Zeitschrift: Mitteilungen aus Lebensmitteluntersuchungen und Hygiene = Travaux de chimie alimentaire et d'hygiène

Herausgeber: Bundesamt für Gesundheit

Band: 92 (2001)

Heft: 6

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DOI: https://doi.org/10.5169/seals-981931

Determination of Lactoperoxidase in Heat Treated Milk: Comparison of a New Rapid Quantitative Reflectometric Test with a Qualitative Reference Test

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Received 6 July 2001, accepted 9 October 2001

Introduction

The detection of lactoperoxidase activity in milk is generally used as an indicator of heat treatment (1, 2) especially for the differentiation between flash and high temperature pasteurization within the range from 70 to 82.5°C (3–6). Inactivation of lactoperoxidase depends on temperature but also on applied heating duration thus resulting in a given heat load. Therefore, different process types need different target temperatures (2) (e.g. a plate heat exchanger in an industrial process and a mobile heater used as a batch process in small dairy plants).

The lactoperoxidase system in bovine milk is an important component of the antimicrobial activity of correctly pasteurized milk (7, 8). Within the EU (directive 92/46) and in Switzerland (9) pasteurized milk has to be phosphatase negative but peroxidase positive to guarantee a sufficient but not excessive heat treatment. UHT (ultra heat treated) and high temperature pasteurized milks have to react lactoperoxidase negative (9, 10). Studies carried out in Switzerland from 1986 to 2000 showed that the average pasteurization temperature decreased from about 84 to 74°C. Consequently, the sensory quality rose (11). It was also shown that higher pasteurization temperatures do not improve the shelf life of the product.

Lactoperoxidase is well described in the literature (12, 13). Activity measurement, kinetics and reaction mechanism of peroxidase have already been studied (14–21). Peroxidases catalyse the oxidation by peroxide of numerous substances to oxidation products which themselves often have strong absorption bands or which

absorb strongly in the visible range (17). A study of lactoperoxidase inactivation in a pasteurizer claimed unsatisfactory variations of the assay used, as well as interferences from milk proteins (5). However, only recently a rapid, easy-to-use quantitative method became available for the measurement of low lactoperoxidase activities in milk (3).

So far, no international reference method has been available (21) for quantitative measurements but a proposal is currently under development (22). Photometric determinations of peroxidase activity have been available for many years based upon different chromophoric donors (3, 6, 15, 17–20, 23, 24). Studies showed that 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is the most sensitive donor, having moreover further advantages (20). Recently a chemiluminescent measurement of peroxidase in medical fields revealed a very low detection limit of 10^{-19} mol/assay (25) but it did not give satisfactory results for lactoperoxidase (24). Additionally a qualitative method exists (26) as well as a commercial semi-quantitative microtest. In Germany, a qualitative test using Traventol® reagent (using guaiacol as donor) is in widespread use (data not shown). Some authors have proposed methods that can be automated (3) using flow injection analysis (24) or a reflectometric methode for the determination of the heat treatment of pasteurized milk using gamma-glutamyltransferase as a marker (33).

In a collaborative study carried out in 1999 *Bosset et al.* (27) demonstrated the suitability of a qualitative lactoperoxidase test for milk based on the qualitative reference method valid in the EU (Storch test) (26). The validation step carried out within this collaborative study made it possible to introduce the method into the Swiss Food Manual. This slightly modified method has achieved official status in Switzerland since 1999 (28).

Concurrently, the prototype version of a new sensitive and rapid quantitative test kit has been developed for the estimation of lactoperoxidase activity in UHT milk. The Reflectoquant® peroxidase test was not available when the collaborative study was carried out. The aim of this study was to compare the Reflectoquant® peroxidase test to the official Swiss qualitative lactoperoxidase test and to check this prototype in order to improve it if necessary. Samples were prepared in the same range as in the collaborative study (27) and were assessed with both tests. In order to obtain data on the lactoperoxidase content in Swiss market milks both procedures were used to analyse several commercial pasteurized, UHT and one ESL (extended shelf life) milk samples from Swiss dairies.

Materials and methods

Preparation of samples

Heat treated milks

A single consignment of raw milk, obtained from a local dairy plant, was thermised at 70° C for 15 s. Aliquots were then pasteurized in the heat range where the

official qualitative method of the Swiss Food Manual is no more sensitive i.e. at 79, 80, 81, 82 and 83° C for 20 s using a tube heat exchanger (Stork equipment, Kundert Engineers, Zurich) and homogenised with a two step homogeniser at 12 MPa.

