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# Fluorescence microscopy methods for the determination of somatic cell count in raw cow's milk

P. Zajac[1], S. Zubricka[1], J. Capla[2], L. Zelenakova[2]

[1]Veterinary and Food Institute Bratislava, Bratislava, Slovak Republic
[2]Slovak Agriculture University in Nitra, Nitra, Slovak Republic

**ABSTRACT**: The direct fluorescence microscopy method with ethidium bromide staining can be used for somatic cell counting in raw cow's milk. However, this method has some limitations that may influence the results of the analysis. We therefore aimed at improving the procedure of somatic cell nuclei staining. We tested the hypothesis that ethidium bromide can better penetrate into the DNA of cells with degraded somatic cell walls or into dead cells. Therefore, we increased the temperature of the sample to 100 °C in order to disrupt the somatic cell wall membrane and to improve ethidium bromide penetration to somatic cell nuclei. In all, 90 samples of raw cow's milk were analysed in this experiment. Three parallel measurements were performed using each of the microscopic methods and the routine flow cytometry method. In all, 810 microscopic smears were analysed. The somatic cells were counted using fluorescence microscopic methods and flow cytometry. The increased temperature during the sample preparation improved ($P < 0.005$) the penetration of ethidium bromide into the somatic cell nuclei. It is concluded that the direct fluorescence microscopy method is suitable for precise laboratory analysis of somatic cell in raw cow's milk.

**Keywords**: microscopy; cow milk; fluorescence; flow cytometry; temperature; somatic cell; wall; temperature; DMSCC

Mastitis is an inflammatory disease of the mammary gland (Pyorala 2003) caused mainly by pathogenic microorganisms (Vasil et al. 2012; Cervinkova et al. 2013; Alekish 2015). A significant relationship can be expected between bulk tank milk somatic cell counts (SCC) and the number of mastitis pathogenic microorganisms in raw cow milk (Rysanek et al. 2007). Determination of somatic cells (SC) in raw cow milk can be used to diagnose mammary gland health and the prevalence of clinical and subclinical mastitis in dairy herds (Idriss et al. 2013). Milk SCC is a key component of European Union regulations for milk hygiene. Food business operators must initiate procedures to ensure that raw cow milk does not exceed a limit of less than or equal to 400 000 SCC/ml calculated as a rolling geometric average over a three-month period, with at least one sample per month (Commission regulation EC No. 1662/2006). Accurate SCC results can be obtained only using laboratory diagnostic methods. In common laboratory practice, an instrumental fluoro-opto-electronic method based on flow cytometry is used for routine SCC determination. This method has to be periodically checked with the reference and calibration samples prepared using the reference method (Zajac et al. 2012). At the present time, the reference method is the international standard ISO 13366-1 (2008) microscopic method. This reference method has two possible procedures for SC staining. Methylene blue or ethidium bromide (EtBr) can be used as the staining agents. The fluorescence microscopy method is based on EtBr staining of somatic cells. In 2013, 45 reference laboratories participated in the European Union Reference Laboratory for Milk and Milk Products inter-laboratory proficiency testing trial for SCC in raw cow milk using ISO 13366-1 (2008). The reference method, based on methylene blue staining, was used by 43 of these laboratories. Only two laboratories used EtBr during the staining (ANSES 2013). The previous version of ISO 13366-1 (1997) contained different procedures for EtBr staining using a modified Newman-Lampert

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stain solution. The staining of cells was performed by dipping the microscopic slide with a fixed smear into the staining solution containing EtBr. The new version of the ISO 13366-1 (2008) staining procedure is based on mixing the staining solution with milk in the reagent tube at a temperature of 50 °C. We set out to determine whether we could improve this staining procedure in order to optimise the penetration of EtBr into SCC nuclei. We have tested the following hypothesis: can EtBr better penetrate into the DNA of cells with degraded somatic cell walls or into dead cells? This effect can be achieved with a detergent such as Triton X-100 that causes creation of pores in the cell wall. We have used a combination of Triton X-100 and a temperature of 100 °C during the sample preparation and tested the effectiveness of EtBr penetration and DNA staining of SC nuclei.

Practical experiences with the fluorescence microscopy method, based on SC nuclei staining with EtBr in milk are not adequately described in the scientific literature and most of the European Union national reference laboratories for milk and milk products are still using the method based on methylene blue staining with the Newman-Lampert stain solution. In this work, we describe our practical experience of SC nuclei staining with EtBr and the fluorescence microscopy technique.

## MATERIAL AND METHODS

**Samples**. The tested materials included raw cow milk samples obtained from individual cows. Sampling was performed according to the ISO 707 (2008). Selection of suitable cows ensured milk samples with different concentrations of SC (from 54 000 to 895 000 SC/ml). Samples were analysed at the State Veterinary and Food Institute in Bratislava in the National Reference Laboratory for Milk and Milk Products. The laboratory is accredited according to standard ISO 17025 (2005). In all, 90 samples of raw cow milk were analysed in this experiment. Three parallel measurements were performed using each of the microscopic methods and the routine flow cytometry method. In all, 810 microscopic smears were analysed.

**Laboratory methods**. We used three microscopic methods and a flow cytometry method in this experiment.

*Method A*: ISO 13366-1 (1997) Microscopic method (old reference method), staining by dipping the microscopic plate with smear into modified Newman-Lampert stain solution (Levowitz-Weber modification) containing chemicals like ethanol, tetrachlorethane, acetic acid glacial; instead of methylene blue, EtBr was used.

