied, ALP removes a phosphate group from the substrate of disodium phenylphosphate. The liberated phenol group is extracted with butanol and then reacts with 2,6-dichloroquinone-chlorimide to form indophenol, a blue-colored substance. The activity of ALP in the milk sample is related to the absorption of the solution[6]. Subsequently, Aschaffenburg and Mullen in 1949 used p-phenylphosphate as a substrate, whose hydrolysis is catalyzed by ALP to form a yellow-colored product (p-nitrophenol) at pH 10.0[7] (Figure 1).

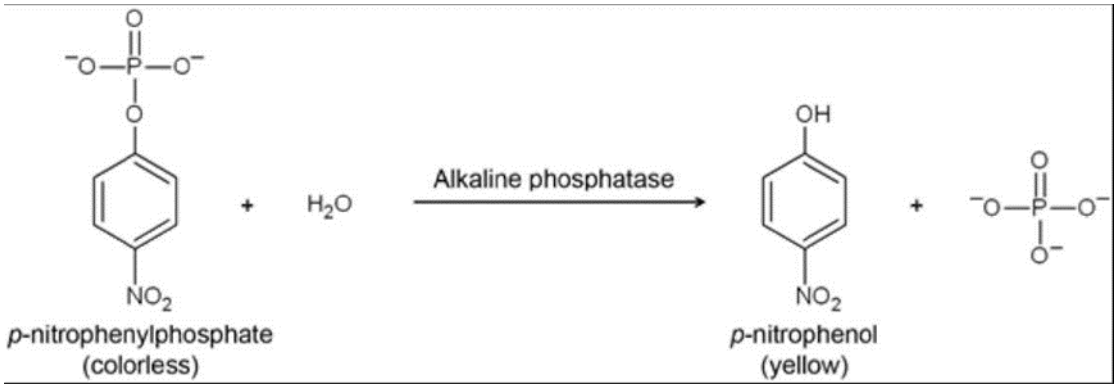

Figure 1. Phenylphosphate hydrolysis in the presence of ALP to form the colored product p-nitrophenol.

II.3. Fluorimetric

In fluorimetric methods, a non-fluorescent aromatic monophosphate ester (fluorophos) is used to determine ALP in milk and milk products. The presence of ALP in the sample causes its hydrolysis and yields a fluorescent molecule, the concentration of which is determined by fluorescence (stimulation: 439 nm, broadcast: 560 nm). Fluorimetric methods are approximately 100-1000 times more sensitive than photometric method's tests[8]. Other common fluorescent substrates are 4-methyl-7-hydroxycoumarinylphosphate (MUP) and 3,6-fluorescein diphosphate (FDP). Sequential hydrolysis, with alkaline phosphatase, of the two phosphate substitutes of FDP yields weak fluorescent monophosphate fluorescein followed by strongly fluorescent fluorescein (stimulation/emission ~ 490/514 nm)[9] (Figure 2) .

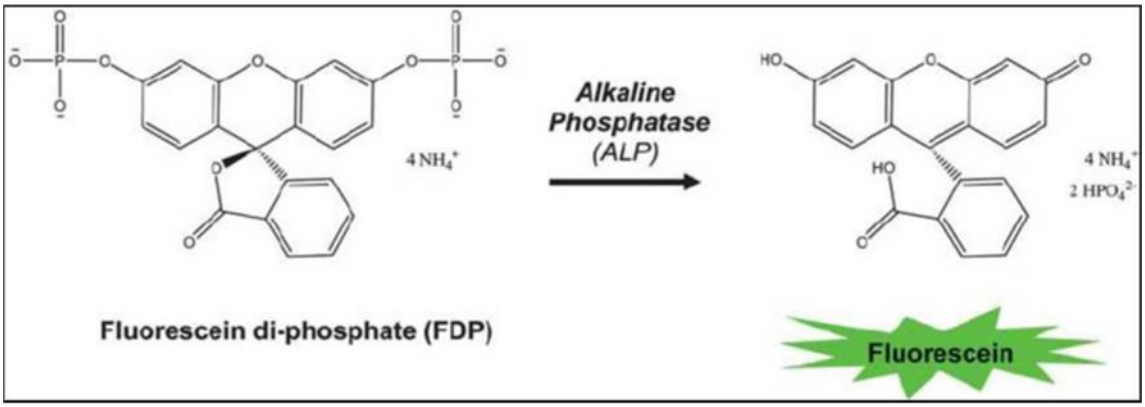

Figure 2. Fluorescein diphosphate dephosphorylation catalytic reaction, ALP presence and subsequent fluorescein production.

