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Chemiluminescent detection of amino acids

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[54] CHEMILUMINESCENT DETECTION OF AMINO ACIDS

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[73] Assignee: The Board of Trustees of the University of Arkansas, Little Rock, Ark.

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[22] Filed: Aug. 4, 1992

Related U.S. Application Data

[63] Continuation of Ser. No. 513,753, Apr. 24, 1990, abandoned.

[51] Int. Cl.[5] G01N 33/68; G01N 33/52; G01N 21/75; G01N 21/76

[52] U.S. Cl. 436/89; 436/86; 436/90; 436/111; 436/166; 436/172

[58] Field of Search 436/86-90, 436/111, 166, 172

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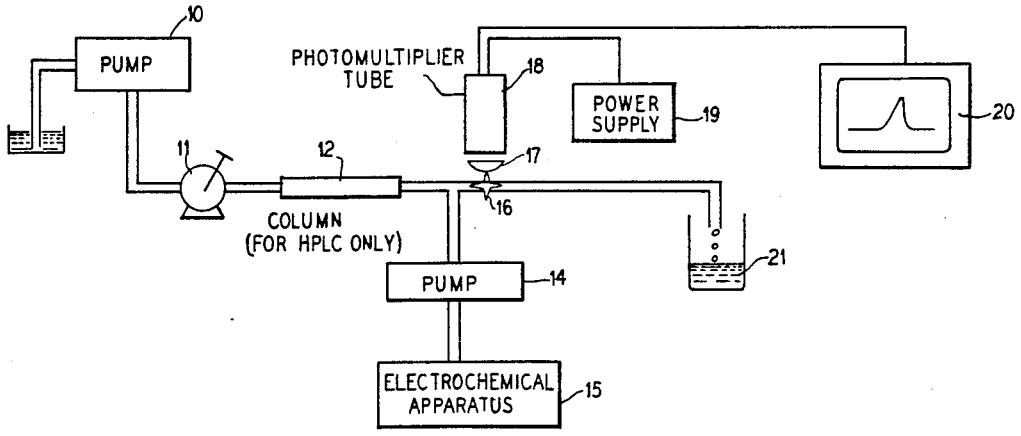
Primary Examiner—James C. Housel
Assistant Examiner—Rachel Heather Freed
Attorney, Agent, or Firm—Hermann Invester

[57] ABSTRACT

A detection system for determining the quantity of amino acid in a sample stream is provided based on the reaction of a buffer at a pH level from 10 to 11, with a reagent $Ru(bpy)_3^{3+}$, which is generated electrochemically on site. The detection system is further characterized by immediate luminescence upon reaction of the buffer in the pH range containing amino acid, with the reagent $Ru(bpy)_3^{3+}$. The detection system is capable of not only immediate detection of the quantity of amino acid in a sample stream, but even in very low concentrations.