*Method B*: ISO 13366-1 (2008) Microscopic method (reference method) staining with EtBr. This standard was corrected with ISO 13366-1:2008/Cor 1 (2009). The principle of staining with EtBr is based on Vermunt et al. (1995). We made some modifications of this method. We changed the temperature of the milk during heating in the reagent tube from 50 °C to 100 °C, followed by the addition of EtBr stain solution and staining for one minute with simultaneous gently mixing. Subsequently, the sample was cooled to 20 °C and spread over the microscopic slide. The EtBr stain working solution was diluted with demineralised water (1 : 9). A calibrated automatic pipette Finpipette 10 µl was used instead of a microsyringe.

*Method C*: Flow cytometry. ISO 13366-2 (2006), we used the Fossomatic 5000 flow cytometer (routine method).

*Method D*: ISO 13366-1 (2008) Microscopic method (reference method) staining with EtBr in the reagent tube at a temperature of 50 °C.

**Instruments and equipment**. The following instruments were used for this experiment: Olympus BX51 fluorescence microscope, WH10x/22 eyepiece, Olympus UPlanFI 60x/125 Oil Iris objective, USH-1030L lamp, fluorescence illuminator with U-25ND25, U-25ND6, NB (blue light) and NG (green light) filters, (Olympus SK, s.r.o., Bratislava, Slovakia), flow cytometer – Fossomatic 5000 (Milcom Servis a.s., Prague, Czech Republic), automatic pipette Finpipette – 10 µl, microsyringe – 10 µl, laboratory glass, extractor hood, Stuart vortex mixer (Stuart, Staffordshire, UK), microscopic slides with pre-marked area of 20 mm × 5 mm (Tekdon, Myakka City, Florida, USA), heat plate (40 °C), and a calibrated micrometre.

**Chemicals and solutions**. All reagents were used according to ISO 13366-1 (1997; ISO 13366-1, 2008) and were of recognised analytical grade or better.

Preparation of solutions for Method A: We followed the procedure described in ISO 13366-1 (1997).

**Working procedure**. *Method A*: We followed the procedure described in ISO 13366-1 (1997). Calculation of results was performed according to the same equation presented below in Method B.

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*Method B*: Sample preparation: Fresh cow's milk was thoroughly and gently mixed by inverting the samples up and down several times (samples with a fat layer on top were heated to a temperature of 40 °C ± 2 °C and mixed for homogenous distribution of fat in the sample).

Working procedure: For the experiment, 1 ml of sample was added to a reagent tube (reagent tube was made of Pyrex and was heat stable). We gently held the reagent tube with a thumb and forefinger and then heated the tube over the burner in a direct flame to a temperature of 100 °C. When the mixture began to boil, the reagent tube was immediately removed from the flame (it is important to prevent the sample from boiling over out of the reagent tube). Immediately, 1 ml of EtBr working stain solution was added and the sample was vortexed for 1 min. Next, the sample was cooled in a refrigerator to a temperature of 20 °C (the micropipette and microsyringe were also calibrated to this temperature). Using the micropipette 0.01 ml of the prepared test sample was taken. It is advisable to pay attention to the foam which may occur; the tip of the micropipette has to be submerged in the milk to prevent suction of the air from the foam. The outside of the tip that had been in contact with the sample, should be carefully and gently cleaned with paper towel. Then, the test portion was placed on a clean microscopic slide with a pre-marked area of 20 mm × 5 mm (1 $cm^2$). Using the tip or needle, the test portion was spread over the entire defined area of the slide to form a compact smear (by holding the pipette like a pen and putting the elbow and wrist on the table for better work). Then, the smear was dried at room temperature until it was completely dry.

Reading optimisation: Using the fluorescence microscope, the cell nuclei in the obtained smears were counted. We used fluorescence light with a wavelength of 450 nm (blue light), eyepiece magnification of × 10, and an objective of × 60. Immersion oil was used for counting the SC nuclei in the smear. Generally, cells in milk are distributed according to a Poisson distribution and the minimum number of cells to be counted in relation to the cell count level is less than 150 × $10^3$ SC/ml, $n$ = 100; 150 to 250 × $10^3$ SC/ml, $n$ = 200; 250–400 × $10^3$ SC/ml, $n$ = 300, greater than or equal to 400 × $10^3$ SC/ml, $n$ = 400.

Counting in successive fields: The nuclei were counted in successive fields in vertical strips in regularly spaced fields, following the instructions in ISO 13366-1 (2008). SC nuclei should be counted only if they are evidently distinguishable and if more than 50% of the nuclear material is visible.

Calculation and expression of results: The length and width of the smear were checked against the 20 mm and 5 mm target values by using the graduations and vernier of the microscope. The total concentration ($c$) of cells was calculated by using one of the equations in ISO 13366-1 (2008). We used this equation:

$$c = \frac{W_s \times L_s \times N_t}{\pi \times \left(\frac{D_f}{2}\right)^2 \times N_f \times V_m} \times \frac{1}{d}$$

where:

$c$ = total concentration, expressed in the number of cells/ml
$W_s$ = width (mm) of the smear
$L_s$ = length (mm) of the smear
$N_t$ = total number of cells counted
$D_f$ = diameter (mm) of the microscope field
$N_f$ = number of fields counted completely
$V_m$ = volume (ml) of the sample smeared

If the EtBr working stain solution is used for staining, $V_m$ = 0.005 ml. Expression of results: the test results were expressed in whole figures of thousands per ml.