II.4. Chemiluminescence

Chemiluminescence assay relies on ALP-mediated dephosphorylation of adamantyl-1,2-dioxetan substrates (e.g.adamadyl-1,2-dioxetan phenylphosphate, CSPD). The aforementioned substrates are specifically identified and hydrolyzed by ALP to give a phenoxide product, which decomposes with prolonged light emission measured by a luminometer. Like previous methods, the higher the light intensity, the higher the enzyme activity (Figure 3).

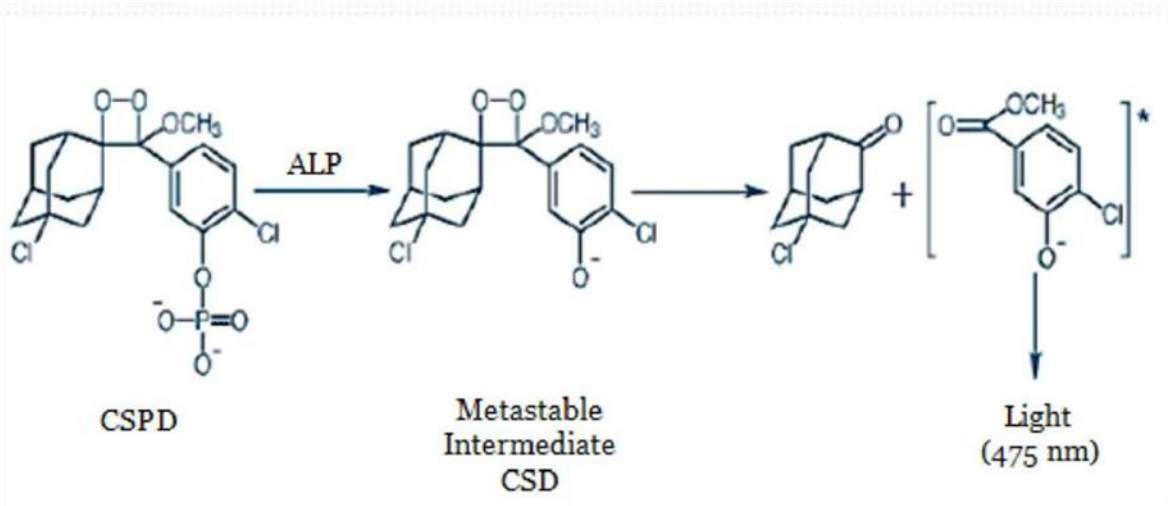

Figure 3. Enzymatic dephosphorylation of deoxetan CSPD in the presence of ALP, resulting in the formation of a phenolic intermediate anion which decomposes with light emission at 475 nm.

II.5. Other Techniques

Other techniques that have been described are:

- Immunochemical: such as competitive ELISA using polyclonal antibodies, specific to identify and differentiate bovine ALP from bacterial[10].
- Dry reagent chip type biosensor: where gold nanoparticles (AuNPs) are used as tracers and the positive reaction is verified by the color change in the control zone due to accumulation of AuNPs[11].
- Chromatography: where, after enzymatic hydrolysis of the substrate, p-nitrophenol is extracted and determined by reversed-phase liquid chromatography and measurement of absorbance at 319 nm [12].