Mixtures of UHT with thermised milks

Commercial UHT milk was used to prepare mixtures with 10 %, 5 % and 2.5 % thermised milk as mentioned above.

Pasteurized, UHT and ESL milks

Six pasteurized, eight UHT and one ESL milk samples, representing all industrial producers, were collected from the Swiss market. Heat treatment of the pasteurized milks ranged from 73 to 75° C and from 12 to 20 s. For the UHT and ESL milks no data were available.

Analytical methods

Qualitative lactoperoxidase test Swiss Food Manual (modified EU-method, Storch test)

The preparation of the reagents and the operating procedure have been described in detail (27). All measurements were performed in duplicates.

Reflectoquant® peroxidase test (prototype)

The Reflectoquant® peroxidase test (fig. 1) is based on test strips containing the chromophoric donor, stabilisers and a buffer system (test patch and blank patch) and a solution of hydrogen peroxide. In the Reflectoquant® peroxidase test, 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of hydrogen peroxide serves as the substrate for the lactoperoxidase in the sample. Quantitation is obtained using a mobile pocket reflectometer (RQflex, also used for other test kits) at 23° C (temperature dependency maximally 5 %/° C). The enzyme activity in the sample is proportional to the concentration of the blue dye formed, which is determined within the wavelength range of 650 to 660 nm. The results are expressed in U/l. The RQflex is calibrated with a barcode strip based on activity values measured by a proposed photometric reference method (22). The measurements of lactoperoxidase range from 5 to 175 U/l (approximately 0.05–1.75 mg/l or 0.6–22 pmol/l using the data given in references (15) and (29)).

This study employed a prototype version of the test kit and was performed according to the manufacturers instructions (30). All duplicates were prepared simultaneously and then tested as follows:

- A sample with a high lactoperoxidase activity was prediluted with UHT milk. If no indication or previous experience is available it is necessary i) to predilute it 1:20; ii) to continue as described below and repeat this procedure until a value in the measuring range is reached.