*Method C*: We followed the procedure described in the operational manual of the Fossomatic 5000 instrument and the instructions from ISO 13366-2 (2006). The instrument was calibrated with calibration samples from Actalia – Cecalait (Poligny, France) and was regularly tested in interlaboratory ring tests organised by this laboratory.

*Method D*: We followed the procedure described in the ISO 13366-1 (2008) microscopic method (reference method) with EtBr staining. Calculation of results was performed according to the same equation presented described for Method B.

**Statistical analysis**. For the evaluation of the results a two-factor analysis of variance (ANOVA) without interactions was used. Calculations were performed using the professional statistical software Statistica 7 CZ (StatSoft CR s.r.o., Prague, Czech Republic). We used a standard main effect ANOVA. For multiple comparisons of methods, Tukey's test and Sheffe's test were used.

## RESULTS

The average results of the SCC determination in raw cow's milk using Methods A, B, C and D

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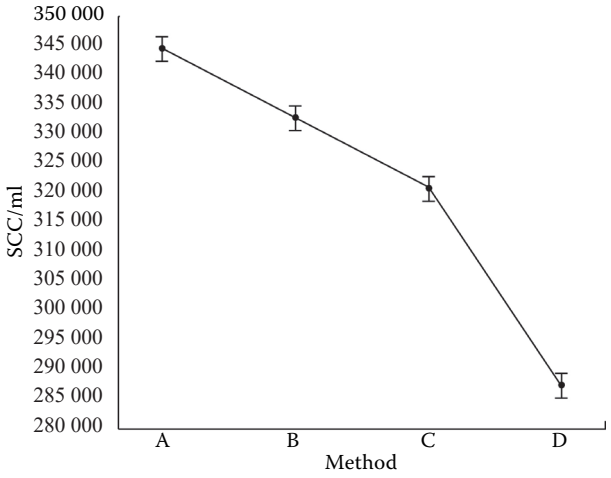


Figure 1. Comparison of Methods A, B, C and D. In this figure, the mean values of SCC/ml for Methods A, B, C and D with 95% confidence bands are presented

Average of set of results, current effect: $F_{3,267} = 555.66$, $P < 0.001$

are presented in Table 1. The descriptive statistics and confidence intervals for the mean SCC values are presented in Table 2. In Figure 1, the 95% confidence intervals are presented for different types of methods. There are significant differences ($P < 0.005$) between the results of all methods: A, B, C and D. In Figures 2–9, images of the microscopic preparations are presented.

The results obtained using Method A were higher by 11 000 SC/ml in comparison with results using Method B, and in comparison to Method C, these results were higher by 22 000 SC/ml. The results using Method B were higher by 10 000 SC/ml in comparison with the results of Method C and higher by 46 000 SC/ml in comparison with results of Method D. The statistically determined differences between Methods A–B, B–C and A–C did not exceed 42 000 SC/ml, representing a repeatability value associated with the Fossomatic 5000 instruments (Method C) at a concentration of 300 000 SC/ml. Also, the inter-laboratory reproducibility value $S_R$ 41 000 SC/ml at a concentration of 245 000 SC/ml, according to ISO 13366-1 (2008), was fulfilled. Thus, in light of the above, both microscopic Methods A and B can be used in laboratory practice. The results of Method D were significantly ($P < 0.005$) lower than results for Methods A, B and C.

## DISCUSSION

The working procedure for smear preparation using Method A was considerably more difficult in comparison with Method B and needs very precise work. It is necessary to use only calibrated micropipettes or microsyringes. We had better experience using micropipettes despite the fact that in general, microsyringes are preferred, as described by Ubben (2004). For better accuracy of the work, it is important to use microscopic slides with fixed premarked areas or templates of a defined size, which have to be checked by micrometre (ISO 13366-1, 2008). In comparison to microscopic slides without pre-marked areas, more precise results are

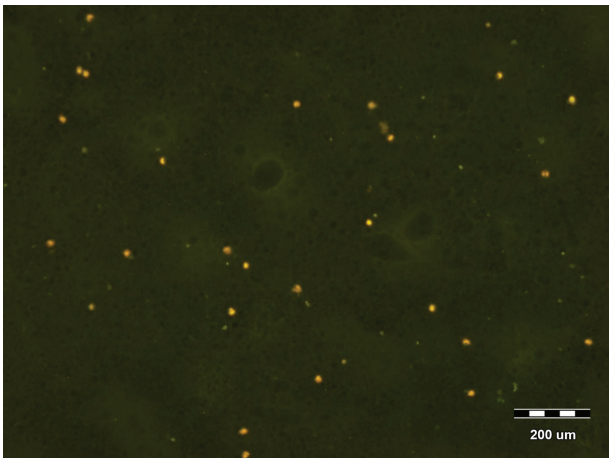


Figure 2. Method A, golden yellow fluorescing nuclei of SC, dark olive green coloured background, smear was prepared from cow milk with 600 000 SC/ml, magnification × 600

