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Validation of a potentiometric peroxide value (POV) assay for analysis of mineral oil with low oxidative content

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ABSTRACT

In vitro human embryo culture is a critical process, which is performed in microdroplets of specialized culture medium with typical physicochemical properties. Since optimal embryo development completely depends on medium stability, microdroplets are covered with mineral oil to maintain pH, osmotic pressure, and temperature during manipulation and culture. Pharmaceutical-grade paraffin oil is widely used as a raw material for the production of mineral oil, used in assisted reproductive technology (ART) procedures. Although suited for pharmaceutical applications, further purification is essential to remove traces of embryotoxicants, including oxidative agents. In view of quality control testing, in-house analysis of oxidative content is routinely performed on each new batch of mineral oil. For this purpose, the European Pharmacopoeia (EP) describes an iodometric titration method with visual end point determination, the so-called peroxide value (POV) assay. Although being the gold standard in the pharmaceutical, food, and fuel industry, the test is not sensitive enough to detect very low levels of oxidative substances. Therefore, several modifications have been made to the EP method, including altered reagents concentration and oil sample quantity, combined with potentiometric end point determination. These efforts have resulted in a very sensitive test method, which is able to reliably detect oxidative content as low as 0.1 mEq/kg. In summary, a potentiometric POV assay, based on the EP method, has been specifically developed for QC analysis of ART mineral oils. A detailed overview of the applied modifications is presented, together with assay validation results, demonstrating excellent sensitivity and reproducibility.

Keywords: Assisted reproductive technology; Embryo culture; Mineral oil; Peroxide value

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INTRODUCTION

In assisted reproductive technology (ART), mineral oil is widely used during *in vitro* embryo culture as an overlay to maintain pH, osmotic pressure, and temperature of culture medium microdroplets [1, 2, 3]. Pharmaceutical grade paraffin oil is a frequently used source for the production of ART mineral oils. Although suited for pharmaceutical applications, the raw entry product must be further processed to remove residual embryotoxicants such as oxidants, eliciting deleterious effects at low levels [4, 5, 6]. Extensive and repeated washing of paraffin oil results in a product with very low oxidative content (<0.1 mEq/kg). In our facility, each newly-produced batch of ART mineral oil is subjected to different quality control (QC) tests, including viscosimetry, toxicity testing (mouse embryo assay), and analysis of oxidative content, as represented by the peroxide value (POV).

The European Pharmacopoeia (EP) monograph for POV assessment in oils describes an iodometric, visual method, being the gold standard in the food, pharmaceutical, and fuel industry [7, 8, 9, 10]. The test is based on the interaction between a saturated solution of potassium iodide and oxidative substances, present in the oil sample. Briefly, oxidation of iodide (I^-) by peroxides will liberate iodine (I_2), which in turn reacts with I^- to form triiodide (I_3^-) anions. In the presence of starch, a typical indicator for iodometric analyses, blue-coloured complexes are formed. Titration against sodium thiosulfate (NaS_2O_3) will reduce I_3^- to I^-. Thus, the blue colour will decrease until all

triiodide anions have been converted, yielding a colourless solution. The consumed volume of thiosulfate is directly correlated to the concentration of hydroperoxides, present in the oil sample [7, 8, 11, 12].

Oil samples with a relatively high oxidative content allow easy interpretation of the colour turnover. In contrast, the very low POV value (<0.1 mEq/kg) of FertiCult™ mineral oil only elicits weak coloration. Hence, end point determination is complicated and negatively affects assay bias.

To increase the sensitivity of the EP method, several modifications have been made, including altered reagents concentration and oil sample quantity. In addition, the redox reaction is measured potentiometrically, allowing an objective interpretation of the experimental results. A detailed overview of the applied changes, and the consequent validation process, is given in the present publication.

