The Clarifying Reagent, or how to make the analysis of milk and dairy products easier

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The Clarifying Reagent, or how to make the analysis of milk and dairy products easier

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Difficulties with the analysis of opaque milk and dairy products can be resolved by rendering the sample transparent. This allows direct photometric measurements of the reaction mixtures, and all sample pre-treatment steps become unnecessary. For this, several reagents have been reported in literature. Among them, the 'Clarifying Reagent' is mainly described here: its composition is given because it is now released from patenting restrictions. Its main property is a low absorbance between 340 and 800 nm. So it can be used in many chemical and enzymatic colorimetric measurements for estimating the quality of milk and dairy products.

Keywords: milk analysis, raw milk, heated milk, cheese, clarification of milk, the Clarifying Reagent.

All raw milk components can be subject of chemical or enzymatic modifications or of degradations during storage, and so it must be processed or consumed promptly. The stability of the mineral and protein equilibrium depends on the conditions of storage, on the presence of bacteria, on the action of endogenous and exogenous enzymes. Processed milk or dairy products have to be analysed to monitor their hygienic quality for the security of the consumer and the overall quality of the product. Incidences of the technological treatment applied to the milk during a process also have to be evaluated by using chemical or enzymatic analysis of the processed milk or manufactured products.

Milk is a complex biological liquid with heterogeneous composition and 3 physical states. It is at once a solution of whey proteins, a colloidal suspension of micellar casein and an emulsion of fat in this aqueous mixture. Consequently, milk is opaque to light. Therefore, in analytical dairy laboratories, milk turbidity due to micelles of phosphocaseinate and fat globules calls for sample pretreatment by precipitation, centrifugation and filtration before spectrophotometric measurement. These preliminary steps are time-consuming, tedious and often costly.

Difficulties in the analysis of opaque milk and dairy products can be resolved by rendering samples transparent. This allows direct photometric measurements of the samples or reaction mixtures, and all the above preliminary sample pre-treatment steps become unnecessary.

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Diverse attempts at the clarification of milk

Clarification or dissolution of samples or of reaction mixtures is not a recent objective. Table 1 reports the major dissolving reagents that have been proposed in the literature. Nakaï & Anh Chi Le (1970) were among the first to directly determine proteins and lipids in total milk. The protein content of clarified milk and dairy products determined from absorbance at 280 nm of the medium is well correlated with the usual Kjeldahl method. Later Bosset et al. (1977a, b) proposed two dissolving reagents, which lead to a 0·200 residual absorbance at 500 nm of clarified milk sample. The major application has been the measurement of total proteins by the Biuret method (Bosset & Blanc, 1977) for which a good correlation is obtained with Kjeldahl method.

From these precedent investigations, clarification may be defined as the result of the combined actions of solvents, detergents and base. The chemical compounds, which produce a rapid and complete dissolution of casein micelles and fat globules responsible for milk turbidity, must be miscible. The aqueous alkaline solutions of Bosset et al. (1977a, b) are responsible of the disruption of disulphide and hydrogenous bonds, this provokes the breakdown of secondary, tertiary and quaternary structures of the proteins. The base shifts the calcium out of the micelles and consequently decreases the cohesion of this

Table 1. Diverse chemical mixtures for the dissolution of milk

Reference	Chemical composition of the reagent	Comments	Applications
Nakai & Anh Chi Le, 1970	(a) Water+acetic acid or (b) urea+imidazole	50 µl milk+5 ml reagent (a) or 50 µl milk+5 ml reagent (a)+2·5 ml reagent (b) Dependence on fat content Clarification within 30 min at room temperature	Protein (reagent a) and fat (reagent a+b) determinations in milk
Bosset et al. 1977a	Water+sodium hydroxide +*n* butylamine	100 µl milk+5 ml reagent Clarification within 2 or 3 min at 60 °C Clarification realised before chemical measurements	Milk protein (see Bosset & Blanc, 1977)
Bosset et al. 1977b	Water+sodium hydroxide +tetrahydrofurane	50 µl milk+5 ml reagent Clarification within 2 min at room temperature Clarification realised before chemical measurements	Milk protein (see Bosset & Blanc, 1977)
Linden & Pâquet, 1981	*n* Butylamine+cyclohexanone +Triton X-100	500 µl milk+1·5 ml buffered substrate+2 ml reagent Reagent: corrosive, viscous, malodorous and interfering with some dosages Dilution depending on enzyme activity in the sample Temperature around 37–40 °C Clarification realised after enzymatic reactions	See table 2
Owen & Andrews, 1984	Water+EDTA+Triton X-100	Variable dilutions of milk; dependence on fat content Variable composition of the reagent Variable conditions of clarification: temperature up to 60 °C within time up to 60 minutes Clarification realised before chemical measurements	SH, NH_2 and protein in milk
Linden et al. 1987† The Clarifying Reagent	Water+SDS+sodium hydroxide+Triton X-100+*n* butanone	Milk dilutions adapted to the measured substance amounts Variable ratio sample/reagent Clarification within 3 to 5 min often at 37 °C, and sometimes at room temperature Clarification realised after chemical or enzymatic measurements See table 3 for more comments	See table 2

