

A COMPARISON OF CERTAIN EXTRACTING AGENTS FOR EXTRACTION OF ADENOSINE TRIPHOSPHATE (ATP) FROM MICROORGANISMS FOR USE IN THE FIREFLY LUCIFERASE ATP ASSAY

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The importance of complete extraction of adenosine triphosphate (ATP) and the ability to assay with minimal inhibition led to a comparison of different ATP extracting agents for use in the firefly luciferase ATP assay. This assay can be used in clinical and industrial applications, such as determination of urinary infection levels, microbial susceptibility testing, and monitoring of yeast levels in beverages.

The optimal extracting agent is one which provides maximal extraction of ATP and minimal inhibition of the luciferase enzyme. Three categories of extractants were investigated for their extracting efficiency. They were ionizing organic solvents, nonionizing organic solvents, and inorganic acids. To represent the ionizing organic solvents, dimethylsulfoxide (DMSO) (Chappelle and Levin, 1964) and formamide were used. For the non-ionizing organic solvents n-butanol (Chappelle and Levin, 1968), chloroform,* ethanol (St. John, 1970), methanol (St. John, 1970), acetone (Chappelle and Levin), and methylene chloride (dichloro-methane) were used. And finally, for the inorganic acids category, nitric acid† and perchloric acid (Picciolo et al., 1971) were chosen. Concentrations used are given in the list below. The references cited for the above agents are for the agent as an ATP extractant, and the procedure used in the reference is not necessarily the one used in this study. These extracting agents were used on certain urinary tract pathogenic bacteria and yeast. They were also used on *Saccharomyces carlsbergensis* (Brewer's yeast).

*Dhople, A.M., J.H. Hanks, and E.W. Chappelle, "Ultrasensitive Method for Detection of Microbial and Mycobacter ATP," *Proc. Amer. Soc. Microbiol. Ann. Meeting*, 1971.

†See Picciolo et al., paper in this document.

Concentration of Extractants Used in Comparative Study

Chloroform 100%
Methanol 100%
Ethanol 100%
DMSO 30%
Formamide 10%
N-Butanol 6%
Methylene Chloride 90%
Acetone 90%
PCA 0.1 N and 1.0 N
HNO₃ 0.1 N and 1.0 N

The urinary tract pathogens used in the study were obtained from a clinical laboratory, and the *Saccharomyces carlsbergensis* used was obtained from a brewery. The urinary tract pathogens consisted of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter species*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, and *Candida albicans*. These were grown at 310 K (37°C) for 16 to 18 hours in trypticase soy broth. The *Saccharomyces* was grown in wort broth for 40 to 42 hours at 310 K (37°C). The bacteria were grown while shaking; the yeast were not. The organisms were centrifuged at 10,500 RCF × G for 5 minutes and the supernatant decanted. The organisms were then treated with the respective extracting reagent in its optimal extracting condition, with the optimal condition having been determined prior to this.

The procedure for each extracting agent was then followed, and the final diluent water was added. The sample was then assayed on the DuPont Biometer using DuPont firefly luciferase-luciferin, reconstituted in TRIS buffer at an optimal concentration and pH complimentary to the respective extracting agents.

The light units of the sample and the light units of the ATP standard that was run with each extracting agent were used to calculate micrograms per milliliter of ATP extracted. These results were then compared for extraction efficiency.

In comparing the organic extractants, the acetone extracted more ATP per milliliter of bacteria than the other organic extractants. It was noted that among the extractants, inhibition of the luciferase was not present with acetone or methylene chloride because these volatile solvents were boiled off.

Figure 1 shows the relative extraction efficiency of nitric acid (HNO₃), acetone, dichloromethane, n-butanol, formamide, and DMSO on *Pseudomonas aeruginosa*, a gram-negative organism. Figure 2 shows the same extractants on *Staphylococcus aureus*, a gram-positive organism.