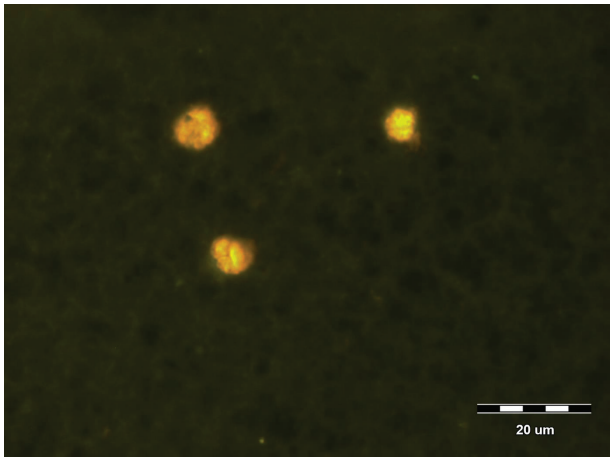


Figure 3. Method A, golden yellow fluorescing nuclei of SC, dark olive green coloured background, magnification × 600

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Table 1. Mean results of SCC, as determined using microscopy methods A, B, D and flow cytometer C in raw cow milk samples. Results of Methods A, B and D are the mean results of three parallel-determined smears. Results of Method C are the mean results of six parallels results

| Sample No. | Method A | Method B | Method C | Method D | Sample No. | Method A | Method B | Method C | Method D |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 371 000 | 345 000 | 392 000 | 305 000 | 46 | 151 000 | 155 000 | 150 000 | 107 000 |
| 2 | 315 000 | 286 000 | 267 000 | 245 000 | 47 | 196 000 | 189 000 | 187 000 | 140 000 |
| 3 | 313 000 | 316 000 | 298 000 | 274 000 | 48 | 183 000 | 178 000 | 167 000 | 128 000 |
| 4 | 348 000 | 316 000 | 321 000 | 272 000 | 49 | 250 000 | 250 000 | 238 000 | 198 000 |
| 5 | 900 000 | 850 000 | 882 000 | 808 000 | 50 | 314 000 | 303 000 | 291 000 | 247 000 |
| 6 | 253 000 | 213 000 | 185 000 | 170 000 | 51 | 254 000 | 245 000 | 230 000 | 187 000 |
| 7 | 232 000 | 216 000 | 191 000 | 177 000 | 52 | 512 000 | 495 000 | 479 000 | 439 000 |
| 8 | 641 000 | 614 000 | 605 000 | 569 000 | 53 | 282 000 | 271 000 | 233 000 | 219 000 |
| 9 | 329 000 | 320 000 | 270 000 | 274 000 | 54 | 248 000 | 245 000 | 201 000 | 191 000 |
| 10 | 113 000 | 106 000 | 101 000 | 65 000 | 55 | 600 000 | 590 000 | 586 000 | 544 000 |
| 11 | 265 000 | 246 000 | 234 000 | 203 000 | 56 | 553 000 | 542 000 | 516 000 | 488 000 |
| 12 | 278 000 | 274 000 | 262 000 | 232 000 | 57 | 244 000 | 240 000 | 230 000 | 198 000 |
| 13 | 800 000 | 763 000 | 784 000 | 714 000 | 58 | 513 000 | 506 000 | 511 000 | 465 000 |
| 14 | 639 000 | 634 000 | 654 000 | 587 000 | 59 | 312 000 | 309 000 | 300 000 | 271 000 |
| 15 | 160 000 | 162 000 | 146 000 | 112 000 | 60 | 606 000 | 591 000 | 587 000 | 532 000 |
| 16 | 224 000 | 208 000 | 210 000 | 168 000 | 61 | 784 000 | 766 000 | 770 000 | 724 000 |
| 17 | 367 000 | 337 000 | 338 000 | 297 000 | 62 | 351 000 | 341 000 | 328 000 | 299 000 |
| 18 | 138 000 | 136 000 | 126 000 | 96 000 | 63 | 688 000 | 676 000 | 647 000 | 628 000 |
| 19 | 241 000 | 226 000 | 217 000 | 185 000 | 64 | 337 000 | 323 000 | 301 000 | 277 000 |
| 20 | 227 000 | 229 000 | 212 000 | 188 000 | 65 | 719 000 | 698 000 | 671 000 | 651 000 |
| 21 | 171 000 | 176 000 | 167 000 | 135 000 | 66 | 220 000 | 193 000 | 189 000 | 143 000 |
| 22 | 293 000 | 301 000 | 276 000 | 255 000 | 67 | 146 000 | 143 000 | 130 000 | 92 000 |
| 23 | 197 000 | 191 000 | 168 000 | 149 000 | 68 | 428 000 | 394 000 | 386 000 | 352 000 |
| 24 | 243 000 | 249 000 | 216 000 | 206 000 | 69 | 342 000 | 321 000 | 300 000 | 279 000 |
| 25 | 222 000 | 226 000 | 200 000 | 185 000 | 70 | 305 000 | 283 000 | 277 000 | 238 000 |
| 26 | 263 000 | 264 000 | 254 000 | 216 000 | 71 | 397 000 | 384 000 | 389 000 | 343 000 |
| 27 | 239 000 | 236 000 | 221 000 | 195 000 | 72 | 351 000 | 342 000 | 337 000 | 289 000 |
| 28 | 730 000 | 703 000 | 678 000 | 659 000 | 73 | 165 000 | 162 000 | 155 000 | 111 000 |
| 29 | 544 000 | 542 000 | 531 000 | 501 000 | 74 | 337 000 | 324 000 | 324 000 | 265 000 |
| 30 | 180 000 | 185 000 | 176 000 | 150 000 | 75 | 587 000 | 591 000 | 572 000 | 551 000 |
| 31 | 371 000 | 345 000 | 392 000 | 299 000 | 76 | 174 000 | 168 000 | 158 000 | 130 000 |
| 32 | 315 000 | 286 000 | 267 000 | 243 000 | 77 | 215 000 | 223 000 | 202 000 | 186 000 |
| 33 | 313 000 | 316 000 | 298 000 | 267 000 | 78 | 753 000 | 723 000 | 716 000 | 691 000 |
| 34 | 348 000 | 316 000 | 321 000 | 274 000 | 79 | 390 000 | 385 000 | 375 000 | 320 000 |
| 35 | 267 000 | 232 000 | 233 000 | 186 000 | 80 | 290 000 | 275 000 | 275 000 | 221 000 |
| 36 | 347 000 | 333 000 | 316 000 | 287 000 | 81 | 143 000 | 141 000 | 139 000 | 94 000 |
| 37 | 64 000 | 53 000 | 51 000 | 12 000 | 82 | 160 000 | 157 000 | 125 000 | 110 000 |
| 38 | 197 000 | 179 000 | 172 000 | 130 000 | 83 | 173 000 | 170 000 | 150 000 | 121 000 |
| 39 | 119 000 | 114 000 | 109 000 | 63 000 | 84 | 296 000 | 284 000 | 259 000 | 239 000 |
| 40 | 286 000 | 258 000 | 251 000 | 208 000 | 85 | 796 000 | 792 000 | 780 000 | 743 000 |
| 41 | 229 000 | 235 000 | 210 000 | 186 000 | 86 | 351 000 | 341 000 | 291 000 | 294 000 |
| 42 | 228 000 | 224 000 | 205 000 | 184 000 | 87 | 597 000 | 585 000 | 559 000 | 540 000 |
| 43 | 143 000 | 149 000 | 138 000 | 108 000 | 88 | 434 000 | 429 000 | 399 000 | 381 000 |
| 44 | 286 000 | 251 000 | 241 000 | 203 000 | 89 | 365 000 | 354 000 | 336 000 | 311 000 |
| 45 | 211 000 | 205 000 | 206 000 | 167 000 | 90 | 530 000 | 527 000 | 504 000 | 478 000 |

