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**MULTILAYER ANALYTICAL ELEMENT CONTAINING PRIMARY AMINE
BUFFER AND METHOD FOR THE DETERMINATION OF ETHANOL**

This invention relates to clinical chemistry. In particular, it relates to a multilayer analytical
5 element and method for the quantitative determination of ethanol.

Ethanol is a commonly encountered toxic substance. Methods for qualitative and quantitative determination of ethanol in body fluids, particularly
10 human body fluids, are used in medicine and in law enforcement. In medicine, the level of ethanol in the blood is significant in diagnosing liver malfunction and alcoholism, as well as for understanding the reason for an emergency room patient being comatose. In law
15 enforcement, such assays are used to determine whether or not an automobile operator is driving under the influence of alcohol.

Ethanol testing can be accomplished using both enzymatic and nonenzymatic assays. The
20 nonenzymatic assays have a number of disadvantages and are being widely replaced by enzymatic assays which are more accurate, highly specific, more sensitive and require less expensive procedures. Enzymatic assays are generally based on the use of alcohol dehydrogenase
25 to catalyze the reaction of ethanol to acetaldehyde. This reaction can be used alone, or in combination with other reactions to produce a spectrophotometric signal which can be related to the amount of ethanol in the tested specimen.

30 An enzymatic assay for ethanol is described in EP-A-0 464 942 which uses nicotinamide adenine dinucleotide (NAD⁺) as a coenzyme with alcohol dehydrogenase to produce the reduced form of the coenzyme. The coenzyme, in turn, reacts with a
35 tetrazolium salt to produce a detectable dye.

One problem that has been encountered in developing a dry analytical element for the assay of ethanol is the strong interference by fluoride ion present in human serum. Fluoride ion is commonly used
5 as a preservative in serum, and interferes in assays possibly by altering the equilibrium between ethanol and acetaldehyde, and causes the assay results to be biased positively compared to the true value of ethanol in the specimen.

10 There is a great need for a sensitive and accurate assay for ethanol which can be carried out using an analytical element which is not affected by fluoride ion.

The problem with fluoride ion interference in
15 assays using analytical elements is overcome with an analytical element for the determination of ethanol comprising a support having thereon, in order and in fluid contact:

a) a first reagent layer,
20 b) a second reagent layer,
c) a third reagent layer comprising alcohol dehydrogenase, and

d) a porous spreading layer containing an oxidized nicotinamide coenzyme,
25 the element characterized wherein:

the first and third reagent layers comprise the same buffer which has a primary amine which is mixed with an uncrosslinked hydrophilic binder, the buffer maintaining the pH at from 8 to 10 during an
30 assay for ethanol and being present in each layer in an amount of at least 25 mmoles/m², and

the second reagent layer comprises a crosslinked hydrophilic binder.

This invention also provides a method for the
35 detection of ethanol comprising:

A) contacting an aqueous fluid suspected of containing ethanol with the analytical element described above, and

5 B) detecting the absorbance of the reduced form of the nicotinamide coenzyme as an indication of the presence of ethanol in the aqueous fluid.

The present invention provides a dry analytical element for the effective and specific detection of ethanol in a relatively short time using a
10 signal generated by the reduction of a nicotinamide coenzyme by alcohol dehydrogenase. Interference by fluoride ion is greatly reduced in assays using the element of this invention. Fluoride interference is reduced by using a high amount of a buffer which has a
15 primary amine. Moreover, in order to use a high amount of buffer, it became necessary to put it into more than one reagent layer. Yet if the hardener typically used to crosslink the binder in standard reagent layers contacts the buffer, binder crosslinking is adversely
20 affected. The hardener also must be kept isolated from the alcohol dehydrogenase. The present invention accommodates all of these requirements with a series of three reagent layers beneath the porous spreading layer. Two reagent layers on either side of a middle
25 reagent layer contain uncrosslinked binders and contain specific amounts of the needed buffer. The middle reagent layer is crosslinked, but contains no buffer.

The element of this invention can be used to determine (that is, detect either the presence,
30 amount or both) ethanol in biological fluids of animals or humans, but preferably in humans. Such fluids include whole blood, plasma, sera, lymph, bile, urine, semen, cerebral spinal fluid, spinal fluid, sputum, perspiration, synovial fluid, lacrimal
35 fluid and stool specimens as well as other biological fluids readily apparent to one skilled in the art.

Fluid preparations of tissues can also be assayed. Preferably, human serum is assayed with this invention.

In its broadest embodiment, the dry element
5 of this invention has an inert support with three reagent layers and a porous spreading layer disposed thereon. The support is generally dimensionally stable, inert to chemical reaction and preferably transparent. However, non-transparent supports can
10 be used if the mode of detection is reflectance spectroscopy instead of transmission spectroscopy. Useful supports are well known in the art.

