remove the chelated metal) with 1.0 $N$ HCl. The amount of acid required is determined by the metal loading on the column. For moderately loaded columns, 2 to 3 column volumes of acid removes more than 95% of the metal, but up to about 10 column volumes (or about 50 ml/g in batch procedures) may be necessary for complete regeneration. The beads are washed again with several column volumes of ultrapure water and dried in air. The material should remain active indefinitely.

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# [8] Use of Ascorbate as Test for Catalytic Metals in Simple Buffers

*By* GARRY R. BUETTNER

Over the years trace levels of adventitious transition metals have provided many problems for researchers studying oxidative processes, leading to the misinterpretation of many experiments.[1] For correct interpretation of these experiments, catalytic metals must be sequestered in an inactive form[2] or be removed from the solution.[1] Chelating resins are commonly employed to remove contaminating metals from buffer, salt, and protein solutions.[1] However, there always remains some doubt about the efficacy of a particular method as well as concern about subsequent recontamination of the solution. Ascorbate can be used to test for the presence of adventitious catalytic metals in simple near-neutral buffer solutions,[1] and a simple method for using this test is presented in this chapter.

## *Ascorbate Autoxidation*

Ascorbic acid is a diacid, $AH_2$, with p$K_a$ values of 4.2 and 11.6.[3] Thus, at near-neutral pH the dominant species is the monoanion, $AH^-$. Ascor-

[1] G. R. Buettner, *J. Biochem. Biophys. Methods* **16,** 27 (1988).

[2] G. R. Buettner, *Bioelectrochem. Bioenerg.* **18,** 29 (1987).

[3] S. Lewin, "Vitamin C: Its Molecular Biology and Medical Potential." Academic Press, London, 1976.


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bate is an easily oxidizable reducing agent. However, the rate of air oxidation of ascorbate in aqueous solution is very pH- and catalytic metal-dependent. In the presence of catalytic metals $AH_2$ oxidizes very slowly, whereas $AH^-$ oxidizes much more rapidly.[1,4] In the absence of catalytic metals, $AH^-$ is quite stable in air-saturated buffer.[1] It appears that metals are absolutely required for this oxidation. Thus, the rate at which ascorbate solutions air oxidize can be used to monitor for the presence of contaminating catalytic metals.

*Method*

For the standard test a 0.100 *M* ascorbate stock solution is prepared using reagent grade ascorbic acid and high-purity water. This results in a colorless solution of approximately pH 2. The low pH stabilizes the ascorbate, so the solution can be kept for days or even weeks for use in the test. Also, once oxygen is consumed oxidation stops, with less than 1% of the ascorbate stock being oxidized. Thus, storage in a relatively air-tight flask helps to increase the shelf life. The appearance of a yellow color indicates significant ascorbate oxidation, and a fresh solution should be prepared.

To perform the test, 3.75 μl of the ascorbate stock is added to 3.00 ml of the solution to be tested. This results in an initial absorbance of 1.8 at 265 nm.[5] The ascorbate absorbance is followed for 15–30 min. In successfully demetaled buffer the loss of ascorbate should be 0.5% or less in 15 min. A greater loss indicates that a significant concentration of catalytic metals remains.[6,7] This test can be used in buffers with pH values ranging from approximately 4 to 8. Below pH 4 ascorbate oxidizes too slowly to provide a sensitive test; above pH 8 the concentration of the dianion becomes significant, and it oxidizes too rapidly to allow for a good test.

When a chelating resin is used to lower the catalytic metal concentration to an acceptable level, some potential problems and considerations should be noted: (1) In the column method, the flow rate should not be too high, because a slow rate of flow is essential to demetal a buffer successfully; (2) in the batch or dialysis methods,[1] the time of stirring should be long enough; (3) the chelating resin may need regeneration (in fact, the

[4] A. E. Martell, *in* "Ascorbic Acid, Chemistry, Metabolism, and Uses" (P. A. Seib and B. M. Tolbert, eds.), p. 124. American Chemical Society, Washington, D.C. 1982.

[5] In my experience the $\varepsilon_{265}$ value for ascorbate in 50 m*M* phosphate buffer (pH 7.0) is 14500 $M^{-1}$ $cm^{-1}$, although values ranging from 7500 to 20400 $M^{-1}$ $cm^{-1}$ have been reported.[3]

[6] If no effort is made to demetal near-neutral buffer solutions, typical ascorbate losses in the standard 15-min test can range from 1 to 30% (or more),[1,7] consistent with approximately 1 μ*M* iron and/or 0.1 μ*M* copper being present. After successful treatment with chelating resin these levels have been estimated to be $<0.1$ μ*M* iron and ~1 n*M* copper.[1]

[7] G. R. Buettner, *Free Radical Res. Commun.* **1**, 349 (1986).

ascorbate test is an excellent method to determine the status of the chelating resin); (4) glassware may recontaminate the solution[8]; and (5) dust, fingers, and phantom sources of contamination may seem to appear magically.

Using this simple, inexpensive method, it is easy to verify that the procedures used to demetal buffer and salt solutions are successful. The method provides a tool for troubleshooting when problems occur, and it is an easy and repeatable way to determine and report experimental conditions.

[8] Standard laboratory glassware is used for solution storage. After this glassware has been successfully cleaned, however, it is never returned to the usual pool of laboratory glassware for general use. Rather, when fresh solutions are required, the glassware is rinsed with high-purity water and refilled with the same solution.

# [9] Spin-Trapping Methods for Detecting Superoxide and Hydroxyl Free Radicals *in Vitro* and *in Vivo*

*By* GARRY R. BUETTNER and RONALD P. MASON

Spin trapping has become a valuable tool in the study of transient free radicals as evidenced by the many investigations in which it has been employed.[1] Oxygen-centered radicals are of particular interest because they have been implicated in many adverse reactions *in vivo*. Their short lifetimes and broad linewidths make many of these radicals difficult, if not impossible, to detect by direct electron spin resonance (ESR) in room temperature aqueous solutions. Spin trapping provides a means, in principle, to overcome these problems, but it is not without its pitfalls and limitations. We discuss some of these problems in this chapter.

## *Choice of Spin Trap*

Two types of spin traps have been developed, nitrone and nitroso compounds. In aqueous solutions, however, oxygen-centered spin adducts of nitroso spin traps such as MNP (2-methyl-2-nitrosopropane) are, in general, quite unstable. Thus, the nitrone spin traps are by far the most popular. The most useful radical trap for the study of oxygen-centered free radicals is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which has

[1] G. R. Buettner, *Free Radical Biol. Med.* **3,** 259 (1987).


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