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(54) ANALYTICAL COMPOSITIONS

(71) We, EASTMAN KODAK COMPANY, a Company organized under the Laws of the State of New Jersey, United States of America, of 343 State Street, Rochester, New York 14650, United States of America, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

The present invention relates to a method, composition and element for the determination of ascorbate in aqueous liquids.

Ascorbic acid is essential in the human body for the normal regulation of the colloidal conditions of intracellular substances, including the fibrils and collagen of connective tissues. Deficiency of ascorbic acid (Vitamin C) results in abnormal development and maintenance of tissue structures, capillary defects and eventual development of scurvy. There is, therefore, a need for determining ascorbic acid in body fluids.

We have developed a composition, element and method for the assay of ascorbate in aqueous liquids such as blood serum which is highly selective (based on an enzyme reaction), very rapid (requires less than 5 minutes), and can be carried out at relatively low temperatures (below or about 37°C).

According to the present invention there is provided a composition for the determination of ascorbate in an aqueous liquid which comprises ascorbic acid oxidase, a coupling substance which interacts with dehydroascorbic acid to produce a detectable product and a buffer.

The term ascorbate is used herein to refer to both ascorbic acid present in its free form as a carboxylic acid or carboxylate ion and in its combined form as an ascorbate ester.

While the present compositions may be used for quantitative determination of ascorbate, they may also of course, be used for qualitative detection.

The present invention also provides a method for determining ascorbate in an aqueous liquid which comprises contacting a sample of the liquid with a composition according to the invention and determining the amount of detectable product thereby produced.

The present invention couples the enzymatic conversion of L-ascorbic acid to dehydroascorbic acid by ascorbic acid oxidase and the reaction of dehydroascorbic acid with certain coupling substances to produce a detectable product.

Ascorbic acid oxidase (L-Ascorbic acid:oxygen oxidoreductase, EC 1.10.3.3) (hereinafter identified as ASO) is known in the art. This enzyme catalyzes the reaction:-

2L-ascorbic acid + O_2 → 2 dehydroascorbic acid + $2H_2O$.

So long as the enzyme catalyzes the foregoing conversion and does not generate products which interfere with the subsequent coupling of the dehydroascorbic acid according to the reaction:-

dehydroascorbic acid + coupling substance → detectable product, the particular enzyme or its source is not critical.

An enzyme extracted from *Cucurbita pepo medullosa* using the technique described in Tables I and II below particularly useful in the compositions and methods described herein. This enzyme has a pH optimum between about 5.7 and 8.7 and hence assay compositions and

media for the detection/determination of ascorbic acid using these methods and materials are buffered in a conventional fashion at this level. Particularly useful buffers include Tris-HCl, Na^+ and K^+ phosphates, Tris-phosphate, Tris (10^{-4} M EDTA)-all at or about 0.05M. Most preferred is sodium phosphate.

It is preferred, in order to optimize the assay, to buffer the composition at a substantially neutral pH, i.e., between 6.8 and 7.2 and most preferred to buffer between 6.8 and 7.0.

The ASO extraction technique described below produces an extract which demonstrates a specific activity of about 3.0 - 5.5 I.U. per mg of protein. One unit of enzyme activity is defined as that amount of enzyme protein which will oxidize 1 μmole of ascorbic acid per minute in 0.05M sodium phosphate buffer at 37°C and pH 6.80 ± 0.05.

TABLE I

Purification Protocol for Ascorbic Acid Oxidase from Green Zucchini Squash (Cucurbita pepo medullosa) (Flow Chart)

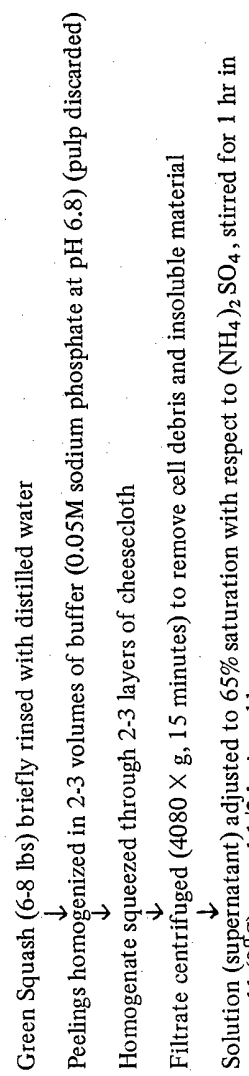

Table II

Summary of Purification of Ascorbic Acid Oxidase

Step	*Preparation*	*Total mg protein*	*Specific Activity (Units/mg)*	*Total Units*	*Activity Recovery (%)*	*Apparent Purification Factor*
1. Crude homogenate of peelings	A	2800	0.67	1876	100	1
	B	12800	0.89	11392	100	1
2. Ammonium Sulphate Precipitation	A	2150	0.82	1760	94	1.22
	B	6042	1.71	10331	91	1.92
3. Acetone Precipitation	A	1278	0.89	1137	61	1.33
	B	5130	1.61	8259	72	1.81
4. Dialysis	A	243	3.4	826	44	5.1
	B	549	5.6	3074	27	6.3

