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Determination of Nitrogen Fractions in Cheese: Evaluation of a Collaborative Study

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This study was carried out within the framework of the European COST 902/FLAIR-programme (European Cooperation in the Field of Scientific and Technical Research). The first objective was the harmonization of certain techniques currently used for the determination of nitrogen fractions of cheese. The second objective was to determine the repeatability (r) and reproducibility (R) of the standardized methods including the determinaiton of total nitrogen (TN), water soluble nitrogen (WSN), nitrogen soluble at pH 4.4 (SN 4.4), ethanol soluble nitrogen (ESN), nitrogen soluble in trichloroacetic acid (TCA12-N) and nitrogen soluble in phosphotungstic acid (PTA-N). The r- and R-values for TN were in the range of 0.3 to 1.4 and 1.4 to 2.5 g/kg, respectively. For all other methods tested, the r-values were in the range of 0.1 to 1.1% of TN, the R-values were below 3.3% of TN except for ESN (5%). For most cheeses tested, the WSN and SN 4.4 results were highly correlated, as were the TCA12-N and ESN results. Therefore, only a limited number of methods should be applied in practice, other aspects should be considered such as practical and environmental aspects (e.g. toxicity of solvents) and future use of the fractions and the kind of cheese. The difference between the TCA12-N and PTA-N values as well as the difference between SN 4.4 and TCA12-N values were used to characterize protein breakdown.

Introduction

The partition of cheese into different nitrogen fractions is a well known technique to follow cheese proteolysis. As early as the 19th century, Bondzynski (1) used water soluble nitrogen (WSN) and phosphotungstic acid soluble nitrogen (PTA-N) as % of total nitrogen (TN). Mogensen (2) evaluated many different fractionation methods and described a method in which the grated cheese sample was dissolved in citrate solution before analyses of TN, soluble nitrogen at pH 4.4 (SN 4.4) and PTA-N. The reason for the precipitation at the constant pH of 4.4 was the pH-dependence of the water soluble fraction. Kuchroo and Fox (3) evaluated different extraction procedures to develop rapid routine methods, while retaining reasonable repeatability and reproducibility. They found WSN and SN 4.6 to be very similar and recommended WSN as being the most simple. They also concluded that 12% trichloroacetic acid soluble nitrogen (TCA12-N) and 70% ethanol soluble nitrogen (ESN) gave similar results and recommended the ethanol fraction if further analysis was to be made on the soluble fraction. The similarities between the methods may not be true of all varieties of cheese.

The different methods have been used in various ways in many laboratories (4). In order to harmonize methods and to enable comparison of the results obtained in different laboratories, standardization is necessary.

The proteolysis subgroup of the FLAIR/SENS-COST 902 project (FLAIR = Food-Linked Agro-Industrial Research; SENS = Relating sensory, instrumental and consumer choice studies) therefore compared five different fractionation methods comprising WSN, SN 4.4, TCA12-N, ESN and PTA-N, and estimated the corresponding repeatabilities and reproducibilities. The group discussed the procedures for each method chosen and agreed on how they were to be performed. Moreover some of the participating laboratories compared the homogenization step by using a Stomacher or an Ultra Turrax homogenizer.

Experimental

Cheese samples

Four AOC (appelation d'origine contrôlée) cheese varieties with different extents of proteolysis were chosen for the tests (**Table 1**). The samples were obtained from the organizations which are responsible for the commercialization of the corresponding cheeses. For Parmigiano-Reggiano, two degrees of ripening were included.

The rind was removed and the samples were grated in order to obtain an homogeneous mixture.

Fractionation methods

After preliminary tests, the participating laboratories agreed upon the fractionation scheme shown in **Fig. 1** and the following procedures:

Total nitrogen. The total nitrogen content of the cheese

Table 1 Cheese samples

Variety	Age (mo)	Origin
Fontina	4	Consorzio Produzione del Fontina, Reggio Valle d'Aosta (Italy)
Comté	6	Comité Interprofessionnel du Gruyère de Comté, Poligny (France)
Parmigiano-Reggiano	12	Consorzio del Formaggio Parmigiano-Reggiano, Reggio Emilia (Italy)
Parmigiano-Reggiano old	18	

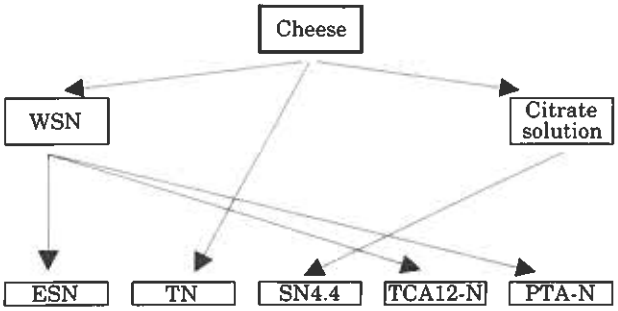

Fig. 1 Cheese fractionation scheme

samples was determined by the Kjeldahl method after mineralization (5).