EXPERIMENTAL SECTION

Chemicals and reagents

Ultrapure water (level 1+) was produced in-house by reversed osmosis on a Milli-RO 60 device (Millipore, Brussels, Belgium) and subsequent deionization and purification using an Elga Purelab Genetic device (Analis, Namen, Belgium). Ultrapure water was boiled and cooled prior to use. This is essential in order to remove traces of oxidative agents. Pharmaceutical grade potassium iodide (KI) was purchased from ABC Chemicals (Wouters-Brakel, Belgium), VWR (Haasrode, Belgium), and Fagron (Waregem, Belgium), respectively. Sodium thiosulfate ($Na_2S_2O_3$) and 2, 2, 4-trimethylpentane (TMP) were obtained from Sigma Aldrich (Diegem, Belgium), while Acros (Geel, Belgium) provided titration-grade potassium iodate (KIO_3) and acetic acid (CH_3COOH). Hydrogen chloride (HCl, 37%) was purchased from Merck (VWR, Haasrode, Belgium). All reagents were freshly prepared before analysis. FertiCult™ Mineral oil (FertiPro NV, Beernem, Belgium) has been used in all experiments.

Equipment

Titration of mineral oil was almost completely automated by using an electronic titrator (Excellence T50; Mettler Toledo, Zaventem, Brussels), coupled to a maintenance-free redox electrode DMi 147SC (Mettler Toledo, Zaventem, Brussels). The latter is specifically designed for iodometric titrations and consists of a platinum ring sensing element, serving as indicator electrode. Dedicated and calibrated burettes were applied to supplement extraction buffer and thiosulfate, respectively. These burettes are able to add extremely low volumes (1 µL) during titration. Addition of water occurred through a calibrated peristaltic pump. Only KI had to be added manually with a calibrated micropipette of 1000 µL. All parameters (incubation time, rotor stir speed, pump speed, buffer volumes & concentrations, potassium iodate purity, calculation formulas…) have been optimized and programmed into one specific program for titer determination, blank titration, and oil sample analysis, respectively. The POV assay is a light-sensitive method, and for that reason, disposable, light-protecting titration vessels (Mettler Toledo, Zaventem, Brussels) have been used for each titration. Also, each can was wrapped with aluminium foil for maximal protection against light.

Assay modifications

Extraction buffer

The EP monograph describes two POV methods, which only differ in the type of extraction buffer: method A = chloroform/glacial acetic acid (ratio 40:60 v/v) and method B = TMP/glacial acetic acid (ratio 40:60 v/v). The extraction buffer is required to dissolve the lipid sample of interest. In view of toxicity issues, extraction buffer B has been used in the present set-up.

Altered parameters

When compared to the EP monograph, the following parameters have been changed:
(1) Replacement of the 0.01M thiosulfate solution by a 0.001M solution for titration.
(2) Oil sample quantity: 10g instead of 5g.
(3) Altered primary standard concentration (0.56074 mM instead of 5.6074 mM KIO_3 solution).
(4) Potentiometric determination of the equivalence point.

A 10-fold higher volume (ml versus µl range) of titrants was required to perform POV analysis because reagent concentrations were reduced with the same factor.

Potentiometric analysis

Instead of a visual end point determination, the equivalence point (equal moles of titrant and analyte) is determined by potentiometric analysis. Briefly, the equivalence point corresponds to the steepest point of the potential curve (fig. 1). At this point, there is a strong inflection of the first derivative dE/dV (fig. 2).

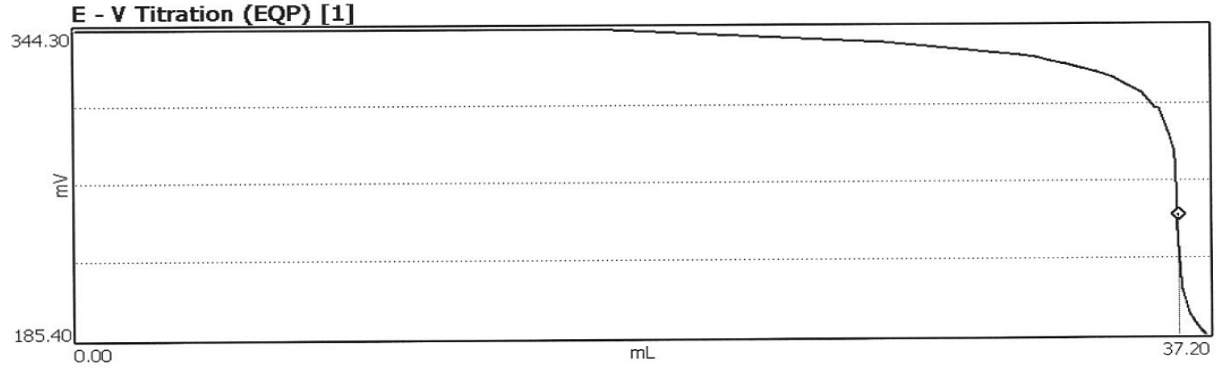

Figure 1: Potentiometric titration curve (X-axis: titration volume (mL); Y-axis: Potential of the solution (mV))