† The Clarifying Reagent of Linden et al. 1987 is now released from patenting restrictions

structure and induces its dispersion. Finally, the base partially hydrolyses polypeptide chains. Milk fat globule membrane is also altered, which facilitates the dissolution of the lipids. The addition of an amphipatic solvent solubilises together lipophilic and hydrophilic components of milk. A non-ionic detergent decreases superficial tension and increases the performances of the organic solvent. All these actions lead to the breakdown of milk suspension and emulsion and milk becomes a homogenous and limpid solution.

In 1981, Linden & Pâquet prepared on these principles a 'dissolving mixture' leading to a residual absorbance of clarified undiluted whole or skimmed milk of 0·100 between 400 and 800 nm. The first application was the measurement of the alkaline phosphatase activity in pasteurised milks and creams. Subsequently, it has been applied to determine activity of 4 enzymes in milk by using specific synthetic substrates (Table 2; Linden et al. 1982; Humbert et al. 1982). It has also been used to enumerate micro-organisms in milk samples with added

Table 2. Chemical and enzymatic protocols using the Dissolving Mixture of Linden & Pâquet, 1981 and the Clarifying Reagent of Linden et al. (1987)

	Protocols using the dissolving mixture Linden & Pâquet, 1981	Protocols using the Clarifying Reagent Linden et al. 1987	Samples
Chemical determinations			
NH_2 groups		Humbert et al. 1990	Milk and cheese
SH groups		Guingamp et al. 1993	Raw and heated milks
Coloured compounds (A_{340})		Guingamp et al. 1999	Raw and heated milks
Ammonia, urea		Guingamp et al. 1994	Milk and whey
Enzymatic determinations			
Alkaline phosphatase	Linden & Pâquet, 1981	Linden & Pâquet, 1981†; Linden et al. 1982†	Milk, cream and cheese
Proteases	Linden et al. 1982; Humbert et al. 1982	Linden et al. 1982†; Humbert et al. 1982†	Milk and cheese
Lactoperoxidase	Linden et al. 1982	Blel et al. 2001	Milk and cheese
β-galactosidase	Linden et al. 1982		Milk
NAGase		Humbert et al. 1995	Milk
Lipase		Humbert et al. 1997	Milk
Plasmine		Saint-Denis et al. 2001a	Raw and heated milk
γ-glutamyltranspeptidase		Blel et al. 2002	Milk and cheese

† See the present text for the modifications of these protocols allowing to substitute the ''dissolving mixture'' of Linden & Pâquet, 1981 by the ''Clarifying Reagent'' of Linden et al. 1987

diverse bacteria strains (Kouomegne et al. 1984). However, it is corrosive, viscous, malodorous and it interferes with measurement of NH_2 groups because of the presence of butylamine.

Later, Owen & Andrews (1984) reported a clarification process independent of pH over the range 5·5 to 10·0. Below pH 5 caseins are not maintained in solution by the reagent of clarification. The method is suitable for colorimetric measurements in the range 400 to 900 nm. However, between 400 and 500 nm, residual absorbance is variable depending on the sample and on the conditions used for clarification. Residual absorbance is around 0·100 at 500 nm for undiluted skimmed milk and 10 times diluted whole milk samples. Owen & Andrews (1984) have proposed its use for the determination of protein by Biuret, the measurement of NH_2 and SH groups with 2,4,6-trinitrobenzene sulphonic acid (TNBS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) respectively, and the quantitation of acid phosphatase activity with NPP in milk. Stead (1987) has proposed a method to measure total proteolytic activity in milk with Hide Powder Azure as substrate after which the reaction mixture is centrifuged and clarified by the protocol of Owen & Andrews (1984) with a delay of 30 min at 37 °C.