6 Claims, 5 Drawing Sheets



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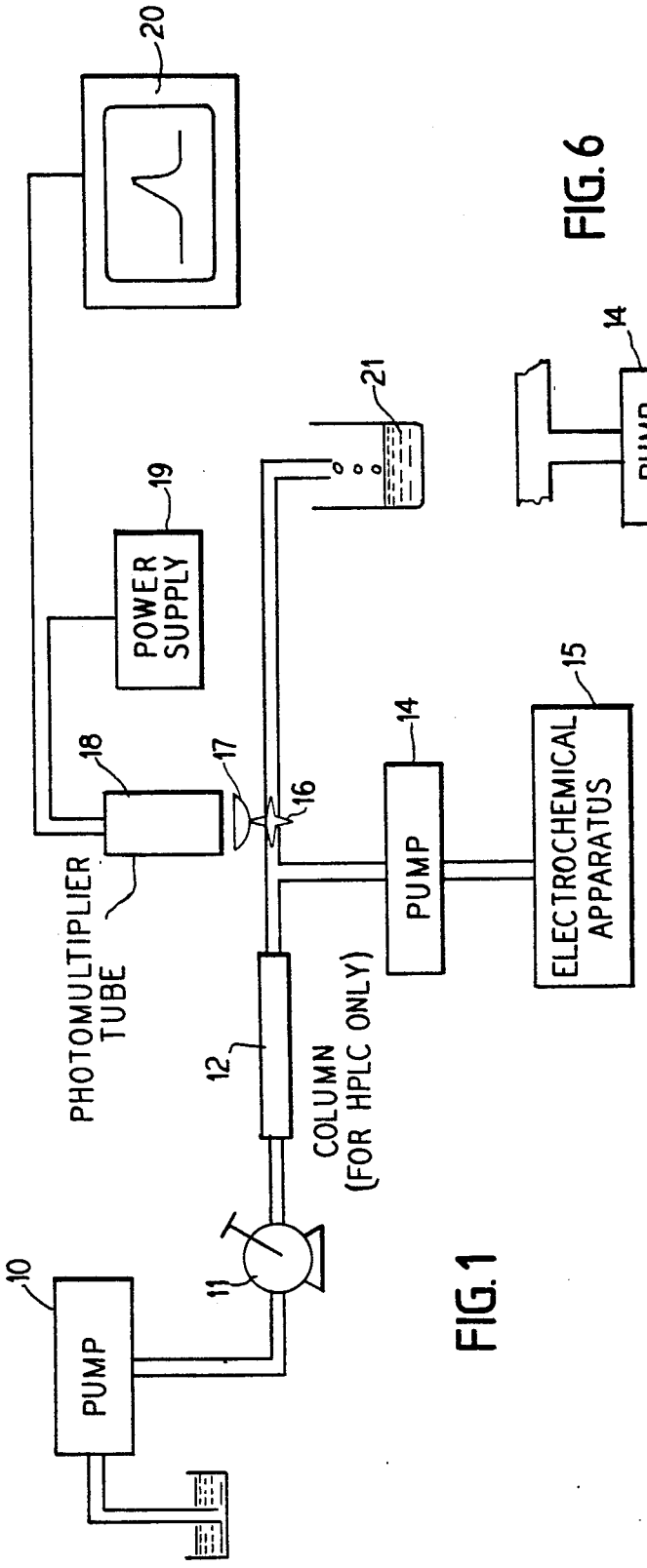

20
21
19
POWER SUPPLY
18
17
16
14
PUMP
15
ELECTROCHEMICAL APPARATUS
PHOTOMULTIPLIER TUBE
12
COLUMN (FOR HPLC ONLY)
11
10
PUMP