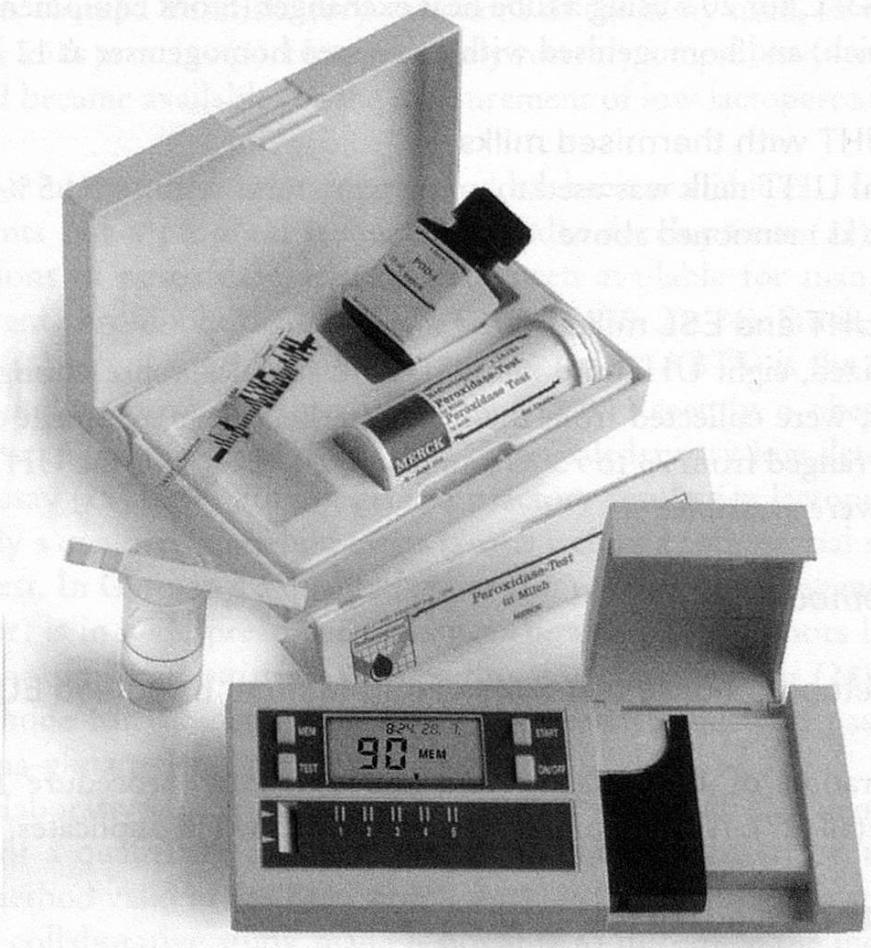

Figure 1 **Reflectoquant® peroxidase test**

- The sample was then diluted 1:5 with distilled water using a glass vessel.
- Five drops of manufacturer reagent "POD-1" (H_2O_2 0.3 ml/l) were added and mixed at room temperature (23° C ± 3° C).
- The test strip was dipped into this solution for approximately 2 s.
- The excess liquid was removed by tapping the long edge of the strip onto a paper towel. The aim is to make the movement in such a way that the exess liquid is removed without mixing the colours on the strip and without removing any of the colours from the strip.
- After 180 s the strip was optically measured with the RQflex. The result read on the display in U/l was automatically corrected using the dilution factor.

Results and discussion

Table 1 shows the results of both methods and figure 2 of the coloured solutions of the qualitative test for raw, thermised, pasteurized and mixtures of UHT with thermised milk. Statistical analysis of the results was performed by ANOVA. Standard deviations and relative standard deviations are presented in table 2.

Table 1
Results of the two methods applied to raw, thermised and mixtures of UHT with thermised milk

No	Milk sample	F2	Replicate	Reflectoquant® peroxidase Test (U/l) a*	b*	c*	d*	e*	mean	Qualitative Lactoperoxidase Test Score	Result of the test
1	*raw (=100 %)*	1:20	1*	1600	1460	1280	1640	1460			
			2*	1440	1640	1360	1520	1400			
			mean	1520	1550	1320	1580	1430	*1480*	2	*positive*
2	*thermised (=100 %)*	1:20	1*	1480	1580	1660	1560	1420			
			2*	1560	1700	1460	1480	1500			
			mean	1520	1640	1560	1520	1460	*1540*	2	*positive*
3	*pasteurized at 79° C*	–	1*	111	126	105	121	114			
			2*	139	119	118	113	129			
			mean	125	123	112	117	122	*120*	1	*trace*
4	*pasteurized at 80° C*	–	1*	8	6	7	7	9			
			2*	9	6	7	7	8			
			mean	9	6	7	7	9	*7*	0	*negative*
5	*pasteurized at 81° C*	–	1*	≤5	≤5	≤5	≤5	≤5			
			2*	≤5	≤5	≤5	≤5	≤5			
			mean	≤5	≤5	≤5	≤5	≤5	*≤5*	0	*negative*
6	*pasteurized at 82° C*	–	1*	≤5	≤5	≤5	≤5	≤5			
			2*	≤5	≤5	≤5	≤5	≤5			
			mean	≤5	≤5	≤5	≤5	≤5	*≤5*	0	*negative*
7	*pasteurized at 83° C*	–	1*	≤5	≤5	≤5	≤5	≤5			
			2*	≤5	≤5	≤5	≤5	≤5			
			mean	≤5	≤5	≤5	≤5	≤5	*≤5*	0	*negative*
8	*90 % UHT+ 10 % thermised*	1:2	1*	172	178	194	208	206			
			2*	164	172	178	204	190			
			mean	168	175	186	206	198	*187*	1	*slightly positive*

No	Milk sample	F2	Replicate	Reflectoquant® peroxidase Test (U/l) a*	b*	c*	d*	e*	mean	Qualitative Lactoperoxidase Test Score	Result of the test
9	95 % UHT+ 5 % thermised	–	1*	124	129	133	121	113			
			2*	122	124	115	132	117			
			mean	123	127	124	127	115	123	1	slightly positive
10	97.5 % UHT+ 2.5 % thermised	–	1*	59	49	51	55	52			
			2*	51	51	50	60	54			
			mean	55	50	51	58	53	53	1	trace
11	100 % UHT+ 0 % thermised	–	1*	≤5	≤5	≤5	≤5	≤5			
			2*	≤5	≤5	≤5	≤5	≤5			
			mean	≤5	≤5	≤5	≤5	≤5	≤5	0	negative

F_2 = predilution factor

* = Replicates 1 and 2 were prepared simultaneously; replicates a to e were not prepared simultaneously.