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Table 2. Descriptive statistics and confidence intervals for the mean values of SCC/ml

| Method | Mean of all results | Standard error | Lower bound (95%) | Upper bound (95%) | Number of results (*n*) |
|---|---|---|---|---|---|
| A | 344 000 | 1 046 | 342 407 | 346 526 | 90 |
| B | 333 000 | 1 046 | 330 940 | 335 059 | 90 |
| C | 322 000 | 1 046 | 319 518 | 323 637 | 90 |
| D | 287 000 | 1 046 | 285 318 | 289 437 | 90 |

obtained because the milk is better spread over the entire defined area of the microscopic slide. Microscopic slides have to be clean and free from fat. If the microscopic slide is not well cleaned or the fat is not thoroughly removed, then separation of the fixed smear from the microscopic slide can occur during the process of washing the staining solution from the smear in tap water. After

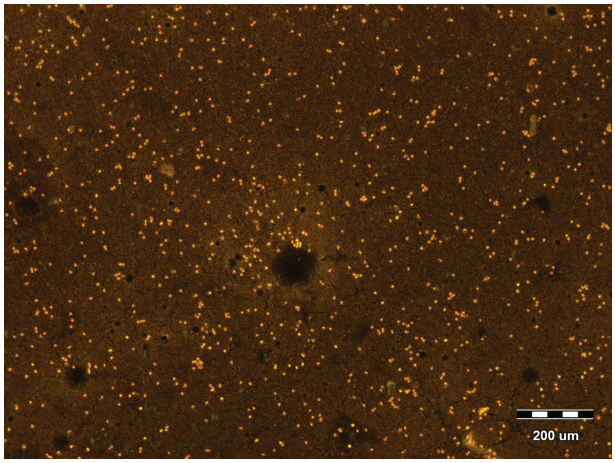


Figure 4. Method B, golden yellow fluorescing nuclei of somatic cells, background is yellow orange coloured, darkened particles are milk fat, smear was prepared from cow milk with 9 000 000 SC/ml, magnification × 40

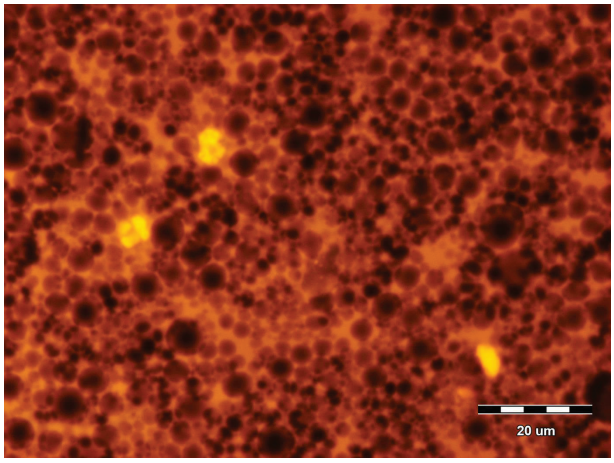


Figure 5. Method B, golden yellow fluorescing nuclei of somatic cells, darkened globular particles are milk fat, background is orange coloured because of the presence of EtBr staining solution added in liquid form directly to the sample during the staining, magnification × 600