The porous spreading zone is prepared from any of the known materials used for such zones as
15 described, for example in US-A-4,292,272, US-A-3,992,158, US-A-4,258,001, US-A-4,430,436, and JP 57(1982)-101760. Preferred spreading zones are those described in US-A-3,992,158 as "blush polymer" zones.

The elements have at least three other
20 layers which can contain one or more reagents needed for the assay. All of the layers are generally in fluid contact with each other, meaning that fluids, reagents and reagent products can pass or be transported between superposed regions of adjacent
25 layers, unless of course, a reagent is immobilized in some manner so it will not migrate within or without a layer.

The reagent layers can be composed of one or more hydrophilic binder materials [such as gelatin
30 and other colloidal materials, hydrophilic polymers such as poly(vinyl alcohol), acrylamide polymers, vinylpyrrolidone polymers and others known in the art, or mixtures thereof] in which reagents are incorporated. The same or different (or mixtures)
35 binders can be in the noted reagent layers. The hydrophilic binder in the first and third reagent

layers is uncrosslinked, while the hydrophilic binder in the second reagent layer is crosslinked using a conventional hardener such as bis(vinylsulfonylmethyl)ether,

5 bis(vinylsulfonyl)methane, glutaraldehyde, succinaldehyde and others known in the art using conventional amounts and procedures. Various materials for such layers are described, for example, in US-A-4,042,335, US-A-4,132,528 and US-A-4,144,305.

10 The buffer used in the first and third reagent layers is one which has a primary amine and maintains the pH of the layer during an assay at from 8 to 10, and preferably at from 8.5 to 9. A number of such buffers are known and commercially available. 15 They include tris(hydroxymethyl)aminomethane, tris(methyl)aminomethane, and their acid forms (for example, acid addition salts of HCl, HF and the like), and tris(hydroxymethyl)aminomethane glutamate. Tris(hydroxymethyl)aminomethane or 20 tris(hydroxymethyl)aminomethane hydrofluoride is preferred.

The amount of buffer in each reagent layer is generally at least 25 mmol/m², and from 30 to 50 mmol/m² is preferred. It is not necessary, but it 25 is preferred, that the amounts in the two layers be the same.

The third reagent layer also contains alcohol dehydrogenase which can be obtained from a number of commercial sources. Generally, the enzyme 30 is present in an amount of from 5000 to 30,000 I.U./m². As used in this application, one I.U. represents the International Unit for enzyme activity and is defined as the amount of enzyme activity required to catalyze the conversion of 1 micromole of 35 substrate per minute under standard pH and

temperature conditions. For alcohol dehydrogenase, the standard conditions are 25°C and a pH of 8.

Within the porous spreading layer is an oxidized nicotinamide coenzyme which can be reduced to provide a detectable colorimetric signal upon reaction with ethanol as catalyzed by alcohol dehydrogenase. Useful oxidized coenzymes include oxidized nicotinamide adenine dinucleotide (NAD⁺) and oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺). For example, in the assay, NADH absorbs at 340 nm, and NADPH absorbs at 340 nm.

A variety of different elements, depending upon the method and equipment for assay, can be prepared in accordance with this invention. They can be configured in a variety of forms and shapes, including elongated tapes of any desired width, sheets, slides or chips. Preferred elements are configured as test slides like those commercially available under the EKTACHEM™ trademark for a variety of clinical assays. Generally, the layers are formed on a suitable support by applying specific aqueous or solvent-based formulations of individual layer compositions in sequence using suitable coating equipment, and procedures followed by drying.

The assay of this invention can be manual or automated. In general, the element is used by physically contacting it with the test specimen (for example, from 1 to 200 µl) suspected of containing ethanol under ambient conditions (although other temperatures can be used). The specimen and reagents become mixed within the layers of the element and any ethanol present in the specimen reacts with the oxidized nicotinamide coenzyme to produce the reduced form which is detectable as described above. Contact can be achieved in any suitable manner, for example by dipping or immersing the element into the specimen

or preferably, by spotting the specimen onto the element by hand, machine or suitable dispensing means.

5 After specimen application, the element is exposed to any conditioning, such as incubation, heating or otherwise, that may be desirable to quicken or otherwise facilitate obtaining a test result.

10 Generally within 5 minutes, a spectrophotometric measurement is made. This measurement can be made using suitable reflection or transmission spectrophotometric equipment and procedures as a measure of ethanol concentration in the test sample. Generally, the detectable signal is
15 measured at a wavelength in the range of from 320 to 360 nm.