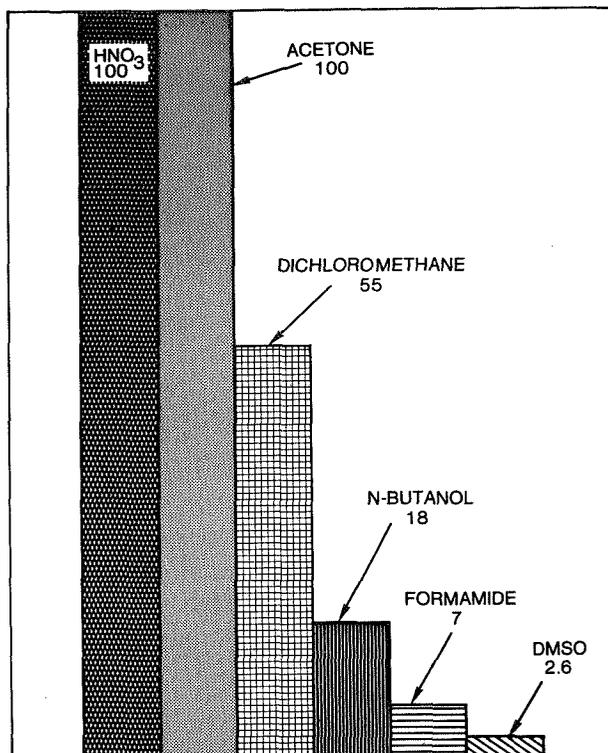


Figure 1. Relative extraction efficiency of various agents on *Pseudomonas aeruginosa*.

In figure 3 is the relative extraction efficiency of nitric acid, acetone, methanol, ethanol, and chloroform on *Klebsiella pneumoniae*, a gram-negative organism.

Figure 4 shows a comparison of acetone and chloroform on *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*, all of which are gram-negative, and on *Streptococcus faecalis*, a gram-positive organism.

Nitric and perchloric acid were found to be comparable in extraction efficiency. The acetone was then compared to the inorganic acid extractants after each procedure had been optimized for both bacterial and yeast ATP extraction.

The results obtained with inorganic acids and the acetone were comparable in extraction efficiency. It was noted that in the two procedures there was a 2 to 5 percent variation in injection values. This would result in the inorganic acid ATP extraction value and acetone ATP extraction value varying 2 to 5 percent.

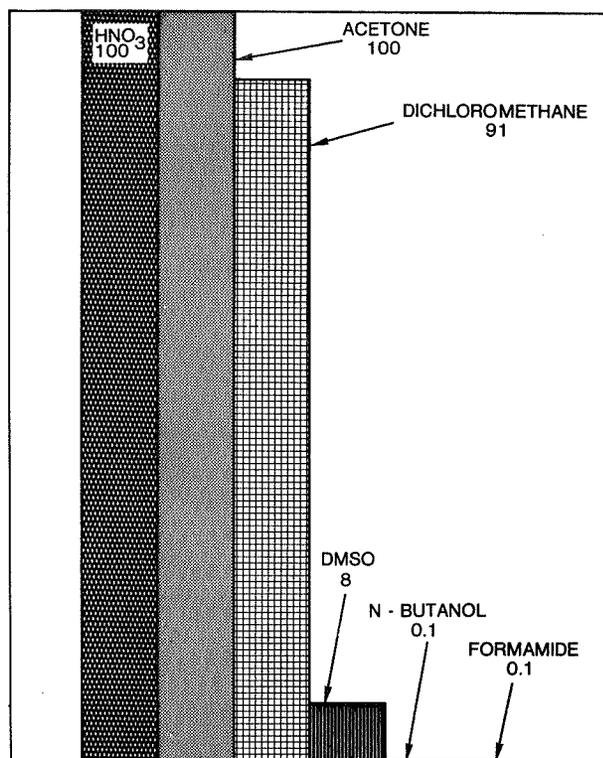


Figure 2. Relative extraction efficiency of various agents on *Staphylococcus aureus*.

In procedure 1, the acetone procedure for both bacterial and yeast ATP extraction is listed. Ten milliliters of sample are centrifuged and the supernatant decanted. The acetone is then added and the sample heated to permit the volatile solvent to boil off. The 0.5 milliliter nonvolatilized is then assayed with DuPont luciferase-luciferin on the DuPont Biometer.