Considering the different reported dissolving reagents and the properties required for obtaining a good clarification, Linden et al. (1987) have proposed and patented a reagent named The Clarifying Reagent (Table 1). Several chemical and enzymatic protocols using this Clarifying Reagent were reported for the analysis of milk and dairy products. The patent being now closed, the details of this reagent are given in the present paper.

Composition and properties of The Clarifying Reagent

The Clarifying Reagent of Linden et al. (1987) is a mixture of organic solvents and detergents which requires preparation of two solutions. The first one is 10 g SDS/l 0·1 M-NaOH. The second is a mixture (1 : 1, v/v) of *n*-butanone and Triton X-100. The Clarifying Reagent is obtained by progressively adding 3 volumes of the second solution to 1 volume of the first.

The Clarifying Reagent has an apparent pH>13 and a very low absorbance between 340 and 800 nm. Clarified milk presents the same spectra as the Clarifying Reagent (Guingamp et al. 1989; Humbert et al. 1990). Absorbances are around 0·140 and 0·090 at 340 nm and 400 nm respectively. Some physical parameters of the Clarifying Reagent have been determined: its index of refraction is 1·4218 at 20 °C, its conductivity is near 158 µSiemens, its viscosity is 9·75 cp at 20 °C and decreases to around 5 cp at 37 °C. It may be stored at +4 °C for 2 weeks or at −20 °C for longer time (at least one year). The Clarifying Reagent is stable about one week at room temperature when it is stored in opaque bottle. When exposed to day light at room temperature, the Clarifying Reagent may become slightly yellow coloured; nevertheless it remains effective and there is only an increase of the absorbance of the blank assays.

Table 3. Comments on some chemical and enzymatic protocols using the Clarifying Reagent of Linden et al. (1987)

	Samples and total volume of reaction mixture	Comments on clarification	Repeatability CV (%)	Correlation with reference method
Chemical determinations				
NH_2 groups Humbert et al. 1990	– 0·5 ml 1/25 to 1/40 aqueous diluted milk or 0·5 ml 1/20 to 1/50 10% (w/v) aqueous cheese slurry (dilution depends on NH_2 concentration) – VR = 2 ml	– Previous acidification with 0·5 ml acid acetic solution before addition of 1·5 ml Clarifying Reagent to avoid alkaline hydrolysis of residual TNBS, clarification at 37 °C within 3–5 min – LS = 30 min	CV = 3·05 n = 32	r = 0·995; n = 32 Ninhydrin (More & Stein, 1954)
SH groups Guingamp et al. 1993	– 0·5 ml milk or 1/1 aqueous diluted milk if high level of SH groups – VR = 1·55 ml	– Reaction with DTNB at room temperature but clarification is processed at 37 °C (3–5 min) with 0·5 ml EDTA solution and 2 ml of Clarifying Reagent – Dependence of fat content – LS = 20 min	CV = 3·2 n = 30	r = 0·994; n = 15 Amperometry (Yoshino et al. 1962)
Compounds (A_{340}) derived of the Maillard reactions Guingamp et al. 1999	– 1·5 ml 1/1 aqueous diluted milk – VR = 1·5 ml	– Clarification with 2·4 ml Clarifying Reagent, at 37 °C (5–7 min) – Necessary to know fat content of the sample because absorbance depends on it – LS = 20 min	CV = 3·1 n = 32 (on a pasteurised milk sample)	No similar method Correlation with lactulose measured with Capillary Electrophoresis on raw and different heated milk samples (Guingamp et al. 1999) r = 0·91; n = 31
Ammonia Guingamp et al. 1994	– 0·4 ml of 1/1 aqueous diluted milk – VR = 1·42 ml	– Enzymatic reaction occurs at room temperature but clarification requires 5 min at 37 °C – Clarification is easier with addition of 1 ml EDTA solution before addition of 1·5 ml Clarifying Reagent – LS = 20 min	CV = 5·4 n = 26	r = 0·99; n = 19 Specific electrode (Hélaine, 1977)
Enzymatic determinations				
Alkaline phosphatase Blel et al. 2002	– 0·5 ml aqueous diluted or not according to the level of activity – VR = 2·0 ml	– Clarification in 3 min at 37 °C with 2 ml Clarifying Reagent – LS = 15 min	nd	r = 0·98; n = 28 *p*-n-itrophenylphosphate (IDF, 1987)