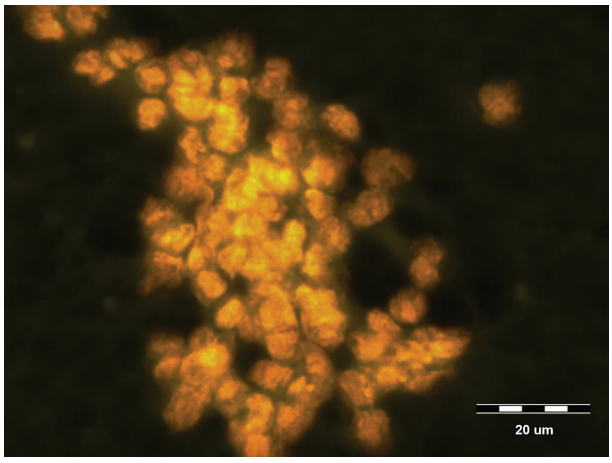


Figure 6. Method A, golden yellow fluorescing nuclei of somatic cells, dark olive green coloured background, smear was prepared with cow milk with 1 500 000 SC/ml; figure shows a cluster of somatic cells that was not countable, magnification × 600

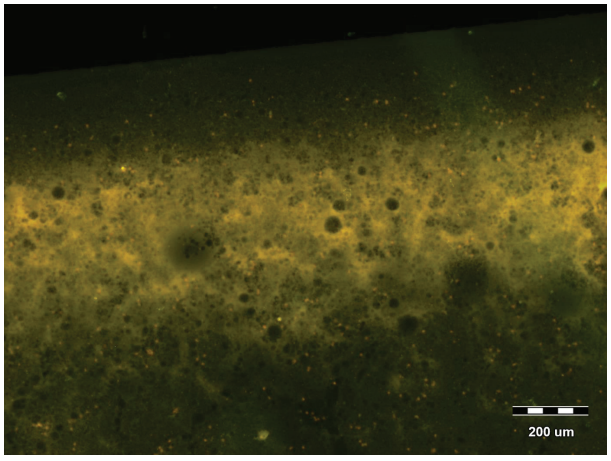


Figure 7. Method A, golden yellow fluorescing nuclei of SC, dark olive green coloured background, smear was prepared with cow milk with 600 000 SC/ml; figure shows the edge of a smear on the top and an intensively fluorescing strip in the middle of smear, magnification × 40

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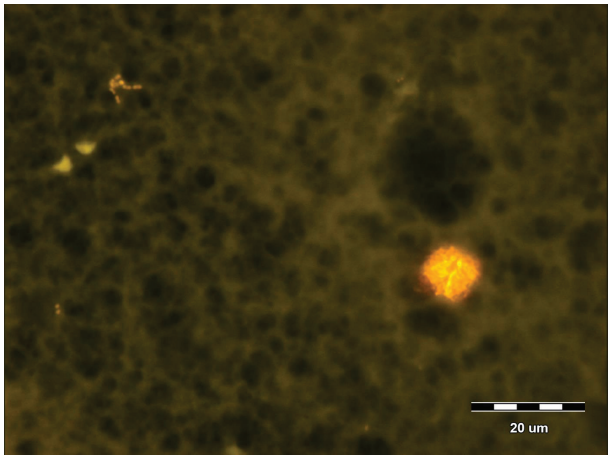


Figure 8. Method A, golden yellow fluorescing nuclei of somatic cells on the right side of the figure, dark olive green coloured background, smear was prepared with cow milk with 1 500 000 SC/ml; in the upper left corner fluorescent rod-shaped bacteria are visible, magnification × 600

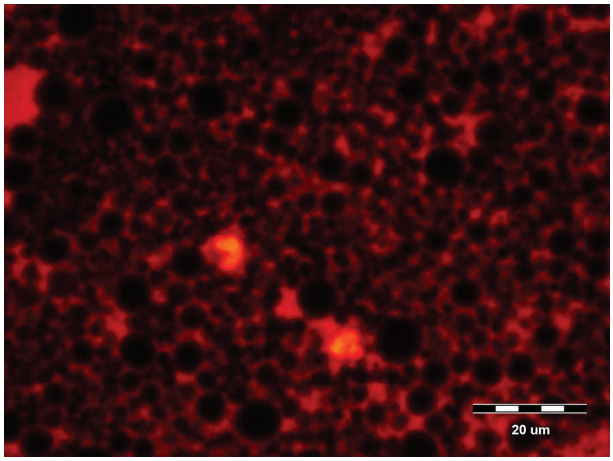


Figure 9. Method B, intensively red fluorescing nuclei of somatic cells, background is red, darkened particles are milk fat, a U-MNG2 lens unit with a filter generating green light, was used, which caused EtBr to emit red light, magnification × 600

spreading the milk over the microscopic slide it is recommended to air-dry the smear. We do not recommend drying the smear on a hot plate, which may lead to the development of cracks in the fixed smear. Subsequently, during staining in Method A dye solution might penetrate these cracks and it is not possible to remove this dye when washing with tap water. Also, longer washing times (ISO 13366-1, 1997) may lead to a separation of the smear from the microscopic slide, and consequently, to smear destruction. On the other hand, if there is not a sufficient washing of dye, then it is not possible to view the microscopic slide under the microscope due to intensive fluorescence. Additionally, longer washing with tap water may lead to excessive removal of dye, and in this case, it is not possible to identify the SC nuclei.