Procedure 2 shows the nitric acid procedure for bacterial ATP extraction. Ten milliliters of sample are centrifuged and the supernatant decanted. The HNO₃ acid is added and, after 5 minutes, the diluent is added. The 0.4 milliliter is then assayed with DuPont luciferase-luciferin on the DuPont Biometer.

It was also noted that the blank value obtained with acetone was much lower than the blank value obtained with nitric acid and perchloric acid.

CONCLUSIONS

With results obtained in the study, it was evident that the acetone can also be used as a bacterial and yeast ATP extractant comparable to the inorganic acids.

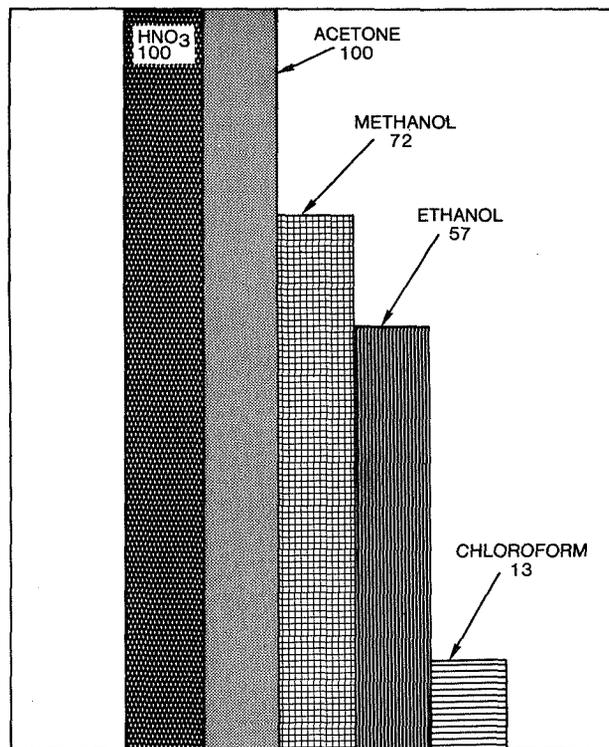


Figure 3. Relative extraction efficiency of various agents on *Klebsiella pneumoniae*.

There were several advantages to the acetone procedure. One of the most important advantages became evident with the yeast. In using nitric acid or perchloric acid it would require an initial acid concentration of 1.0 N to completely extract the ATP from the yeast. The result of using this concentration of acid required a dilution factor of 10 to allow for assay with the luciferase enzyme uninhibited. This dilution factor resulted in a loss of sensitivity. The acetone procedure was adequate for extracting bacteria or yeast with no adjustment needed.

Another advantage in the acetone procedure was the blank value obtained. The lower value obtained with the acetone procedure would allow for a wider range of ATP to be measured, thus lending to the ability to detect fewer microorganisms.

It was also shown that the advantage of having the acetone boiled off left no reagent to inhibit the luciferase and allowed for a lower molarity of TRIS buffer to be used which would increase luciferase activity.

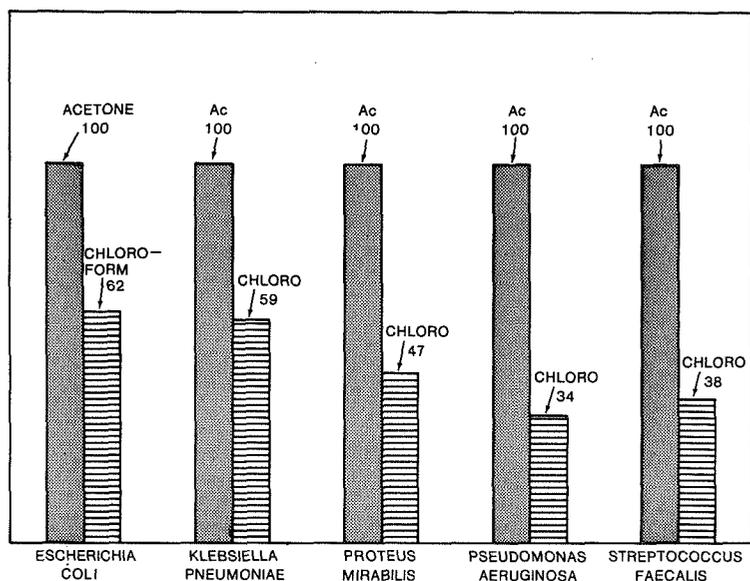


Figure 4. Relative extraction efficiency of acetone and chloroform on various bacteria.