Note: These two preparations started from 3.2 kg of purchased Zucchini squash. The actual *dry* weight of each preparation after lyophilization of the dialysate was approximately 2 to 3 times the gram weight as determined by the procedure of Lowry. Thus, preparation (A) yielded a dry weight of ~0.69 grams while (B) 1.7 grams. It is this dry weight which is quoted in this work.

The second component of the assay compositions hereof comprises a coupling substance which interacts with the dehydroascorbic acid to form a detectable product, preferably a coloured or fluorescent product in proportion to the amount of ascorbic acid contained in a sample under analysis. Thus, substantially any coupling substance so interactive in a medium which permits analysis of ascorbic acid by ASO is considered useful in the successful practice of the invention.

Such coupling substances include by way of example, *o*-, *m*- and p-phenylenediamines substituted or unsubstituted which interact with dehydroascorbic acid to yield a blue fluorophor, which can be monitored spectrophotometrically or fluorometrically. *o*-Phenylenediamine is specifically preferred from among this group of coupling substances. Certain of these coupling substances perform more satisfactorily in the presence of small amounts of organic solvents such as methanol which appear to impart increased solubility.

Other examples of useful coupling substances are:

N,N-dimethyl *p*-phenylenediamine dihydrochloride,
N,N-dimethyl *p*-phenylenediamine monohydrochloride,
2,3,5,6-tetramethyl *p*-phenylenediamine dihydrochloride,
hydroquinone,
2,4-diaminophenol dihydrochloride,
4-amino-N-ethyl-N-(β-methylsulphonamidoethyl)-m-toluidine sesquisulphate monohydrate,
p-aminophenol monohydrochloride,
N-ethyl-N-(β-hydroxyethyl)-*p*-phenylenediamine sulphite and
p-phenylenediamine dihydrochloride

The compositions of the present invention may be provided either as a dry mixture of lyophilized enzyme and powdered buffer and coupling substance readily reconstitutable with water or as an aqueous solution. Furthermore, assay compositions as described herein can be incorporated into any of the wide variety of test elements well known to those skilled in the art. Such elements generally comprise an absorbent matrix, for example paper, impregnated with reagents. Typical such materials and elements produced therewith which can be adapted for the assay of ascorbic acid using the compositions described herein are those described, for example, in the following U.S. Patents: 3,092,465, 3,418,099, 3,418,083, 2,893,843, 2,893,844, 2,912,309, 3,008,879, 3,802,842, 3,798,064, 3,298,739, 3,915,647, 3,917,453, 3,933,594, 3,936,357, etc.

Such compositions may also be incorporated into multilayer analytical elements of the type described in British Specification 1,440,464. A more detailed discussion of such elements is presented below.

In whatever physical state the reagent composition is initially provided it should be such as to produce, at the time of use, an aqueous medium comprising at least 10μg/ml of ASO, from 5×10^{-3} to 10^{-2}M coupling substance and sufficient buffer to provide a pH of between 5.7 and 8.7, preferably between 6.8 and 7.2 and most preferably between 6.8 and 7.0. Lowering of the coupling substance concentration below this range reduces the dynamic range of the test rather significantly. However, coupling substance levels below this range may be useful for qualitative ascorbic acid detection. Although the determination can be carried out at temperatures of between 16 and 40°C, best results are obtained at a temperature of 20° ± 2°C. The time required for any given determination will, of course, vary depending upon the particular reagents used, the concentration of reagents and the temperature of the determination. Using the preferred materials and conditions, optimum results are obtained in about 5 minutes.

As alluded to hereinabove, the compositions described herein may be incorporated into single- or multi-layered analytical elements of the type described in the extensive prior art in this field.

It is preferred to incorporate the compositions described herein into analytical elements of the type described in aforementioned British Specification 1,440,464.

Accordingly a highly preferred embodiment of the present invention comprises an element for determining ascorbate in an aqueous liquid which comprises a liquid-impermeable transparent support bearing a reagent layer containing the composition according to the invention and superposed thereover, a porous layer adapted to spread liquid applied to it.