Comparison of the qualitative with the Reflectoquant® peroxidase test

The Reflectoquant® peroxidase test, which has been designed to detect low lactoperoxidase activities, is significantly more sensitive than the qualitative lactoperoxidase test. Raw and thermised milks give high values for lactoperoxidase, respectively positive scores, thus leading to samples (diluted with UHT milk) which are still rich in lactoperoxidase. In case of pasteurized milks, the qualitative test shows a trace positive value at the lowest treatment temperature (79° C) (fig. 2), whereas a differentiation between the different pasteurization temperatures is possible with the Reflectoquant® peroxidase test. A heat treatment at 79° C, and even at 80° C, can be differentiated from higher heat treatments such as 81–83° C.

Mixtures of 10 %, 5 % and 2.5 % thermised milk in UHT milk result only in slight and trace positive values with the qualitative test. With the Reflectoquant® peroxidase test, the mixtures result in more sensitive and significantly different values.

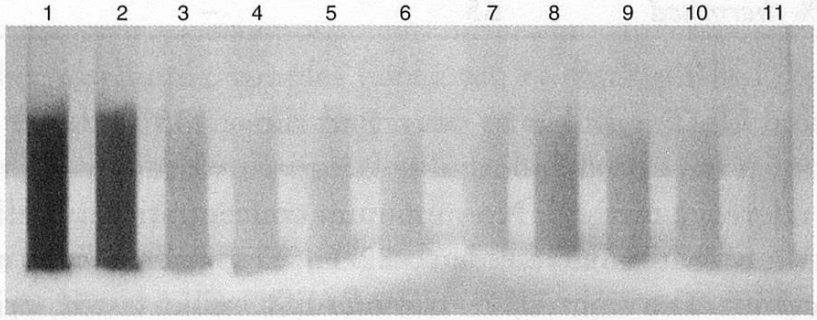

Figure 2 **Coloured solutions of qualitative test.** Sample numbering in table 1

Analytical data from the Reflectoquant® peroxidase test

The standard deviations (table 2) were comparable with those of a first collaborative study organised by DIN (8–49 U/l for mean values in the range from 290 to 480 U/l, (data not shown). The standard deviation at a very low value such as 7 U/l is approximately 1 U/l. The quantification limit of a method is usually defined as ten times the detection limit (31). This results in a quantification limit of 50 U/l. The definition of the quantification limit is valid in all cases where the presence of an analyte has to be positively determined and the analyte has to be quantified. However, the Reflectoquant® peroxidase test was also designed for cases where clear absence of lactoperoxidase (negative test) is questioned. The test has its highest sensitivity in the range from 5 to 50 U/l with an expected relative standard deviation of ±10 % (data not shown). At this concentration the qualitative lactoperoxidase test gives only slightly positive or negative results. Reflectoquant® peroxidase test calibration is cut off at 5 U/l due to the instability of the reflectometer.

Table 2
Standard deviation and relative standard deviation of Reflectoquant® peroxidase test

Milk sample	Reflectoquant® peroxidase test mean (U/l)	standard deviation s (U/l)	relative standard deviation RSD (%)
raw (=100 %)	1480	120	8.1
thermised (=100 %)	1540	89	5.8
pasteurized at 79° C	120	10	8.2
pasteurized at 80° C	7	1	14.5
pasteurized at 81° C	≤5	–	–
pasteurized at 82° C	≤5	–	–
pasteurized at 83° C	≤5	–	–
90 % UHT+10 % thermised	187	16	8.5
95 % UHT+5 % thermised	123	7	5.6
97.5 % UHT+2.5 % thermised	53	4	7.1
100 % UHT+0 % thermised	≤5	–	–

In Switzerland UHT milk has to be treated above 135° C. UHT and high pasteurized milks must have no demonstrable lactoperoxidase activity, measured with the qualitative reference method. No minimum concentration is defined. The data presented here show that milk treated at >82° C (20 s) will show an absence of lactoperoxidase activity. The eight UHT and one ESL milks tested, originating from eight Swiss dairy plants, did not show any detectable lactoperoxidase activity (showing the reading "Lo") with the Reflectoquant® peroxidase test, and therefore contained less then 5 U/l.