20 Example 1 Preferred Analytical Element for Ethanol Determination

The preferred element of this invention and amounts of components (as coated) are illustrated in the structure:

		Dry Coverage (g/m ²)
Spreading Layer	Barium sulfate	105
	Cellulose acetate	8
	ESTANE™ 5715 polyurethane	1.1
	TRITON™ X-405 nonionic surfactant	2.1
	NAD ⁺	8
Subbing Layer	Poly(N-isopropylacrylamide)	0.4
Third Reagent Layer	Gelatin (unhardened)	6
	Tris(hydroxymethyl)- aminomethane	5
	Ottasept	0.02
	TRITON™ X-200E anionic surfactant	0.01
	TRITON™ X-100 nonionic surfactant	0.02
	Alcohol dehydrogenase	10,000*
	Bovine Serum Albumin	1.75
Second Reagent Layer	Gelatin	1.1
	Bis(vinylsulfonylmethyl) ether	0.14
	TRITON™ X-200E anionic surfactant	0.01
	TRITON™ X-100 nonionic surfactant	0.02
	Ottasept	0.003
First Reagent Layer	Gelatin	6
	TRITON™ X-100 nonionic surfactant	0.02
	TRITON™ X-200E anionic surfactant	0.01
	Ottasept	0.02
	Tris(hydroxymethyl)amino- methane	5
Poly(ethylene terephthalate) Support		

*I.U./m²

Example 2 Determination of Ethanol

This example demonstrates the use of the element of this invention and shows the reduction of interference by fluoride ion achieved by the practice
5 of this invention.

Interference by fluoride ion was evaluated by using serum specimens containing known amounts of ethanol. To portions of the specimens, certain quantities of sodium fluoride were added. The amount
10 of ethanol was then determined in both a portion of the specimen containing no fluoride ion (identified as a "blank"), and in the portion of the specimen containing the fluoride ion. The difference between the two results of predicted values of ethanol
15 concentration is then defined as the interference caused by fluoride ion.

In this particular experiment, specimens containing 60, 130 and 220 mg/dl of ethanol, respectively, were used. To a portion of each
20 specimen was added 2000 mg/dl of sodium fluoride. Each specimen portion was assayed using analytical elements like that of Example 1 except the amount of buffer and pH were varied. The results of the assays are shown below in the table. They indicate that
25 interference by fluoride ion is reduced by increasing pH, increasing the amount of buffer, or both.

Amount of Ethanol (mg/dl)		Difference in Predicted Ethanol Values (mg/dl)	
		pH 8.25	pH 8.75
5	60		
	82 mmoles/m ² buffer*	1.7	-1.6
	49.2 mmoles/m ² buffer*	3.7	1.7
130			
10	82 mmoles/m ² buffer*	10.4	3.6
	49.2 mmoles/m ² buffer*	16.1	9.8
220			
15	82 mmoles/m ² buffer*	17.0	7.2
	49.2 mmoles/m ² buffer*	30.0	16.5

*Buffer = tris(hydroxymethyl)aminomethane.



CLAIMS:

1. An analytical element for the determination of ethanol comprising a support having thereon, in order and in fluid contact:
 - 5 a) a first reagent layer,
 - b) a second reagent layer,
 - c) a third reagent layer comprising alcohol dehydrogenase, and
 - d) a porous spreading layer containing an
- 10 oxidized nicotinamide coenzyme, the element characterized wherein:

the first and third reagent layers comprise the same buffer which has a primary amine which is mixed with an uncrosslinked hydrophilic binder, the
- 15 buffer maintaining the pH at from 8 to 10 during an assay for ethanol and being present in each layer in an amount of at least 25 mmoles/m², and
- the second reagent layer comprises a crosslinked hydrophilic binder.
- 20 2. The element as claimed in claim 1 wherein the hydrophilic binder in each of the reagent layers is gelatin.
3. The element as claimed in either claims 1 and 2 wherein the buffer in the first and third
- 25 reagent layers is tris(hydroxymethyl)aminomethane, tris(methyl)aminomethane or an acid form thereof or tris(hydroxymethyl)aminomethane glutamate.
4. The element as claimed in any of claims 1 to 3 further comprising a subbing layer between the
- 30 third reagent layer and the porous spreading layer.
5. The element as claimed in any of claims 1 to 4 wherein the nicotinamide coenzyme is nicotinamide adenine dinucleotide.
6. The element as claimed in any of claims
- 35 1 to 5 wherein the amount of buffer in each of the



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first and third reagent layers is from 30 to 50
mmoles/m².

7. A method for the detection of ethanol
comprising:

5 A) contacting an aqueous fluid suspected of
containing ethanol with the analytical element as
claimed in any of claims 1 to 6.

10 B) detecting the absorbance of the reduced form
of the nicotinamide coenzyme as an indication of the
presence of ethanol in the aqueous fluid.

8. The method as claimed in claim 7 wherein
the detection step B) is carried out within 5 minutes
of the contacting step A).

15 9. The method as claimed in either claims 7
and 8 wherein the aqueous fluid is human whole blood,
serum or plasma.