Proteases, adapted from protocol of Humbert et al. 1982	– 0·5 ml milk or dilution of 5% (w/v) aqueous cheese slurry – VR = 2·0 ml	– Clarification in 3 min at 37 °C with 2 ml Clarifying Reagent – LS = 15 min	nd	nd
NAGase Humbert et al. 1995	– 0·2 ml milk – VR = 0·5 ml	– Enzymatic reaction at 50 °C – The reaction is better stopped and clarification is improved with addition of 1·5 ml solution EDTA before 1 ml Clarifying Reagent, 2–4 min at room temperature or short time at 37 °C – LS = 20 min	CV = 3·0 n = 33	r = 0·96; n = 52 Spectrofluorimetric enzymatic method (Kitchen et al. 1978)
Lipase Humbert et al. 1997	– 0·5 ml aqueous diluted or not according to the level of activity, or aqueous 2% (w/v) cheese slurry – VR = 2·55 ml	– Required 15 min pre-incubation at 37 °C before addition of substrate for the enzymatic reaction – The reaction is better stopped with 0·4 ml addition of an inhibiting mixture (EDTA, PMSF, DMF) – Clarification with 2 ml of Clarifying Reagent within 3–5 min at 37 °C – LS = 15 min	CV = 3·25 n = 20	r = 0·89; n = 29 Tributyrin (Castberg et al. 1975)
Lactoperoxidase Blel et al. 2001	– 0·25 ml 1/5 raw milk diluted with boiled milk (more dilution if high activity) – VR = 2·45 ml	– Very short time of reaction: 20 sec for milk and 40 sec for cheese – Clarification with 2 ml Clarifying Reagent, after 2 min at 37 °C – LS = 10 min	CV = 5·2 n = 33	r = 0·98; n = 52 Rothenfusser reagent (Resmini et al. 1985), and r = 0·99; n = 21 ABTS method (Barett et al. 1999)
γ-glutamyl transpeptidase Blel et al. 2002	– 0·1 ml milk, diluted with boiled milk if high activity, no dilution for aqueous 5% (w/v) cheese slurry – VR = 2·1 ml	– Clarification with 2 ml Clarifying Reagent at 37 °C within 2 min for milk and 5 min for cheese slurry – LS = 10 min	CV = 3·0 n = 20	r = 0·98; n = 40 γ-Glutamyl p-ranitroanilide, gly-gly (Patel and Wilbey, 1994)

– VR = Total volume of chemical or enzymatic reaction mixture
– LS = Length of stability of the absorbance of clarified mixture allowing spectrometric measurement

General procedure for the Clarifying Reagent use

Here we present the general principle of the use of the Clarifying Reagent of Linden et al. (1987) and the required major precautions when improving one protocol.

First, a small volume of buffer containing the chemical reagent (for example TNBS for NH_2 groups measurement) or the enzyme substrate (for example *p*-nitrophenyl butyrate for determination of lipase activity) is added to an aliquot of milk sample. The choice of the buffer, its molarity and pH are very important for protocol optimisation; these conditions must be suitable for the reaction and for the clarification step. Buffering power and pH are of great significance because the Clarifying Reagent is not effective when the final pH of the clarified mixture is below 8·8–9·0.

Often, the reaction mixture is incubated at 37 °C to allow the chemical or enzymatic reaction that lead to the formation of a coloured product in the medium to take place.

The Clarifying Reagent is added at the end of the chemical or enzymatic reaction. It contributes to stop these reactions by raising the pH. Principal requirements are that the Clarifying Reagent does not hydrolyse the excess of chemical reagent or of enzyme substrate and does not destroy the reaction product that will be measured in the protocol. The advantage of the Clarifying Reagent is to render casein micelles and fat globules soluble which allows direct spectrophotometric measurements in milk without preliminary separation and extraction steps usually required in the common protocols. Nevertheless, it is sometimes necessary to add EDTA to aid the clarification (see NAGase protocol, Humbert et al. 1995) or EDTA with an enzyme inhibitor mixture both to aid the clarification and to completely stop the enzymatic reaction (see lipase protocol, Humbert et al. 1997). Generally after 3 or 5 min at 37 °C, the reaction mixture is limpid and can be used for direct spectrophotometric measurement. The clarified reaction mixture is stable for at least 10 to 30 min which allows time to make series of assays or to automate procedures. The loss of stability is shown by a turbidity that disturbs spectrophotometric measurement. Table 3 presents some comments on the procedures of some chemical and enzymatic protocols using the Clarifying Reagent.

Chemical and enzymatic protocols using the Clarifying Reagent

The Clarifying Reagent has been used in the chemical or enzymatic protocols reported in Table 2 and 3. The protocols for NAGase, lipase, plasmin and γ-glutamyl transpeptidase activities measurements reported in these tables have been established with the Clarifying Reagent of Linden et al. (1987).