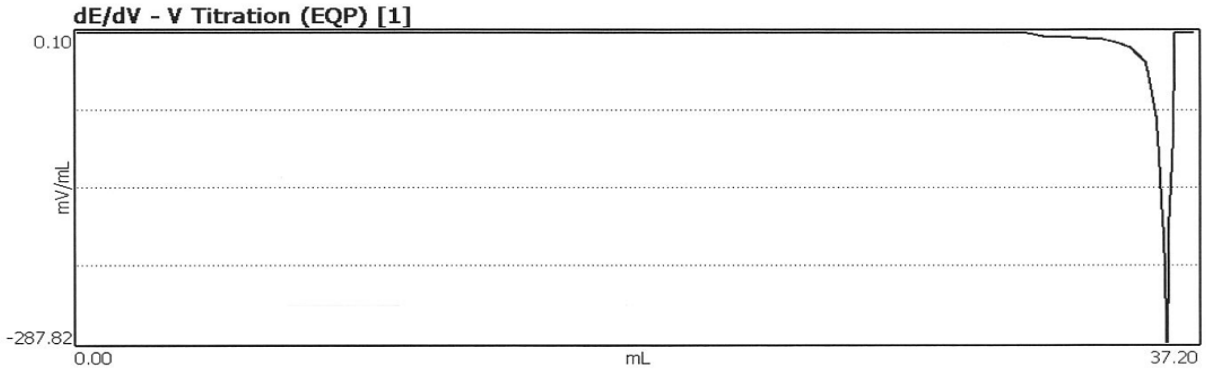

Figure 2: Inflection of the first derivative (dE/dV) at the equivalence point of the titration

The titrant volume, corresponding to the equivalence point, is used for calculation of the analyte concentration. All applied formulas are described in detail in the chapters below. Potentiometric measurement of the redox reaction allows objective sample analysis, in contrast to the visual method, where interpretation is subjective and complicated in samples with low oxidative content.

Thiosulfate molarity

Importantly, for each analysis, the exact molarity of the thiosulfate solution must be determined by titration with a primary standard. A potassium iodate (KIO_3) solution is widely used for iodometric titrations. Briefly, an exact amount (0.03 g per 250 ml) of the primary standard was weighed and analytically dissolved in boiled, ultrapure water to a final concentration of 0.56074 mM. Under acidic conditions (addition of 5mL of 4M HCl), potassium iodide (0.5mL) was added to a fixed volume of iodate solution (10 mL). The formed triiodide anion (I_3^-) was then titrated with thiosulfate according to the following formula:

$$\mathbf{IO_3^- + 6H^+ + 8I^- \rightarrow 3I_3^- + 3H_2O}$$

$$\mathbf{2S_2O_3^{2-} + I_3^- \rightarrow S_4O_6^{2-} + 3I^-}$$

Molarity of the thiosulfate solution was automatically calculated by the T50 device, according to the formula [7, 8, 11, 12]:

$$\mathbf{M_{Thio} = (1.125 \times m \times (\%\ purity\ KIO_3)/ (100 \times V_{Thio})}$$

With m = amount of KIO_3 (g); % = purity of potassium iodate, and V_{Thio} = average volume of thiosulfate (mL).

Blank and oil analysis

A blank titration was performed in triplicate for each new analysis. Briefly, 500 µL of a saturated KI solution was added to 50 ml of extraction buffer and incubated for exactly 60 seconds while gently stirring. Next, 30 ml of ultrapure water was added and titrated with the calibrated 0.001M thiosulfate solution until equivalence point was reached. The average volume was automatically calculated by the electronic titrator.

Oil sample analysis was also performed in triplicate. The titration procedure was almost identical to the blank method, except that 10 g of oil was carefully weighed in a titration vessel before addition of extraction buffer. Following titration, POV content was automatically calculated by the device, using the following formula [7, 8, 11, 12]:

$POV = (1000*(V_S-V_{Bl})*C) / m$

Where V_S = average volume of thiosulfate, required for oil sample analysis, V_{Bl} = average volume of thiosulfate, required for blank analysis, C= molarity of the thiosulfate solution, and m = weight of the oil sample.