A generally useful range of concentration for the ascorbic acid oxidase preparation in the elements of this invention is between 0.10 and 0.60 g/m^2. Coupling substances such as *o*-phenylenediamine are generally included at levels of from 0.01 to 1.0 g/m^2.

The following Examples illustrate the present invention.

Example 1

Mixtures were prepared containing 10-12 μg of squash ascorbate oxidase prepared as described in our Copending Application and *o*-phenylenediamine at a level of 5×10^{-3}M.

Ascorbate was added to these mixtures at varying levels immediately after formulation and the reaction at 20° ± 2°C monitored by following the increase in absorbance at 350 nm. The figure of the accompanying drawings is a graph of replicate ascorbate determinations obtained at various levels of ascorbate at a five minute endpoint. As shown in Figure 1 the five minute endpoint colour changes demonstrate a linear response in the ascorbate range of 0-10 mg/dl which encompasses the known normal (0.5-1.5 mg/dl) and abnormal (1.4-2.8 mg/dl) ranges in human serum.

Example 2

A multilayered element for the determination of ascorbic acid was prepared as follows: a 0.25 mm thick cellulose acetate support was first coated with an indicator layer comprising; gelatin (10.7g/m^2), sodium phosphate buffer (pH 6.0) and o-phenylenediamine (0.10g/m^2). After drying this layer was overcoated with a reagent layer comprising gelatin (5.4g/m^2), sodium phosphate buffer (pH 6.8), bis (vinylsulfonylmethyl) ether (0.06g/m^2), oleic ether of polyethylene glycol (0.3g/m^2) and ASO (0.6g/m^2). This layer in turn was coated with a spreading layer comprising microcrystalline cellulose (86g/m^2) (commercial product available from FMC Corporation under the trade mark Avicel) and polyvinylpyrrolidone (2.2g/m^2) in a water/isopropyl alcohol solvent mixture.

This element was evaluated by applying 10μl aliquots of freshly prepared samples of aqueous ascorbic acid solution (0-5mg/dl) in 0.05M phosphate buffer at pH 6.8 to the spreading layer. The change in relative fluorescence as a function of the change in millivolts, Δmv, was monitored at 340mμ excitation and 400-600mμ emission. The results of this testing are shown in Table I

Table I

Fluorometric; 3-minute end-point

Ascorbic Acid Concentration mg/dl	*Δmv*
0	0
1	26
2	54
3	73
5	122

The results show that the element is useful for determining the concentration of ascorbic acid in an aqueous sample only three minutes after application of the sample.

WHAT WE CLAIM IS:-

1. A composition for the determination of ascorbate in an aqueous liquid which comprises ascorbic acid oxidase, a coupling substance which interacts with dehydroascorbic acid to produce a detectable product and a buffer.
2. A composition as claimed in Claim 1 wherein the ascorbic acid oxidase is from *Curcurbita pepo medullosa*.
3. A composition as claimed in Claim 1 or 2 in which the coupling substance is a *o*-, *m*- or *p*-phenylenediamine.
4. A composition as claimed in any of Claims 1-3 in which the buffer is capable of buffering an aqueous liquid at a pH between 5.7 and 8.7.
5. A composition as claimed in any of Claims 1-3 in which the buffer is capable of buffering an aqueous liquid at a pH between 6.8 and 7.2.
6. A composition as claimed in any of Claims 1-5 which is an aqueous solution containing at least 10μg/ml of ascorbic acid oxidase.
7. An aqueous composition as claimed in Claim 6 which contains from 5×10^{-3} to 10^{-2}M coupling substance.
8. A composition according to Claim 1 substantially as described herein and with refer-

ence to the Examples.

9. A method for determining ascorbate in an aqueous liquid which comprises contacting a sample of the liquid with a composition according to any of Claims 1-8 and determining the amount of detectable product thereby produced.

10. A method according to Claim 9 substantially as described herein and with reference to the Examples.

11. An element for determining ascorbate in an aqueous liquid which comprises a liquid-impermeable transparent support bearing a reagent layer containing the composition according to any of Claims 1-8 and superposed thereover, a porous layer adapted to spread liquid applied to it.

12. An element as claimed in Claim 11 in which the reagent layer contains 0.10 to 0.60 g/m^2 of ascorbic acid oxidase and 0.01 to 1.0 g/m^2 of coupling substance.

13. An element according to Claim 11 substantially as described herein and with reference to the Examples.

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1579040 COMPLETE SPECIFICATION

1 SHEET This drawing is a reproduction of the Original on a reduced scale