Water soluble nitrogen. Ten grams of cheese were mixed with 50.0 mL deionized water and homogenized by using a Stomacher (5 min at 40 °C) or an Ultra Turrax (1 min at 10 000 rpm, after 1 min the suspension was again homogenized for 1 min). The homogenate was then held for 1 h at 40 °C. The samples were centrifuged at 3000 *g* for 30 min at 4 °C or 30 min at 20 °C and then cooled to 4 °C. The suspension was finally filtered through glasswool. The nitrogen content was then determined using the Kjeldahl method.

pH 4.4 soluble nitrogen. Five grams of cheese were dispersed in 90 mL of 0.1 mol/L trisodiumcitrate solution (pH 7.0), for 30 min at 30 °C by gentle stirring with a magnetic stirrer. The volume was adjusted to 100 mL with citrate solution and the pH rectified if necessary to 4.4 at 30 °C with 1 mol/L hydrochloric acid solution (the volume of acid added was taken into account for the calculation of the dilution). The suspension was held at 30 °C for 30 min and then filtered through Whatman No. 40 filter paper. The nitrogen content was again determined using the Kjeldahl method.

12% trichloroacetic acid soluble nitrogen. Twenty-five millilitres of WSN extract was added to 25.0 mL of 240 g/kg trichloroacetic acid solution. The suspension was held at room temperature for 2 h and then filtered through Whatman No. 40 filter paper. The nitrogen content was determined using the Kjeldahl method.

Phosphotungstic acid soluble nitrogen. Ten millilitres of WSN extract was added to 7.00 mL of 3.95 mol/L sulphuric acid solution and 3.00 mL 330 g/L phosphotungstic acid solution. The mixture was equilibrated overnight at 4 °C and then filtered through Whatman No. 40 filter paper. The nitrogen content was determined using the Kjeldahl method.

Ethanol soluble nitrogen. Thirty-five millilitres of WSN extract was added to a 100 mL beaker. The pH value was measured and, if necessary (>5.5), adjusted with 1 mol/L hydrochloric acid solution to 5.5. 15.0 mL of 960 g/kg ethanol was then added to the WSN extract. The suspension was held at 25 °C for 1 h and then filtered through Whatman No. 40 filter paper. The nitrogen content was determined using the Kjeldahl method. This method was applied to only one cheese sample (Older Parmigiano-Reggiano, see **Table 4** and **Fig. 2**).

Statistical analysis

Original values were recalculated as g nitrogen per kg of cheese (g/kg) for TN and as relative content (% of TN) for all other fractions. SYSTAT/SYGRAPH (6) programs were used to draw box plots.

Repeatabilities and reproducibilities were calculated according to ISO 5725 (7).

Results

Outliers

Values were considered as outliers if they were outside $Q_{25\%} - 1.5 * IQ$ (IQ = interquartile range) to $Q_{75\%} + 1.5 * IQ$ and $Q_{25\%} - 3 * IQ$ to $Q_{75\%} + 3 * IQ$ see **Fig. 2**, marked with * and o, respectively).

Homogenization procedures

The homogenization procedures were compared only for the older Parmigiano-Reggiano cheese sample. Stomacher and Ultra-Turrax homogenization revealed no significant differences between the mean value for WSN, TCA12-N, ESN and PTA-N fractions (**Table 2**). However, homogenization with a stomacher gave more precise results for the WSN method, most probably because of the longer homogenization time of 5 min compared to 2 min for the Ultra Turrax (8).

Repeatability and reproducibility

Tables 3 to **5** summarize the relative repeatabilities, reproducibilities and the coefficient of precisions of all methods tested. **Figure 3** shows the median values of four nitrogen fractions together with the intervals covered by the r- and R-values.

Discussion

Determination of total nitrogen

The TN content of the cheeses analysed was in the range of 40 to 55 g/kg. In this range, the repeatability