Importantly, after each titration (either blank or sample analysis), the stirrer was rinsed with 100 ml of boiled, ultrapure water to remove traces of the reaction solution.

Data integration and statistical analysis
General statistics were calculated using MedCalc statistical software (MedCalc, Ostend, Belgium). The relative standard deviation (RSD) or coefficient of variation (CV), expressed as %, was calculated using the following formula: 100*(standard deviation (SD)/average).

Method validation
Method validation was based on EP17 guideline of the Clinical & Laboratory Standards Institute (CLSI) [13]. Besides specificity, the following parameters have been assessed: limit of blank (LOB), limit of detection (LOD), limit of quantification (LOQ), linearity and range, precision, accuracy, and robustness, based on the International Conference on Harmonization Results (ICH) guidelines [14, 15].

RESULTS AND DISCUSSION

Blank titration
Blank values are required to measure the background signal of POV analyses. The complete mix of reagents (extraction buffer, boiled water, KI), without oil sample, was titrated against thiosulfate. The EP POV monograph states that blank values, obtained with the 0.01M thiosulfate stock, must be lower than 100 µL. As the concentration of the thiosulfate and KIO_3 solutions have been reduced by a factor 10, ten-fold higher volumes of the titrant were required to reach the equivalence point. For this reason, the maximum allowable blank volume has been set to 1 ml for titrations with a 0.001M thiosulfate stock. If the consumed amount of thiosulfate was higher than 1 ml, all reagents had to be prepared freshly and tested again.

Limit of blank (LOB)
Although the common principle of the POV assay is well-known and approved by the EP and other instances, we have chosen to perform a complete internal validation in view of the important modifications to the original assay. The EP17 guideline defines the limit of blank (LOB) as the highest apparent analyte concentration expected to be found when replicates of a blank sample, containing no analyte, have been tested [13, 14]. As advised by the guideline, 60 samples have been included and tested at different time points. The average thiosulfate volume and the corresponding standard deviation were incorporated in the following formula:

$LOB\ (ml) = mean_{blank} + 1.645 \times SD_{blank}$

The calculated LOB value of 0.578 ml corresponds to a POV of 0.0104 mEq/kg, taking into account that M_{thio} = 0.001M and 10g of sample were used. A general summary of the blank data is presented in table 1.

Table 1: General summary experiment LOB determination

Parameters	Blank measurements
Sample size	60
Arithmetic mean (mL)	0.4747
95% CI for the mean (mL)	0.4584 to 0.4909
Standard deviation (mL)	0.06286
Relative Standard Deviation	13.26%

As recommended, we have chosen to assign a value of 0.05 (5%) for type I (α) and type II (β) errors. In this case, the LOB contains 95% of all observed values. The remaining 5% corresponds to values which could be found when samples with very low analyte concentrations are tested, thus being false positive (type I error, α).

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) is the lowest analyte concentration which can reliably be distinguished from the LOB and at which detection is reasonable. We have calculated the LOD of both methods using the following formula, as described in the EP17 guideline [13]:

$LOD = LOB + 1.645 \times SD_{low\ concentration\ sample}$

In this case, 95% of the results will be higher than the LOB, thus only 5% of the low concentration samples will yield values below the LOB and result in a false negative (β) error.

For LOD determination, it is advised to analyze samples of which the analyte content lies within the proximity of LOD. In the present experiment, 10 samples of mineral oil were spiked with 10uL of 0.1% H_2O_2, and assessed for POV content. The LOB and SD of the observations were used to calculate LOD, yielding a value of 0.0356mEq/kg. The limit of quantification (LOQ) was determined according to the formula below, yielding a value of 0.1067 mEq/kg:

LOQ = 3 * LOD

Specificity

The POV assay is widely recommended as a reliable analysis method to assess the presence of oxidative substances in oils and fats. Official guidelines (pharmacopoeia monographs, ISO directives) are available for the pharmaceutical, fuel, and food industry [7, 8]. Therefore, no additional testing has been performed to confirm specificity of the POV assay, except for the spiking experiments (see below), which confirm specific analysis of oxidative content in mineral oil.

Linearity and range

Mineral oil samples have been spiked with 5 different volumes (10-100 µL) of a 0.1% hydrogen peroxide solution and peroxide value was determined. As shown in figure 3, there is an excellent correlation (R^2 = 0.996) between spike volume and peroxide value.