FIG. 1

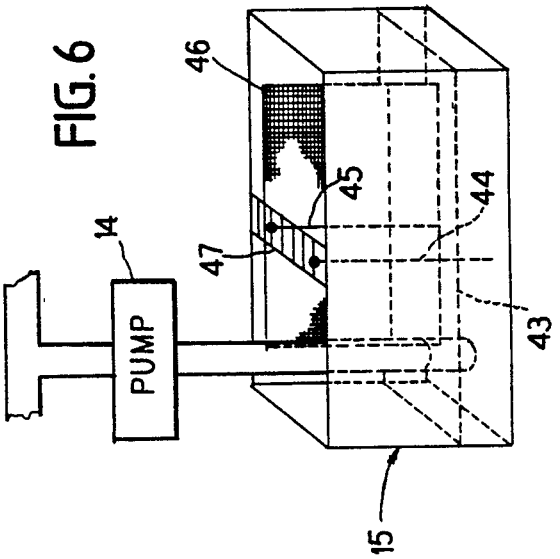

46
45
44
47
14
PUMP
43
15

FIG. 6

FIG. 2

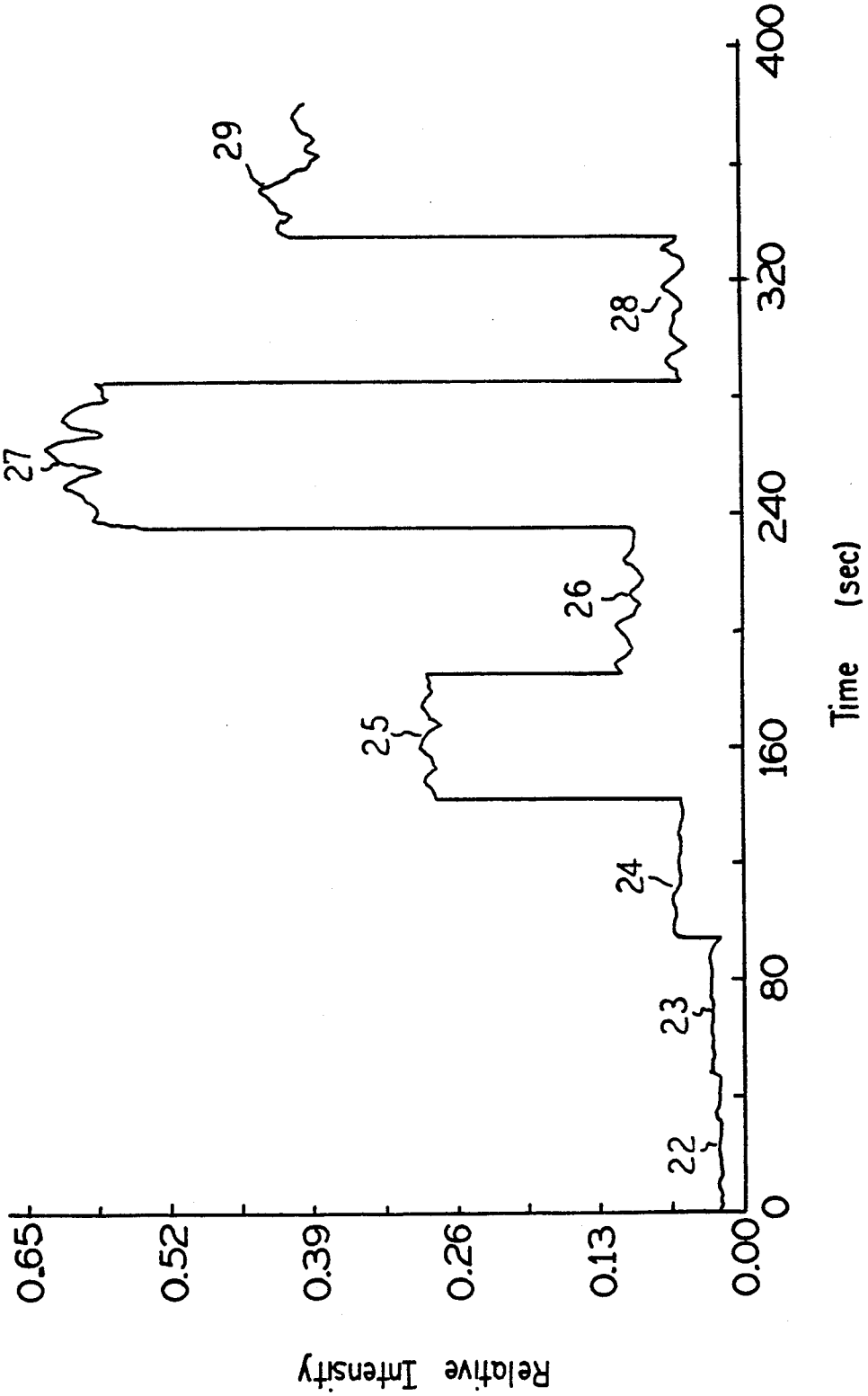

Relative Intensity
0.00
0.13
0.26
0.39
0.52
0.65
0
80
160
240
320
400
Time (sec)
22
23
24
25
26
27
28
29