On the other hand, correctly pasteurized milk must show lactoperoxidase activity, measured using the qualitative reference method. The activity values depend to a large extent on the original activity of the raw milk and are therefore difficult to compare and reproduce from study to study. Based on the activity of the raw milk used in this study, thermised milk in UHT milk is theoretically detectable at a concentration as low as 0.3 % with the Reflectoquant® peroxidase test because the value for a mixture containing 2.5 % thermised milk is still 10 times higher than the detection limit of the test (5 U/l). Unintended mixtures of milks with different heat treatments are therefore easily detectable.

Determination of the applied heat treatment

Heat treatments at 79 and 80° C (both for 20 s) showed a mean lactoperoxidase activity ranging from 7 to 120 U/l (table 1) while no activity could be detected (<5 U/l) in milks treated at 81 to 83° C. The data where compared with the qualitative values of *Bosset et al.* (27, table 3). They showed similar values in the overlapping temperature range. The quantitative test is more sensitive than the qualitative test.

Table 3
Comparison of values from the current study with those of *Bosset et al.* (27)

heat treatment	*qualitative lactoperoxidase test (this study)*	*qualitative lactoperoxidase test (Bosset et al.)*	
temperature (20 s each)	*key/description read of after 60 s*	*key/description read of after 30 s*	*key/description read of after 60 s*
77° C		2/positive	2/positive
78° C		1/slightly positive[1]	1/slightly positive
79° C	1/trace	0/negative	0/negative
80° C	0/negative	0/negative	0/negative
81° C	0/negative	0/negative	0/negative
82° C	0/negative		
83° C	0/negative		

[1] The majority of the participants in the collaborative study found slightly positive values, however some obtained negative results.

Raw and thermised milk samples could not be distinguished (table 2) by their mean activity values but were distinguishable by their standard deviations. This may be due to the double homogenisation step included in the thermisation procedure.

Table 4 lists the activities of six pasteurized market milks. The values range from about 200 U/l to about 3000 U/l. All milk samples were correctly treated. In the milks of plants nos. 2 to 5 no substantial loss in activity could be detected.

Table 4
Lactoperoxidase content of pasteurized milks from the Swiss market

Dairy plant no.	*Replicate*	*Reflectoquant® peroxidase test (U/l)*			
		F_2		*mean (U/l)*	*relative standard deviation (RSD %)*
1	1	1:5	200		
	2	1:5	242	*221*	13.4
2	1	1:20	2600		
	2	1:20	3200	*2900*	14.6
3	1	1:20	2740		
	2	1:20	3100	*2920*	8.7
4	1	1:20	2240		
	2	1:20	2340	*2290*	3.1
5	1	1:20	2080		
	2	1:20	1960	*2020*	4.2
6	1	1:5	390		
	2	1:5	340	*365*	9.7

F_2 = predilution factor

Table 5
Lactoperoxidase activities for thermised and mixtures of UHT with thermised milks. Comparison of measured with expected values

Sample	*Reflectoquant® peroxidase test*			
	expected activity (U/l)	*F_2*	*measured activity (mean) (U/l)*	*measured-expected activity (U/l)*
thermised milk	1540	1:20	*1540*	±0
10% thermised milk	154	1:2	*187*	+33
5% thermised milk	77	–	*123*	+46
2.5% thermised milk	39	–	*53*	+14
UHT (0% thermised milk)	0	–	*≤5*	~0

F_2 = predilution factor

Detection of thermised in UHT milk

All mixtures (10%, 5%, 2.5%) showed detectable lactoperoxidase activity, UHT milk gave negative results (<5 U/l, table 1). The measured lactoperoxidase activities were higher than the expected (calculated) activities (table 5 and fig. 3) but no explanation could be found.

In the literature, various factors decreasing the activity or thermostability of lactoperoxidase have been described (12, 13, 15, 16).