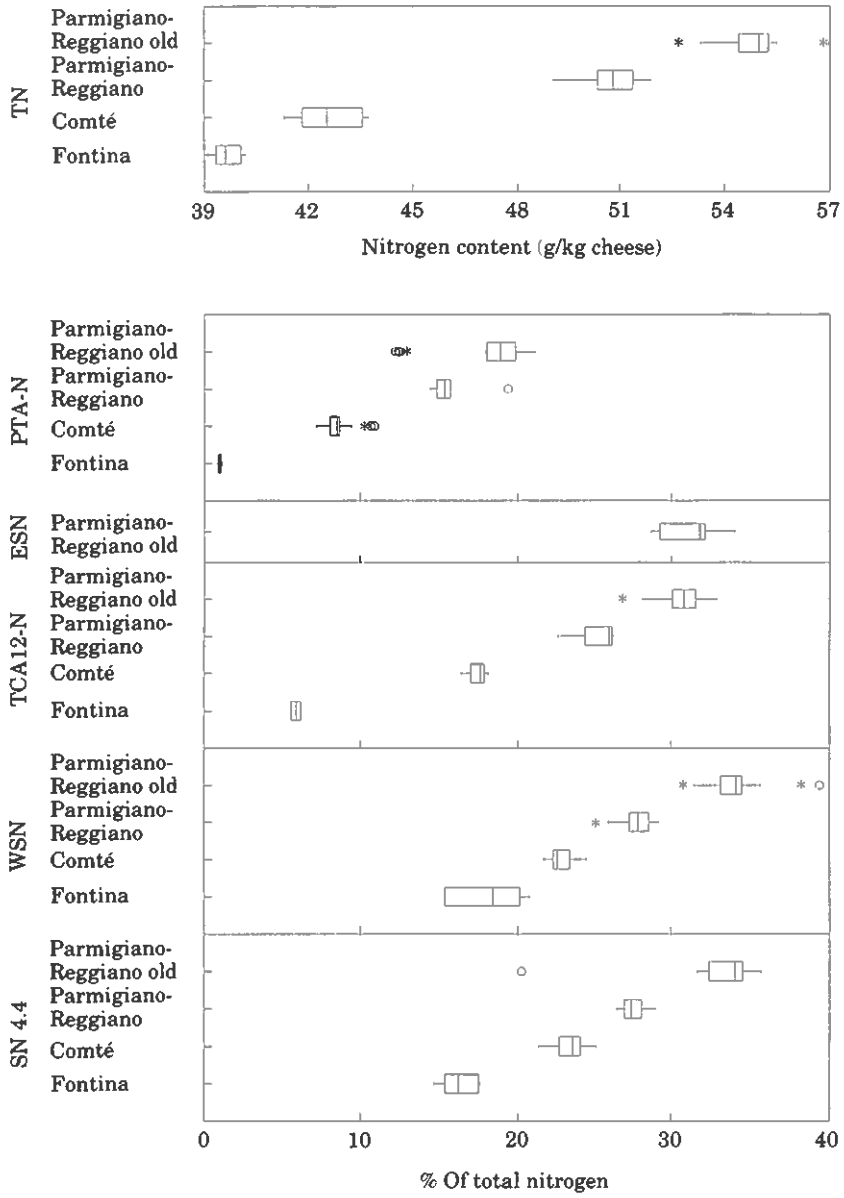

Fig. 2 Box plots of the different nitrogen fractions

Table 2 Comparison of two homogenization procedures: mean value and standard deviation (SD) of nitrogen fractions (% of TN)

	Stomacher			Ultra Turrax		
Fraction	*n*	Mean	SD	*n*	Mean	SD
WSN	7	33.4	1.1	4	33.9	1.5
TCA12-N	6	30.8	1.4	4	30.6	1.6
ESN	3	32.1	0.2	3	31.9	1.5
PTA-N	5	19.1	1.2	4	19.6	3.1

and the reproducibility were in the range of 0.3 to 1.4 and 1.4 to 2.5 g/kg, respectively.

Determination of ethanol soluble nitrogen

The determination of ESN was for most laboratories a new method. This could explain the poor reproducibility of about 5% of TN. The repeatability, on the other hand, was below 0.7% of TN. This method has two important advantages over the TCA12-N fraction. Firstly, ethanol can easily be evaporated to yield a residue for use with further separation procedures or for sensory analysis. Secondly, ethanol is a much less polluting solvent than trichloroacetic acid.

Determination of the WSN, SN 4.4, TCA12-N and PTA-N

The repeatability for these fractions was in the range of 0.1 to 1.1% of TN. The reproducibility usually was below 3.3% of TN for all fractions. The WSN-values were found to depend on the pH of the cheese extract. It has been shown, that the water soluble fraction is only independent if the pH of the cheese extract is below 6.0 (9).

One laboratory used a slightly different fractionation scheme, where the TN, SN 4.4 and PTA-N fractions were obtained from a citrate solution and the TCA12-N in a further step from the SN 4.4 solution (2). These values did not differ from those obtained by the other laboratories using the fractionation scheme shown in **Fig. 1**. Further information on the nitrogen fractions

Table 3 Mean value, repeatability (r) and reproducibility (R) of the method for the determination of TN (N, g/kg)

Cheese variety	*n*	Mean	r	R	R/r
Fontina	3	39.67	0.53	1.40	2.64
Comté	7	42.54	0.47	2.39	5.08
Parmigiano-Reggiano	5	50.82	1.33	2.51	1.89
Parmigiano-Reggiano old	7	54.80	0.34	1.79	5.26

n = Number of laboratories (outliers eliminated).
r = Repeatability (within laboratories).
R = Reproducibility (between laboratories).
R/r = Coefficient of precision.