The correct measurement of phosphatase in milk is done either quantitatively by using specific analytical apparatus (luminometer or spectrophotometer) or qualitatively to ascertain the level of phosphatase in pasteurized milk or its general presence in it immediately after completion of pasteurization. For proper pasteurization, the established limit is at 350 mU/l but this had been established in past decades when research and analysis regarding the consequences of the presence of phosphatase in milk were inadequate. Moreover the available commercial detection tests were minimal and without proper sensitivity. Currently, the accepted limit of phosphatase must be almost zero, since as a natural enzyme in milk it must be destroyed during pasteurization and be practically undetectable. Consequently, dairy farms are guaranteed that phosphatase, as a measurement indicator, ensures the destruction of pathogenic microorganisms during pasteurization. The higher the concentrations of phosphatase in pasteurized milk (in the quantitative measurements), or close to the detection limit (in the qualitative measurements), this constitutes a warning of improper pasteurization process, since the concept of pasteurization is the inactivation of phosphatase.

Two categories, quantitative and qualitative analysis of phosphatase and their key commercial or experimental detection tests, are listed below.

II.6. Quantitative determination of phosphatase in milk

II.6.1. Using a Luminometer:

II.6.1.1. CHARM F-AP: Product from CHARM SCIENCES Inc. – China

It is a one-step procedure without sample preparation and gives results in 45-90 seconds (depending on the nature of the sample). Limit of detection of phosphatase is set by the manufacturer at 20 mU / l. This product is certified by US National Conference of Interstate Milk Shipments (NCIMS), New Zealand Ministry of Agriculture and Forestry (MAF), Tasmanian Dairy Industry Authority (TDIA). This test utilizes a reagent incubated at room temperature before the addition of the sample, gives a quantitative phosphatase value to pasteurized milk and requires the use of a luminometer.

II.6.1.2. ZymoSnap ALP: Product of HYGIENA -US
It measures phosphatase levels below 350 mU/l and specifically at 222 mU/l. The test requires room temperature, incubation of the samples and reagent at 37°C in a water bath or in a Peltier plate, a luminometer device and variable volume pipettes. It gives quantitative results in 15 minutes.

II.6.2. Using a spectrophotometer
II.6.2.1. CHARM PASLite: Product from CHARM SCIENCES Inc. – China
Requires room temperature, preparation of samples and reagents in a centrifuge, incubation of samples and reagents for 3 minutes at 37 °C in a water bath or Peltier plate, spectrophotometer apparatus and variable volume pipettes. It gives results in 45-90 seconds (depending on the nature of the sample). The sensitivity limit for phosphatase detection in a sample is 20.5 mU/L. This product is ISO 22160/IDF 209 certified.

II.7. Qualitative determination of phosphatase in milk.

II.7.1. SENSOBIZ APT: Product from NANObiz Nano BiyoTeknolojik Sistemler – Turkey
The supplier indicates that incubation of the sample and reagents is not required, gives results in 5-10 minutes, works at room temperature and has a sensitivity limit for detection of phosphatase in pasteurized milk of 120 mU/l. This method is in line with the 91/180/EC which states that pasteurized milk must give a negative result to the presence of phosphatase and, on the contrary, heated milk must give a positive result to phosphatase. Uses color-coded test strips.

II.7.2. Phospatesmo Mi: Product of MACHEREY-NAGEL – Germany
It requires incubation for 60 minutes at 36°C in a water bath or Peltier plate, yields a result immediately after incubation and takes place at room temperature. Sensitivity limit for detection of phosphatase in a sample is 1750 mU/l. Uses color-coded measurement strips.

II.7.3. BIOO Scientific MaxSignal: Product of BIOO Scientific – USA
It is a colorimetric method. Its reagents are maintained at a temperature of 2-8°C and have a shelf life of 12 months. To perform the test, incubation for 30 minutes at 37°C in a water bath or in a Peltier plate is necessary, gives a result immediately after incubation and takes place at room temperature. Sensitivity limit for detection of phosphatase is 700 mU/l.

II.7.4. Lactognost III: Product from Heyl – Germany
It is one the oldest detection tests on the market. Incubation of 60 minutes at 37 °C in a water bath or Peltier plate is required, gives a colorimetric result after incubation and takes place at room temperature. Sensitivity limit for detection of phosphatase in a sample is 350 mU/l.