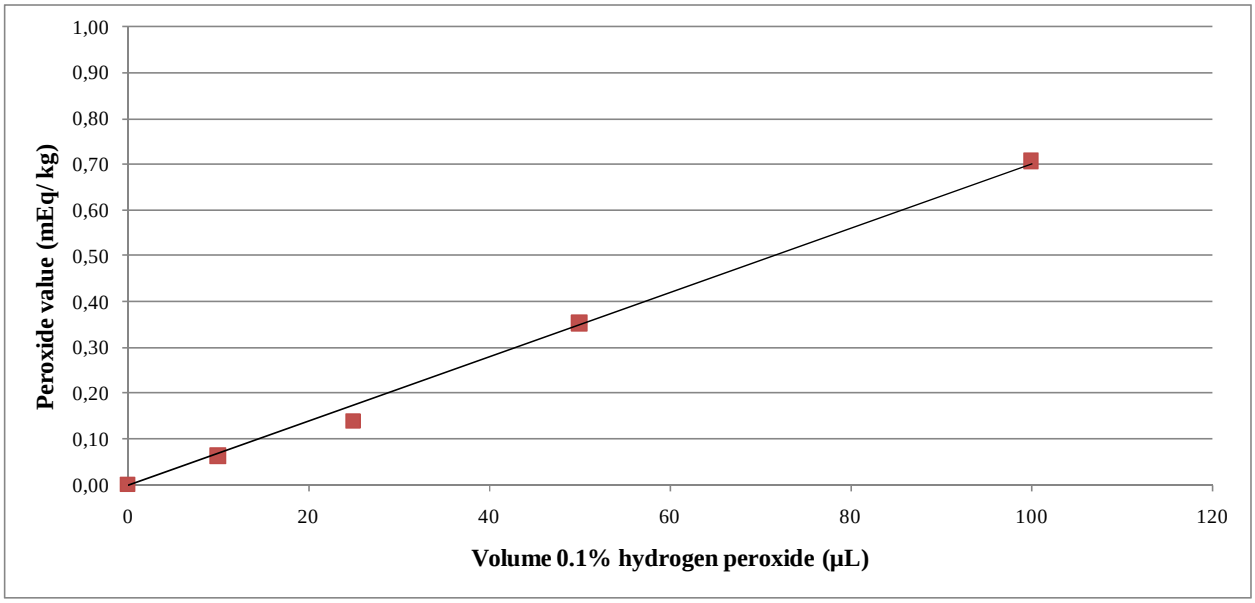

Figure 3: Linearity of the POV assay, after spiking with different peroxide concentrations

For the modified assay, the range lies between 0.0356 (LOD) and 0.7076 mEq/kg, which perfectly fits our requirements for the in-house QC assay. Indeed, POV of FertiCult™ Mineral oil must be lower than 0.1 mEq/kg to allow product release. However, linearity is also guaranteed at higher POV values (data not shown), allowing correct POV analysis of mineral oil samples with a higher oxidative content.

Accuracy

Recovery of different spikes of 0.1% H_2O_2 (10-100 µL) was calculated to determine accuracy of the POV assay. Briefly, the measured POV values were compared with their corresponding theoretical values. An average

overestimation of 5.1% was calculated. Only the lowest spike was underestimated by 3.9%. Recovery values are presented in table 2.

Table 2: Recovery (%) of different spikes

Spike (µL/POV)	Recovery (%)
10 / 0.065	96.1
25 / 0.163	106
50 / 0.325	108.8
100 / 0.646	109.5
Average	105.1
Stdev	6.2
RSD	5.9

Precision (inter- and intra-assay CV)

Inter-assay and intra-assay coefficient of variation (CV) for POV analysis were calculated using data from 4 independent experiments. The number of samples per run varied between 6 and 10. All experiments have been performed using mineral oil, with and without a peroxide spike (20 µL of a 0.1% H_2O_2 solution). The reason why two different oil samples were used is because unspiked mineral oil has a POV content that lies between the LOD and LOQ value (or even below LOD). Therefore, a significantly higher deviation will be obtained during sample analysis. The peroxide spike has been chosen in such a way that the resulting POV lies in the proximity of the LOQ, hereby allowing correct determination of precision parameters. Results are shown in table 3.