Procedure 1

Acetone Procedure for Extraction of Yeast and Bacteria

1. 10 ml sample.
2. Centrifuge 10,500 RCF × G 5 minutes.
3. Decant supernatant 5 minutes.
4. Add 5.0 ml, 90 percent acetone (diluted with H₂O)—vortex.
5. Heat 40 minutes at 363 K (90°C) (until odor of acetone not present).
6. Let cool—vortex (0.5 ml should be remaining in tube after acetone has boiled off).
7. Assay: Inject 0.1 ml of above into 0.1 ml luciferase reconstituted with 1.5 ml of 0.05 M TRIS with 0.01 M MgSO₄ pH 7.75 per vial DuPont luciferase.

Procedure 1 (Continued)

8. An ATP standard of 1.0 $\mu\text{g/ml}$ or 10^{-1} $\mu\text{g/ml}$ should be used. Use 0.05 ml of ATP standard plus 5.0 ml of acetone and heat. Then assay.
9. Blanks should be run on the media in which the bacteria are suspended.
10. Calculation of micrograms ATP/ml should be done with consideration for the final volume of sample (0.50 ml) and for the ATP standard (0.55 ml).

Procedure 2

HNO_3 Procedure for Extraction of Bacteria

1. 10 ml sample.
2. Centrifuge 10,500 RCF \times G 5 minutes.
3. Decant supernatant 5 minutes.
4. Add 0.2 ml, 0.1 N HNO_3 .
5. Wait 5 minutes.
6. Add 0.2 ml of sterile, distilled, deionized water—vortex well.
7. Assay: Inject 0.1 ml of above into 0.1 ml luciferase reconstituted with 1.5 ml of 0.20 M TRIS with 0.01 M MgSO_4 pH 8.3 per vial DuPont luciferase.
8. An ATP standard of 1.0 $\mu\text{g/ml}$ or 10^{-1} $\mu\text{g/ml}$ should be used. 0.05 ml of ATP standard plus 0.2 ml 0.1 N HNO_3 , then add 0.2 ml of HOH and assay.
9. Blanks should be run on the water used and the media the bacteria are suspended in.
10. Calculation of micrograms ATP/ml should be done with consideration for the final volume of sample (0.40 ml) and for the ATP standard (0.45 ml).

There are also disadvantages to the acetone procedure. The most evident one is the 40-minute heating time as compared to the nitric acid and perchloric acid extraction time of 5 minutes. It was felt that the flammability and requirement of a hood for safe ventilation of the fumes were also disadvantages.

Over all, the acetone procedure is equivalent to the inorganic acid procedure in ATP extraction efficiency and can be used in applicable situations to an advantage.

REFERENCES

- Chappelle, E. W. and G. V. Levin, "The Use of the Firefly Bioluminescent Assay for the Rapid Detection and Counting of Bacteria," *Biochem. Med.*, 2, 1968, p. 49.
- Chappelle, E. W. and G. V. Levin, "Rapid Microbiological Detection," Navy Contractor Report 178-8097, 1964.
- Picciolo, G. L., B. N. Kelbaugh, E. W. Chappelle, and A. J. Fleig, "An Automated Luciferase Assay of Bacteria in Urine," NASA TM X-65521, 1971. (Also in *Proc. Chemical Abs.*)
- St. John, J. B., "Determination of ATP in Chlorella with the Luciferin Luciferase Enzyme System," *Analytical Biochem.*, 37, 1970, p. 409.