II.7.5. Alkaline Phosphatase: A product from Hardy Diagnostics – USA
Reagents are used. An incubation of 30-60 minutes at 37°C in a water bath or Peltier plate is required, yields a post-incubation result and is performed at room temperature. Sensitivity limit for detection of phosphatase is 700 mU/l.

II.7.6. AP test: Experimental test newly developed by Gerokomou et al. 2022[13]
"AP test" was developed with the main objective of implementing a simple hypersensitive rapid test for the detection of alkaline phosphatase in pasteurized milk. It simplifies the testing procedure and is not affected by exogenous factors (e.g. ambient temperature, humidity) and is not limited by the use of specific accompanying equipment. At the same time, an effort was made to reduce the sensitivity limit for phosphatase detection from 20 mU/L to 3.5 mU/L. AP test consists of three (3) reagents that are refrigerated at 2–8 ^{0}C and have an expiration date of two (2) years from the production date. The composition of the three individual reagents is as follows: **Reagent R1** - [Diethanolamine (DEA) 175 mL/L and Silver Iodide (AgI) 4 g/L]. **Reagent R2** - [p-nitrophenyl phosphate (pNPP) 18 g/L].**Reagent R3** - [Trypsin 1.5 ug/ L], used in volumes of 40, 10 and 5 mL respectively. The method using these three (3) reagents is kinetic enzymatic with colorimetric visualization of the result.

The chemical action mechanism used by the "AP test" is the following: Alkaline phosphatase (ALP) catalyses the hydrolysis of p-nitrophenyl phosphate at pH 10.4, releasing p-nitrophenol and phosphate. The percentage of p-nitrophenol is compatible with the catalytic concentration of phosphatase in the test sample (with a sensitivity limit of 1.5 U/L) and the sensitivity of this percentage is enhanced by the enzyme catalyst contained in AP reagent R3.

III. Results and Discussion

A comparison of various applied commercial or experimental pasteurization methods with the recently developed AP test is presented in table 1 with details regarding incubation time, required time for results, appropriate temperature for the conduction of each method, necessary equipment for each method, limit of detection and potential samples that can be evaluated by any commercial or experimental method.

Table 1. *Comparative table of advantages of the "AP test" with other commercially available tests (both quantitative and qualitative ones).*

	E. Incubation	Time for result	Temperature (^{o}C)	Required equipment	Limit of detection	Samples
AP Test	No	5 sec	2-42 ^{o}C	No	3,5 mU/L	Pasteurized milk, HTST, chocolate milk, milk cream, whey milk, cheese, butter
CHARM F-AP	No	45 - 90 sec	Room Temperature	Luminometer, centrifuge	20 mU/L	HTST, chocolate milk, whey milk
CHARM PASLite	3 min	45-90 sec	Room Temperature	Luminometer, spectrophotmeter, centrifuge	20 mU/L	HTST, chocolate milk, whey milk
SENSOBIZ APT	No	5-10 min	Room Temperature	No	120 mU/L	Pasteurized milk, HTST
MACHEREY-NAGEL Phosphatesmo MI	60 min	Results after incubation	36 ^{o}C	Incubator	1750 mU/L	Pasteurized milk
BIOO SCIENTIFIC MaxSignal	30 min	Results after incubation	37 ^{o}C	Incubator	700 mU/L	Pasteurized milk, cheese
Heyl Lactognost III	60 min	10 min after incubation	37 ^{o}C	Water bath, centrifuge, incubator	350 mU/L	Pasteurized milk, whey milk, milk cream, butter
HARDY Alkaline Phosphatase	30-60 min	Results after incubation	37 ^{o}C	Water bath, incubator	700 mU/L	Pasteurized milk, butter, whey milk, cheese
HYGIENA ZymoSnap	10 min at 20-25 ^{o}C + 5 min at 37^{o} C	Results after incubation	Room Temperature	Water bath, luminometer	222 mU/L	Pasteurized milk, whey milk, HTST

Under standard conditions of assay, the ALP method has been shown to be a remarkably valuable tool for the routine assessment of milk pasteurization validation. Between the quantitative and qualitative methods and their corresponding commercial or experimental test kits, the newly developed AP test by Gerokomou et al has crucial benefits, such as:

Incubation: All the quantitative and quantitative kits require from 45 seconds (Charm F-AP) to 60 minutes (e.g. Phosphatesmo MI) to incubate. "AP test" and Sensobiz APT need no incubation.