Method A, in comparison to Method B, has other disadvantages. More aggressive and toxic chemicals are used for preparation of staining solution. The acute and chronic effects of tetrachlorethane were observed in humans (ATSDR 1996). Working procedures have to be performed in an extractor hood. Fixation of smear takes at least 15 min in Method A and further staining procedures are required. Fixation of the smear for Method B takes the same amount of time, but no further action is required and the smear can be analysed immediately after drying. An advantage of Method A over Method B is a more comfortable viewing of the smear under a microscope. The smear does not contain excessive dye, only the SC nuclei fluoresce with brightly golden yellow or orange colour, and the background of the smear is a dark olive-green colour (Figures 2 and 3). This is observed only when the blue fluorescence light of 450 nm wavelength is used. The accuracy of results is strongly affected by the quality of the prepared smear. There are more working operations within Method A in comparison to Method B. Consequently, there is an increased risk of possible mistakes or damage of the smear with Method A. In Method A, staining of the smear is performed by dipping the microscopic slide with the dried smear in the dye solution. Subsequently, the smear is gently dipped in tap water until all of the surplus dye is washed away and then the smear is dried again (ISO 13366-1, 1997). These operations increase the risk of smear damage. It is recommended to use only a gentle flow of tap water.

The advantage of Method B is that the dye solution is directly added to the milk that has been boiled to 100 °C. Also, the milk is diluted with a dye solution at a 1 : 1 ratio. The smear contains excessive dye, there are fluorescing SC nuclei with brightly golden yellow or orange colour and the background of the smear is gently fluorescing with orange colour due to the presence of EtBr in the whole volume of the sample (Figures 4 and 5). Also, we can observe dark fat globules of different sizes. This is the scenario if the blue fluorescence light at a 450 nm wavelength is used. There are fewer working operations within Method B than Method A,

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and consequently, there is a lower risk of possible damage to the smear. A disadvantage of Method B is that there is one more pipetting step in comparison to Method A. Each mistake caused by pipetting may influence the results of the analysis. In Method B, this is the most important mistake because damage to the smear by creating cracks during drying does not influence the results of the analysis because staining is performed before drying.

A common problem associated with Method A is the clustering of SC (Figure 6); in this case the counting of cells is problematic. A very frequent problem with Method A is the creation of a very intensive fluorescing stripe lengthwise to the edge of the smear (Figure 7). At the edge of the smear, it is not possible to count the SC nuclei due to the very intensive fluorescence. This effect is caused by drying a larger volume of milk at the edge of the smear, and due to the increased number of cracks created during the process of drying (drying proceeds from the edges to the centre of the smear). Other parts of the smear are well-visible.

We found that it is important to use only non-preserved cow's milk or milk preserved by bronopol. We do not recommend the use of milk preserved by potassium dichromate because there is a decreased adherence of the milk smear to the microscopic slide, as well as insufficient penetration of EtBr into SC nuclei. In comparison to our working procedure, Gonzalo et al. (2003) used poly-L-lysine to improve adherence of the smear to the slide. Ubben (2004) recommended analysing the smear immediately after preparation. We agree with this suggestion; however, we have found that smears can also be analysed several months after preparation without the results being affected. These smears should be protected from light and dust. We recommend analysing the smear using a microscope at a × 600 magnification and with immersion oil. Lower magnification can lead to problems with identification of the SC nuclei present in clusters. The use of higher magnification is not necessary; furthermore, it is time-consuming. In general, it takes approximately 20 min for one smear to be analysed by a skilled worker.

The advantage of both Methods A and B is that EtBr stains only the SC nuclei. EtBr forms chemical complexes with the DNA of the cell nuclei (Raugel 1999). An exact determination of SCC in the sample is possible and mistakes due to counting formations that are not SC are eliminated. This mistake may occur when SC are stained by a methylene blue-modified Newman-Lampert stain solution (Levowitz-Weber modification). Also, some authors recommend the replacement of the methylene blue-based stains with the DNA-specific pyronin Y-methyl green stain PMG for determination of DMSCC in sheep milk (Petersson et al. 2011). According to our findings, the identification of fluorescing SC nuclei is simple and a laboratory technician can concentrate only on the fluorescing SC nuclei.

According to Raugel (1999) bacteria are stained with EtBr. We also found that EtBr stained the DNA of microorganisms present in the sample. In Method A, it is possible to distinguish microorganisms from SC nuclei. Microorganisms like bacteria are smaller in comparison to SC nuclei and also have characteristic shapes. In Figure 8, it is possible to see yellow colour fluorescing rod-shaped bacteria on dark olive green backgrounds. In Method B, microorganisms are practically not viewable because their identification is not possible due to the presence of fat globules and excessive concentration of EtBr in the whole volume of milk (Figure 9). According to Gallier (2010), the diameter of milk fat globules ranges from 0.1 to 20 μm. We found that these fat globules make viewing of the smear harder in Method B to a certain extent, because they can overlap with the SC. The diameter of somatic cells ranges from 6 to 15 μm (Varzakas and Tzia 2015). In Method A, this problem does not occur because most of the fat globules are destroyed by tetrachlorethane.