Table 3: Inter- and intra-assay CV values of mineral oil analyses

Intra-assay CV (%)	Interassay CV (%)
21.83	19.31
4.70	12.94

Indeed, CV values are much higher in non-spiked oil when compared to spiked oil, showing very low inter- and intra-assay CV values. In conclusion, the assay is able to determine POV values in samples with very low and low oxidative content in a reproducible manner.

Robustness

The adapted POV method must be able to detect extremely low oxidative content in mineral oil. Although it is a generally accepted and widely used assay, several parameters may influence assay performance. It is therefore essential to take into account the following guidelines to guarantee reproducible and reliable results.

Ultrapure water

Ultrapure water must be boiled and cooled prior to use. This is essential in order to remove traces of oxidative agents. Water of lower quality (e.g. reversed osmosis water, tap water) cannot be used for this type of analysis, as it may contain substances which can interfere with the electrochemical redox titration.

Potassium iodide

Different suppliers (VWR, Fagron, and ABC Chemicals) of pharmaceutical grade KI have been evaluated. Freshly prepared solutions yielded comparable blank values. However, if solutions of 1 or 2 days old were used, a higher volume of thiosulfate was required to titrate blank samples. Taking into account that the maximal allowable volume for a blank titration is 1 ml, several preliminary experiments exceeded this limit, especially with Fagron and in a lower extent, with VWR KI. This was confirmed by colour formation into the solution. Normally, freshly dissolved KI must be colourless. However, the colour of 1 day-old VWR and Fagron solutions varied from pale yellow to mediate yellow. This can be explained by the formation of I_2, although solutions were still saturated. These data are somewhat confusing, as all products were approved for pharmaceutical use and should yield comparable results, at least theoretically. This phenomenon was not observed with KI from ABC Chemicals. As shown in the Box-and-Whisker plots below (figure 4a and 4b), no significant difference ($P>0.05$) was observed between fresh and 1 or 2-day old KI solutions (n=60). Nevertheless, "old" KI solutions yielded a wider range of thiosulfate volume, indicating a slight decrease in quality. For this reason, all optimization and validation analyses have been performed with ABC Chemicals KI solution, prepared immediately prior to use.

Extraction procedure

Preparation of the extraction buffer must be performed analytically. Changes in the ratio of acetic acid and TMP content can alter blank values. Furthermore, solvents must be titration-grade (or comparable). Since the redox

reaction is measured electrochemically, the presence of trace elements may disturb the optimal functioning of the electrode, and thus alter assay outcome. For example, during assay optimization, we have experienced problems with glacial acetic acid. The initial batch, purchased at Merck chemicals (VWR, Haasrode, Belgium), yielded normal experimental results. However, extremely high blank values (1-2 ml) were obtained following introduction of a new batch. Finally, similar to ultrapure water and KI solution, extraction buffer must be prepared immediately before use, as results may also change in function of buffer age.

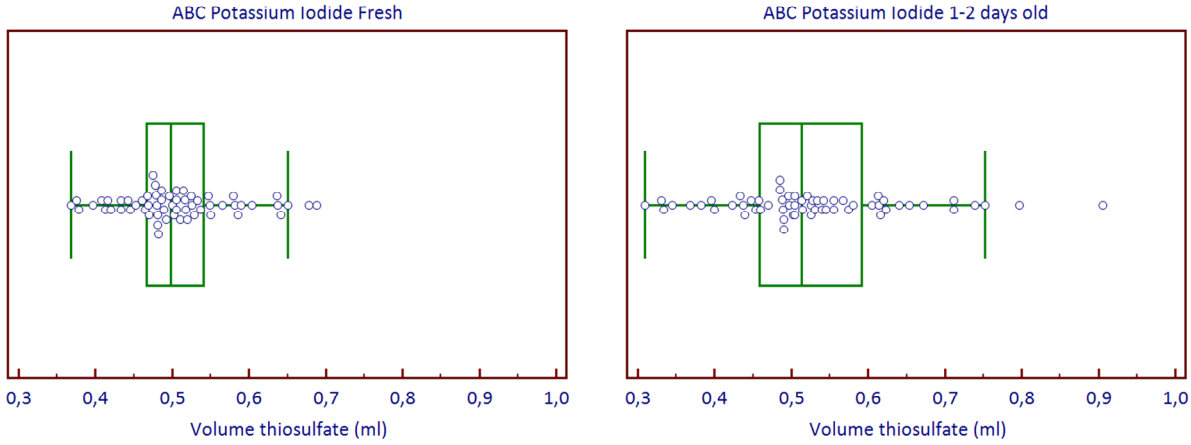

Figure 4 and b: Box-and-Whisker plot of blank thiosulfate volumes, obtained with fresh (left) and 2-3 days old (right) KI (left)