Time to results: Charm F-AP gives results in 45-90 seconds, Lactognost Heyl gives results 10 minutes after the end of the incubation period, Sensobiz APT gives results in 5 to 10 minutes and "AP test" gives results in 5 seconds.

Temperature: Alkaline phosphatase test from Hardy is performed at 37°C, Charm PAS-Lite at room temperature and "AP test" is performed in both room and outdoor temperatures, ranging from 2 to 42°C.

Equipment: All commercial methods need a range of equipment to carry out. Sensobiz APT and "AP test" need no equipment.

LoD (limit of detection): The detection limit of Lactognost Heyl is at 350 mU/L, of Sensobiz APT at 120 mU/L, of Charm F-AP at 20 mU/L and of the "AP test" is at 3.5 mU/L.

Sample type: Each commercial kit is ideal for specific sample types but only AP test is suitable for pasteurized milk, HTST milk, chocolate milk, whey milk, cream, cheese, butter.

"AP test" is the most sensitive, state-of-the-art phosphatase detection test available. It meets the global demand as it is modern, affordable (without the use of accompanying equipment), fast, ultra-sensitive, easy to use and it can be performed anywhere, over a wide range of temperatures. Comparative analysis between "AP test" and all other marketed tests worldwide showed that the sensitivity limit for detecting phosphatase in pasteurized milk is 3.5 mU/L for "AP test", whereas the next most sensitive test is CHARM at 20 mU/L. By

introducing the "AP test" into the milk handling process, any cases of bacterial contamination are minimized producing excellent quality dairy products fit for consumption.

In a dairy processing plant, a significant loss of time has been observed from the time the pasteurization control process ends until the result (correct or not) of the control is received. With marketed tests from other companies, the time loss can reach up to 60 minutes.

One of the many advantages of the AP test is that it practically compensates for the time lost (up to 70 minutes) in the production process when the other tests were used. A direct consequence of this is the optimal management, redeployment and/or reallocation of human resources to other tasks, increasing the production line and reducing the cost of electricity in each dairy unit. As for the increase in production process time per year, it amounts to (approximately) 365 days x 1 hour = 365 hours. So for example, in a small dairy factory pasteurizing fifteen (15) tons of milk per day, 365 hours per year are gained and added to the production process

IV. Conclusion

"AP test" is the only test that has been successfully tested in both room and outdoor ambient temperatures, ranging from 2°C to 42°C. Therefore, there is practically no geographical limitation associated with local environmental temperatures. These properties render "AP test" ideal for commercial exploitation and easy application in the dairy process. Using "AP test", the work of a food technologist and a chemist are simplified.

No special equipment (such as centrifuges, luminometers, spectrophotometers, water baths, Petri plates) is required to perform the "AP TEST". The only required equipment is three (3) pipettes of variable volume 0.5 - 10 µl, 10 - 100 µl, 100 - 1000 µl. This leads to a dramatic reduction in capital investment costs for each dairy plant. The test can be carried out effortlessly using cutting-edge technology and it is affordable for all dairy plant sizes.

"AP TEST" was tested with 100% result accuracy for two (2) years with 100% result repeatability, in four (4) different dairy units in different geographical areas across Greece. In this way, we were able to certify the accuracy of the test, taking into account all the different variables (type of milk, type of product, temperature variations per region, type of breeding animal, different breeds per species, different fat concentration in raw milk, time period of intake of milk). The method of this test makes it an ideal export product of 100% Greek origin and know-how.

In conclusion, the use of the "AP TEST" is suggested as the most valid method to detect the correct pasteurization of milk, for the following reasons: sensitivity, repeatability, ease of use and user-friendliness for a food technologist or laboratory chemist. Moreover, it is economically beneficial for a dairy unit and it can be easily used in many geographical areas around the world.

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