Table 4 Mean value, repeatability and reproducibility of the method for the determination of the ESN (% of TN)

Cheese variety	*n*	Mean	r	R	R/r
Parmigiano-Reggiano old	8	31.15	0.7	4.97	6.72

Table 5 Mean value, repeatability and reproducibility of some methods for the determination of nitrogen fractions (% of TN)

	WSN					SN 4.4					TCA12-N					PTA-N				
Cheese variety	*n*	Mean	r	R	R/r	*n*	Mean	r	R	R/r	*n*	Mean	r	R	R/r	*n*	Mean	r	R	R/r
Fontina	3	17.95	0.96	8.33	8.68	2	16.30	0.95	4.59	4.83	2	5.92	0.51	0.88	1.73	3	1.00	0.06	0.30	5.00
Comté	7	22.92	0.95	2.37	2.49	6	23.61	0.57	3.18	5.58	3	17.48	0.85	1.85	2.18	6	8.32	0.37	1.74	4.70
Parmigiano-Reggiano	5	27.88	0.94	3.23	3.44	4	27.56	0.65	2.48	3.82	3	25.25	0.28	5.01	17.89	4	15.23	0.78	1.50	1.92
Parmigiano-Reggiano old	10	33.75	0.81	3.18	3.93	7	34.25	0.36	3.04	8.44	10	30.92	0.83	3.34	4.02	8	19.38	1.11	2.94	2.65

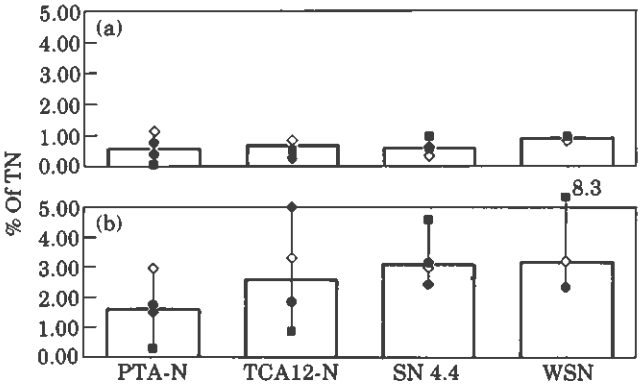

Fig. 3 (a) Repeatability and (b) reproducibilities of some methods for the determination of nitrogen fractions. (□) Median; (■) Fontina; (●) Comté; (◆) Parmigiano-Reggiano; (◇) Parmigiano-Reggiano old

can be obtained by deducing TCA12-N, PTA-N and SN 4.4-TCA12-N (**Fig. 4**).

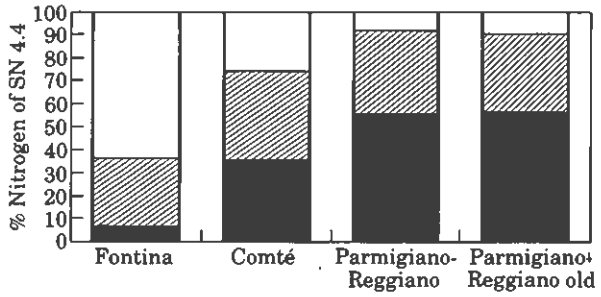

Fig. 4 Estimation of peptide distribution from nitrogen fractions. (■) PTA-N; (▨) TCA12-N–PTA-N; (□) SN 4.4–TCA12-N

Conclusions

Homogenization with Stomacher and Ultra-Turrax equipment gave similar results for medium and hard type cheeses. Because of the high correlation between the various methods, only a limited number of methods should be applied in practice.

WSN and SN 4.4, as well as TCA12-N and ESN, gave similar results for most cheese varieties. When choosing methods, practical considerations should be taken into account, e.g. the use of the fractions for chromatography or sensory evaluations. Other aspects such as toxicity and environmental pollution by the solvents should also be considered. For cheese flavor research, the WSN, PTA-N, TCA12-N and ESN fractions correlate well with sensory analysis of flavor development (10). The water soluble fraction and the residue from the ethanol soluble nitrogen fraction can be used for sensory analysis (11).

Two different fractionation schemes can be used in practice. The first (see **Fig. 1**) uses WSN while the second uses citrate solution for further nitrogen fractionation. Further work is recommended to standardize these methods.

Participating laboratories

The following laboratories participated in the collaborative studies: Anna Polychroniadou, Aristotelian University of Thessaloniki (Greece); Rosina Lopez-Fandiño, Inst. Fermataciones Industriales, C.S.I.C., Madrid (Spain);¹Leo Bertozzi, Consorzio del Formag-

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