DETERMINATION OF TRUE PROTEIN CONTENT BY THE AMIDO BLACK METHOD
Description and critical points

The amido black method is a practical method allowing the determination of the true protein content of milk [(NT-NPN) x 6.38]. This method is standardised at the AFNOR level as No NF V 04-216.
This method is applicable to cow, goat and ewe raw milk, and also processed milk if the protein composition (caseins-seric proteins report) has not been modified.

***Note**: NF V 04-216 standard contains also information annexes to determine the true protein content in products with contents different from milk and also for products with different protein composition.*

- Analytical principle:

The general principle of this method consists to add a amido black solution to a test sample of milk. This operation will create an insoluble complex between the proteins of the samples and the amido black. The complex is then eliminated by centrifugation (or filtration) and the optical density of the supernatant amido black solution is measured using a colorimeter (at λ of 578 or 620 nm), and transforms into true proteins concentration thanks a calibration equation.

- The reagent: the amido black solution

The amido black solution is constituted of:

- Amido black 10 B dye
- Sodium hydrogen orthophosphate (di-hydrated)
- Citric acid (monohydrated)

This solution can be bought in ready to use solution or realised by the laboratory according to the protocol described in the § 5.2.1 of the standard.
In the both cases, this solution has to be verified by the laboratory:

- pH must be of 2.4 ± 0.1. This characteristic is important for the formation of the insoluble complex when the solution is added to the milk test sample. To realise this verification, the pH-meter must be adjusted on the measurement range (from pH 2 to pH 7 for example).
- The optical density at 620 nm (c = 10 mm) of the solution at 1 % must be between 0.695 and 0.735. The accuracy of the dilution must be controlled to have a correct measurement (the distributor used for the milk sample test should be used to exactly deliver 1 ml of the amido black solution in a 100 ml graduated flask). The measurement should be performed with an optical path of 10 mm (± 0.1 mm), only a SOG (special optical glass) enables this dimension. A single-use plastic flask does not allow to guarantee this type of specification (its measurement is also very difficult due to the characteristics of the plastic used).

  The optical path directly affects the measurement of the optical density and this verification is necessary to ensure that the measurements will be made in the linearity field of the colorimeter.

- The material:

The amido black method uses many types of materials, which, must meet standardised or not requirements:

- **Tubes** allowing the distribution of milk and amido black solution. There is no real specification for these tubes. However, after distribution of milk and amido black solution, a significant "empty volume" must be available for a good stirring of the mix.
- **System(s) of distribution**
  The system(s) of distribution must meet NF V 04-216 requirements, that is:
  - 1 ml ± 0.02 with CV % < 0.15 % for the milk system of distribution
  - 20 ml ± 0.05 with CV % < 0.20 % for the amido black system of distribution

This verification must be performed according to the ISO 8655-6 standard requirements: Piston-operated volumetric apparatus: gravimetric methods for the determination of measurement error (on 10 measurements).

A fault to these requirements could cause, either repeatability problems (CV %), or a measurement zone out the linearity zone of the colorimeter (mean volume).
Many systems exist and may be used to distribute milk and amido black solution (single or combined) if they fulfill recommendations described above: automatic distributor for distribution of milk and amido black, positive-displacement pipette for milk and piston distributor for amido black.

- **Stirring system**

The objective of the stirring system is to permit the mix of the tubes and then the fixation of amido black on the proteins, a turning system is appropriate (wheels, mechanical system,…).
The tubes should be stir 10 minutes to ensure the optimal fixation of the dye on the proteins.

NB: As the number of « places » available on the stirrer is limited, amido black (or the milk-amido black mix) should be only distributed in tubes, which will be immediately stirred after the distribution.

- **Centrifuge**

The objective of the centrifugation is the separation of the pellet (proteins and amido black) and the supernatant (residual amido black solution) to measure its absorbance (concentration) using the colorimeter. This instrument has to produce an acceleration of about 350 g to allow the separation of the precipitate and the supernatant obtained after 5 minutes. In practice, the majority of laboratories use the centrifuges used for the « Gerber » method.

- **Photometer**

It must be equipped with a measurement cell with an optical path from 0.2 to 1 mm (in practice, we exclusively meet cells of 1 mm). The wavelengths must be between 550 and 620 nm (in practice, the wavelengths used, corresponding to the maximum of the amido black sensibility, are 578 and 620 nm).
At the liquid-conductive level, the pipe length in the cell must be limited, because it generates tracing (marking by a value of the 1st tube different from the other tubes of a same sample)
The apparatus piloted by a computer may allow an adjustment of the instrument.

NB: 2 instruments are authorised for use in the milk payment: ATL33 and CECIL 2031/2041

- **Calibration (adjustment) and verification:**

To predict results in g of true proteins per liter or by kg of milk, the photometer has to be calibrated using milk standard (realised from dried milk) setting the measurements (3 standards per range: Rich (R), Medium (M) and Poor (P) are available).

The software of the instrument enables to realise a prediction model between the absorbance of the supernatant solution and the concentration of proteins of the standard

According to the instruments, many mathematical models are possible: the curvilinear model is the most adapted (it is the nearest to the instrumental response), if it cannot be done, a linear model must be suitable (it could be then possible to recalculate the model externally (Excel) from absorbance obtained for the standard and their respective concentrations in proteins).

Concerning the procedure: after the realisation of a blank sample from a dilution of the amido black solution (prediction in « equivalent proteins in the order to 40 to 42 g/liter for a cow milk type range), analysis of at least 3 samples per standard to calculate the calibration function.

A verification must be then realised analysing the control milk (cow whole raw milk), whose the value obtained in proteins (3 samples at least) must be in accordance to the reference value of the control milk (maximum limit ± 0.15 g/liter). If the value of the control milk is non-concordant inside this limit, the initial adjustment is not validated.

- **Stability monitoring:**

It is necessary to verify the stability of the measurement for each analytical set. Indeed, many factors can influence the prediction using the initial calibration function: temperature of the samples and amido black solution (affecting the distributed volume), evolution of the distributors, intrinsic value of zero, drift of the photometer…).

This verification can be realised with transfer milks(1) (3 samples with contents near standards contents realised from raw milk and a control whole milk immediately defined after calibration) or standard milks and the control milk.

In practice, these milks are analysed at least in duplicate in each analytical set and the values observed are compared to the target values defined after the initial adjustment:

- If the values observed for the transfer milks remain in a limit ± 0.15 g/liter in relation to the target values, the stability of the measurement is confirmed
- If the deviations between the values obtained for one or many transfer milks and the target values are higher than ± 0.15 g/liter, calculate a mathematical correction of the results, either using a curvilinear correction equation, or using a mean factor of correction (on the basis of the values observed for the transfer milks). This mathematical correction is first applied to the « crude » value of the control milk: the mathematical correction calculated with the values of the transfer milks will be validated if the target is found with a tolerance of ± 0.15 g/liter.
- When the correction is validated, it is applied to the « crude » values of the other milks of the analytical set..

[(1)] *On prendra soin de vérifier la stabilité des laits de transferts sur la période d'utilisation*

Conclusion:

The amido black method is a practical and quick method enabling to predict results on milks equivalent to results obtained by the Kjeldahl method.
Its implementation is relatively easy but its procedure, particularly the adjustment and the verification operations of stability have to be control to ensure the quality of the results