We also tried to stain the yeast *Candida albicans* with EtBr. This organism may be an aetiological agent in cow mastitis (Dworecka-Kaszak et al. 2012; Sartori et al. 2014). The size and shape of these yeasts are similar to somatic cells and in order to distinguish them from SC we inoculated *Candida albicans* into distilled water and stained them with EtBr according to Method B. We found that the intensity of fluorescence decreased rapidly after illumination with fluorescent light. Thus, the presence of *C. albicans* is not problematic for microscopic determination of SC.

Changes in the permeability of blood vessels and mammary epithelium lead to the leakage of blood components into milk during mastitis (Harmon 1994). Sometimes, erythrocytes can appear in the milk of *E. coli*-inflamed quarters as a result of dramatic alterations in mammary blood flow and

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microvascular integrity during mastitis (Burvenich et al. 2003). We added bovine erythrocytes to pasteurised milk without somatic cells and stained the sample with EtBr. No fluorescence was observed.

Also, we determined that the EtBr standard stock solution can be kept in a dark place in an airtight flask between 0 °C and 5 °C for more than one year. Sample No. 90 was stained with working staining solution prepared from either a 3-year-old or new solution. The results using Method B were 527 000 SC/ml (new solution) and 529 000 SC/ml (old solution).

The main differences in Method B in comparison with the reference method ISO 13366-1 (2008) included a dilution of the EtBr working staining solution at a ratio of 1 : 9 with demineralised water. We did this because the sample contained a large concentration of dye, which complicated the process of microscopic counting. There was an increased intensity of reflected light from the whole smear.

The most important modification in this experiment was the change in the temperature of the milk before staining. The temperature during staining was changed from 50 °C, as utilised in Vermunt et al. (1995) and reference method ISO 13366-1 (2008), to 100 °C. This modification improved the penetration of EtBr into SC nuclei through the denatured cell wall membrane. According to Pelvan and Unluturk (2015), EtBr can only penetrate and stain the DNA of dead cells. Also, in previous experiments, we found a statistically significant difference in the $P$-value < 0.005 (–79 000 SC/ml) between the test results when temperatures of 50 °C and 100 °C were used (Zajac 2007). This is an explanation for the temperature modification in Method B. In our previous experiment we determined a difference between Methods A and B of –21 000 SC/ml, which is in contrast with the 11 000 SC/ml determined in this experiment (Zajac 2007). This difference was probably caused by changes in the method of calculation in ISO 13366-1 (2008). In the previous experiment, we used a calculation with a constant working factor based on the number of strips counted completely. Because of the higher difference we do not recommend using the formula based on the constant working factor and the number of bands counted completely.

Gonzalo et al. (2003) analysed SCC in sheep milk and compared three microscopic methods – staining with methylene blue (MB), staining according to May-Grunwald-Giemsa (MGG) and staining with pyronin Y-methylene green (PMG) – and calculated the correlation coefficients between these methods. They found that correlations between MB staining and MGG and PMG stainings were 0.981 and 0.982, respectively. The correlation coefficient for MGG and PMG stainings was 0.990. The correlation coefficients between variants of the direct microscopy somatic cell count reference methods (DMSCC) and Fossomatic instruments in different analytical conditions were consistently very high (0.957–0.996). The authors demonstrated that the correlation coefficients between the three DMSCC stainings were very high (almost 1.00), so they could all be considered FSCC reference methods (Gonzalo et al. 2003). These results are in agreement with the results of our experiments. We calculated the following correlation coefficients: 0.998 between Methods A and B, 0.996 between Methods A and C, and 0.996 between Methods B and C. These results closely match the results of several other authors (Grappin and Jeunet 1974; Heeschen 1975; Schmidt-Madsen 1975; Heald et al. 1977; Schmidt-Madsen 1979), who calculated high correlation coefficients close to 1.00.

We calculated the expanded uncertainty of measurement $U$ according to Ellison and Williams (2012). The expanded uncertainty $U$ for both Methods A and B was 8% SC/ml.

In conclusion, fluorescence microscopy methods are suitable for precise laboratory analysis of somatic cells in raw cow milk. We determined the statistical difference between the results of different fluorescence microscopy methods to be 11 000 SC/ml. It was necessary to change the temperature of the milk from 50 °C to 100 °C during SC nuclei staining. This elevated temperature improved the penetration of EtBr into SC nuclei when the EtBr staining solution was added directly to the milk. The methodology of smear preparation by dipping the fixed smear of milk on the microscopic slide into staining solution was more problematic; toxic chemicals were used, the smear was often damaged during preparation and this method was time-consuming. Our results are characterised by a high degree of accuracy due to the skills, talent and experience of the laboratory technicians. In the future, we plan to focus on the application of substances that enhance the penetration of EtBr into the somatic cell nucleus and to determine the influence of different preserving agents on the penetration and binding of EtBr to the DNA of SC nuclei.

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Corresponding Author:

Ing Peter Zajac, PhD., Veterinary and Food Institute Bratislava, National Reference Laboratory for Milk and Milk Products, Hlohovecka 5, 951 41 Nitra-Luzianky, Slovakia
E-mail: zajac@potravinarstvo.com