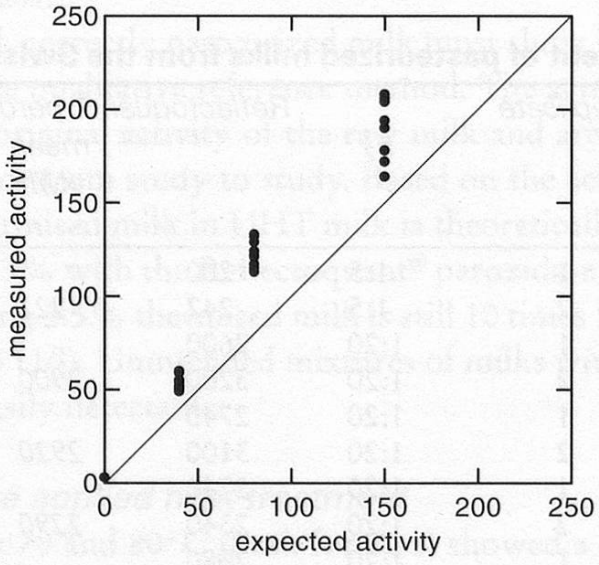

Figure 3 **Comparison of expected vs. measured lactoperoxidase values**
From left to right, the measuring sequence is the following: samples no. 11, 10, 9 and 8 (see table 1)

Concentration and dilution factor (12, 13, 15, 16)

When lactoperoxidase solutions were diluted to reach concentrations more appropriate for an assay, a loss of activity with time was observed, probably due to adsorption of the enzyme onto the glass surface of the cuvette. This may also lead to aggregation and turbidity. It was observed that a 50 times more concentrated solution (25 mg lactoperoxidase/l) did not loose activity over 3 h. On the other hand, at higher concentrations an aggregation of lactoperoxidase monomers to catalytically more active trimers and higher species was observed.

pH value and protein influence (12, 13)

Thermostability in permeate and in buffer was lower than that in whey or in milk. The largest decrease in activity was observed at pH 5.4, amounting to a loss of 15 % of activity in 15 min, indicating that analysis should be carried out quickly following the final dilution of lactoperoxidase solutions. The lactoperoxidase activity varied linearly with a protein concentration (12.5–125 µg/l) in a 0.1 mol/l sodium acetate buffer at pH 4.4, 5.5 and 6.5, respectively. All this indicates that lactoperoxidase is less stable during heating at acid pH.

Light influence (13)

Light has also been found to have a deleterious effect on lactoperoxidase activity in milk and in whey. The photochemical inactivation was irreversible.

Influence of calcium on the previous factors (13)

Monget and Laviolette claimed that several of their observations changed due to Ca^{2+} binding to lactoperoxidase. Calcium ion activity seems to be a key factor for the activity and thermostability of lactoperoxidase. A decrease in the structural stability of lactoperoxidase was found on lowering the pH, and a dilution of milk with water or buffer has a destabilising effect.

None of these factors, however, can explain the higher activities measured compared to those calculated. One would expect lower values for the mixtures in case of a higher activity of thermised milk if it were a polymeric, dilution or a photochemical effect. Moreover, most of the samples in this study were treated in the same manner. Raw, thermised and the 10 % mixture all had to be prediluted with UHT milk but not using the same predilution factor. All samples were diluted with distilled water in the assay and all of them were prepared for measurement in glass vessels within a short time. They were all exposed to light for a short time.

Dry reagent strip tests are known to have sources of systematic error (32). For the Reflectoquant® peroxidase test these are: i) too long delay between dipping the strip in the milk and starting the RQflex, ii) reagent-patch contamination caused by failure to remove excess sample solution by tapping the strip the wrong way onto a towel or when shaken off, iii) failure to position the strip correctly in the RQflex, iv) contamination of the optical system by a strip containing excessive sample solution,

Table 6
Lactoperoxidase activities for different milks, data from literature

sample	*original lactoperoxidase concentration*	*activity[1] (U/l)*	*source*
buffalo whole milk	7315±0.134 U/ml	*7300±130*	(20)
milk	30 mg/l	*3000*	(13)
milk	10–30 µg/ml	*1000–3000*	(7)
milk	738–3889 mU/ml	*700–3900*	(7)
milk	0.21–1.51 U/0.1 ml	*2100–15100*	(18)
pasteurized milk (photometric method)	392 U/l	*400*	[2]
pasteurized milk (reflectometric method)	373 U/l	*400*	[2]
raw milk	0.42–0.93 U/ml	*400–900*	(4)
whole milk	5724±0.274 U/ml	*5700±270*	(20)

[1]=calculated using the data given in (15) and (31), values rounded
[2]=data not published

and v) dipping a new strip into a previously tested milk sample (contamination with strip reagents).

Finally the order of magnitude of the values found with raw and thermised milks in table 1 are approximately 1000 U/l lower than those found with pasteurized milk reported in table 4. These data were compared with those from the literature (table 6). The comparison is confounded by the various chromogens used. None the less, the data from this study do not seem to be abnormal.