Light sensitivity

As POV analysis is a light-sensitive reaction, the disposable titration vessel must be protected from light during analysis. For this purpose, Mettler-Toledo provides orange-coloured vessels. Our experimental data showed a significant difference between transparent and coloured-vessels. However, wrapping cans with aluminium foil yielded even better results when compared to the orange vessels (data not shown). Therefore, each analysis must be performed in the presence of an aluminium cover.

Stirring speed

The oil/reagent solution is continuously stirred during titration to allow optimal dispersion of thiosulfate. However, one must take into account that stir speed must be relatively slow. High speed mixing introduces oxygen into the reaction mixture, which in turn, may affect assay outcome in a negative manner (data not shown).

POV and ART mineral oil

Pharmaceutical grade paraffin oil is an important source for the production of mineral oil for ART purposes. The latter serves as an overlay for *in vitro* embryo culture to maintain pH, temperature, and osmotic pressure, being crucial parameters for optimal embryo development [1, 2, 3]. In comparison to pharmaceutical and food oils, the concentration of oxidants and other toxic agents, must be extremely low to minimize embryotoxic effects [5, 6]. For this reason, paraffin oil is further purified by performing an extensive washing procedure, yielding an end product with very low POV value (<0.1 mEq/kg) [4]. Before batch release, FertiCult™ Mineral oil is subjected to a battery of QC tests, including viscosity assessment, mouse embryo assay (toxicity), and POV analysis. Since years, the EP POV assay is the gold standard for analysis of oxidative content in pharmaceutical and food oils, and implies an iodometric titration with visual end point determination [7, 8].

Based on our experience, the EP method is not suited for analysis of ART mineral oils. Due to the their low oxidative content, only limited iodine/I_3^- anion will be produced from iodide. Consequently, blue complex formation with starch is rather weak and thus, colour turnover is difficult to interpret during titration against thiosulfate. In addition, the sensitivity of the classic EP method is too low to reliably detect oxidative content in ART oils. This observation is confirmed by the data of Semb [16], reporting a detection limit of 2 mEq/kg.

In order to increase assay sensitivity, the EP method has been modified at several levels. First, instead of a visual end point determination, the redox reaction is constantly monitored using a specific, silver indicator electrode. The equivalence point is reflected by a strong inflection of the first derivative (dE/dV) during titration and does not require visual determination anymore. Thus, potentiometric analysis allows objective and reproducible end point interpretation.

Next, assay reagent concentration (primary standard and thiosulfate) was reduced with a factor 10. In the classic method, only small volumes of thiosulfate were required to reach the equivalence point in ART oils, hereby yielding large deviation amongst experimental results. In the present set-up, 10-fold higher reagent volumes are required to perform the redox reaction, hereby reducing the chance of assay errors. Finally, analyzing 10 g of oil sample (instead of 5g) doubles the total amount of oxidants in the reaction mix.

CONCLUSION

We have developed and validated an improved version of the EP method for POV analysis in mineral oil for ART purposes. By taking into account several factors, one can avoid several pitfalls, which may result in false negative (or positive) results. All applied changes have resulted in a very sensitive method with a detection limit of 0.0356 mEq/kg, being approximately 56x lower than the value, obtained with the classic method [16]. The measurement range of the assay lies between 0.0356 mEq/kg and 0.7076 mEq/kg and perfectly fits our requirements for the QC assay. FertiCult™ mineral oil is only released for sale purposes if POV<0.1 mEq/kg. However, the modified assay is also able to measure higher POV content (e.g. in UV-exposed samples) in a reliable and reproducible manner.

It is important to stress that many factors may influence assay performance, including the source of KI, the purity of extraction buffer ingredients, the use of (boiled) ultrapure water, light exposure, and stirring speed. However, if one takes into account all of these factors, a very reliable and reproducible protocol can be designed, allowing sensitive POV measurement in ART oils.

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