Other protocols concerning lactoperoxidase, proteases and alkaline phosphatase had been first developed with the dissolving mixture of Linden & Pâquet (1981). Thereafter, activity of these enzymes was measured by using the Clarifying Reagent of Linden et al. (1987), which required some modifications or adaptations of the previous protocols. The lactoperoxidase method established with the dissolving mixture (Linden et al. 1982) has been modified for pH, buffer, substrate concentration and wavelength in order to use the Clarifying Reagent (Blel et al. 2001). For the determination of different proteolytic activities in milk with specific synthetic substrates, the only difference was the replacement of the 0·3 M-triethanolamine/HCl buffer, pH 8·0 (Linden et al. 1982; Humbert et al. 1982) by 0·1 M-imidazole buffer, pH 8·0. To measure alkaline phosphatase activity, the molarity of the diethanolamine buffer (pH 10·6) has reduced from 0·15 to 0·1 M for use with the Clarifying Reagent.

Chemical protocols for the measurement of the NH_2 groups with TNBS and of the SH groups with DTNB have been developed with the Clarifying Reagent (Humbert et al. 1990 and Guingamp et al. 1993 respectively). Preliminary studies using a NADH/NAD reaction have been carried out for ammonia and urea measurements in milk and whey (Guingamp et al. 1994). A rapid and simple protocol has also been proposed to estimate the effect of heat treatment on milk by measuring absorbance at 340 nm of the some coloured compounds formed during the Maillard reactions (Choukri et al. 1991; Guingamp et al. 1995).

In comparison with the other previously published methods, protocols using the Clarifying Reagent are time saving; for instance the lactoperoxidase protocol (Blel et al. 2001) is 5 times faster than the Rothenfusser reference method (Resmini et al. 1985), or the protease activity measurement is easier than that of Stead (1987) which requires a centrifugation step. Furthermore, all these protocols are repeatable with a good coefficient of variation (3–5%), and are well correlated to the official reference methods (Table 3).

While all the protocols are established with bovine milk, NAGase and alkaline phosphatase protocols have also been applied to goat milk (Le Mens et al. 1994) and camel milk (Ramet & Humbert, 1998) respectively.

Analytical applications in the dairy industry of protocols using the Clarifying Reagent

Analysis of raw milk

It is very important to rapidly determine the overall quality of raw milk after its arrival at the processing dairy. The first aspect of this analysis is to determine the integrity of the protein matter by measuring the NH_2 groups, urea or ammonia in the milk. The second concerns endogenous and exogenous or bacterial protease and lipase activities present in milk. In addition, NAGase allows the detection of mastitis and to classify milks according to their quality (Humbert et al. 1995, 1996). Such analyses will

indicate the potential of the milk to render high quality products.

Analysis of heated milk

Protein denaturation and Maillard Reactions are among the most significant and undesirable consequences of the heat treatment of milk. The Clarifying Reagent was used to quantify SH groups (Guingamp et al. 1993) and to measure absorbance at 340 nm (A_{340}; Choukri et al. 1991; Guingamp et al. 1995) which reflects the presence of coloured compounds derived from the Maillard reactions. Levels in SH and A_{340} values allow discrimination between raw, pasteurised, UHT-treated and sterilised milks and then to classify heated milk samples (Guingamp et al. 1999).

Due to their thermosensitivity, some indigenous enzymes are good indicators of the severity or effectiveness of heat treatment of milk and milk products. Clarifying Reagent has been used to measure alkaline phosphatase, lactoperoxidase and γ-glutamyltranspeptidase residual activities in heated milk and especially after pasteurisation (Blel et al. 2001, 2002). Plasmin activity has also been determined with the Clarifying Reagent to study its heat denaturation (Saint-Denis et al. 2001a, b).

Analysis in cheese

Ripening of cheeses can be followed either by the analysis of the protein and lipid fractions or by the measurement of enzyme activities such as total lipase or protease activities. Ammonia measurement protocol in milk (Guingamp et al. 1994) might also be adapted for monitoring cheese ripening. It is of interest to know if a cheese is manufactured with raw, thermised or pasteurised milk. The appearance of NH_2 groups during ripening has been monitored using the Clarifying Reagent; to differentiate untreated Brie-type cheese and pasteurised Brie-type cheese (Humbert et al. 1990). Recently, the ''Clarifying Reagent'' has also been used to measure residual γ-glutamyltranspeptidase in addition to alkaline phosphatase activities to differentiate Camembert made with raw milk from Camembert made with heated milk (Blel et al. 2002).

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