None of this information can explain the higher measured activities in the mixtures (fig. 3). It is not clear if the original activity of the thermised milk was underestimated or the activities of the mixtures overestimated. Other causes may be responsible, such as the non-linearity of the measuring system (Lambert-Beer like law) or a lack of substrate when measuring thermised milk. As a consequence of these results the calibration of the Reflectoquant® peroxidase test kit was adjusted in the meantime by the manufacturer and is done with samples measured with the above mentioned quantitative reference method (22).

Conclusions

High pasteurization is defined by the legislator using the lactoperoxidase content of the heat treated milk, measured with the qualitative reference method. Dairy practice needs a complementary sensitive and fast test system for process control. The prototype version of the Reflectoquant® peroxidase quick test was tested in this sense. The results showed a need for improvement. The improved version will be a helpful instrument for dairy practice and meet the requirements for use as a routine method in dairy plants and laboratories. This test is easy to use needs no clearifying reagent and gives quantitative values.

Acknowledgements

We wish to thank *Lychou Abbühl-Eng*, *Roland Gauch*, *Claude Hegel*, *Doris Mühlethaler*, *Marie-Therese Raemy*, *Franziska Schärer*, *Verena Sennrich* and *Andreas Spahni* (Swiss Federal Dairy Research Station) for they technical help in preparing the samples and for the measurements as well as *W.J. Looney*, *Walter Siegenthaler* and *Gerda Urbach* (Australia) for their kind linguistic assistance as well as for the final reviewing of this article.

Summary

The prototype of a new quantitative reflectometric rapid test for the estimation of lactoperoxidase activity in heat treated milk, Reflectoquant® peroxidase test, was compared with the official qualitative method of the Swiss Food Manual within the upper temperature range of the denaturation of the enzyme (79–83° C). Raw, thermised, pasteurized and UHT milk from the Swiss market, as well as mixtures of thermised and UHT milks were measured. The reflectometric test gave reproducible results in the range of 5–175 U/l with relative standard deviations around 5 %. With a detection limit of 5 U/l this test was more sensitive than the official qualitative method. Some validation data are presented. The Reflectoquant® peroxidase test is easy to use and suitable for both in dairy plants and laboratories.

Zusammenfassung

Der Prototyp eines neuen quantitativen reflektometrischen Schnelltests zur Bestimmung der Lactoperoxidaseaktivität in hitzebehandelter Milch, der Reflectoquant® Peroxidasetest, wurde mit der offiziellen qualitativen Methode aus dem Schweizerischen Lebensmittelbuch im oberen Temperaturbereich der Denaturation des Enzyms verglichen. Rohe, thermisierte, pasteurisierte und UHT-Milchproben aus dem Schweizer Markt sowie Mischungen aus thermisierter und UHT-Milch wurden analysiert. Der reflektometrische Test zeigte reproduzierbare Aktivitäten im Bereich zwischen 5 und 175 U/l, mit relativen Standardabweichungen um 5 %. Mit einer Nachweisgrenze von 5 U/l ist dieser Test empfindlicher als die offizielle qualitative Methode. Einige Validierungsdaten sind aufgeführt. Der Reflectoquant® Peroxidasetest ist einfach zu handhaben und geeignet für den Einsatz in Molkereien und Laboratorien.

Résumé

Le prototype d'un nouveau test reflectométrique quantitatif rapide pour l'évaluation de l'activité lactoperoxydasique de laits traités thermiquement (Reflectoquant® peroxidase test) a été comparé à la méthode qualitative officielle du Manuel suisse des denrées alimentaires dans le domaine de température supérieur ou cet enzyme est dénaturé. Des laits cru, thermisé, pasteurisés et UHT du marché suisse ainsi que des mélanges de laits (thermisé/UHT) ont été mesurés. Ce test reflectométrique donne des valeurs reproductibles entre 5 et 175 U/l avec un écart-type d'env.

5 %. Avec une limite de détection de 5 U/l, il est plus sensible que le test qualitatif susmentionné. Quelques données de validation y sont indiquées. Le Reflectoquant® peroxidase test est d'un emploi aisé tant en fabrication qu'au laboratoire.

Key words

Lactoperoxidase, Rapid test, Pasteurization, UHT

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