FIG. 3

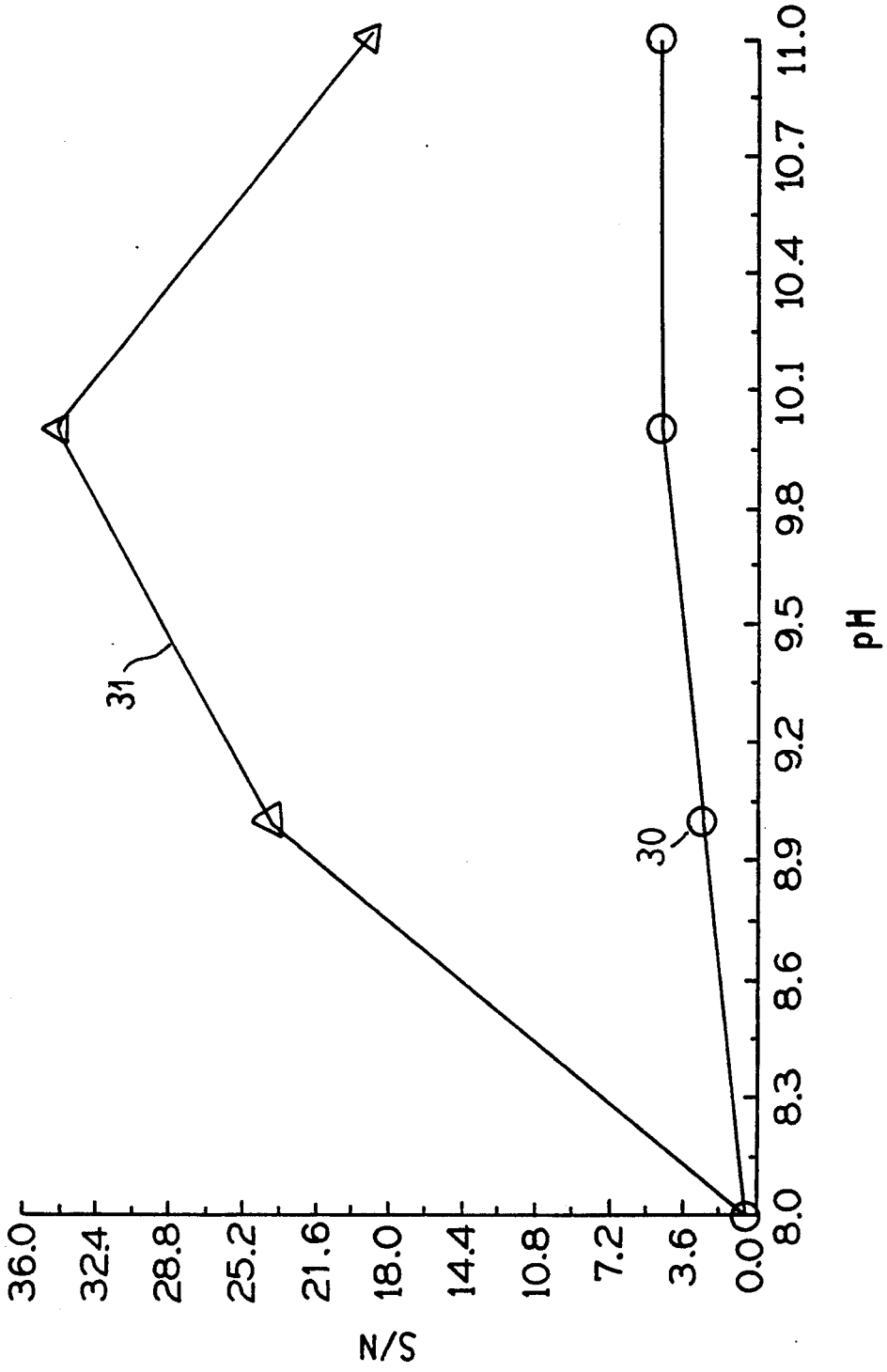
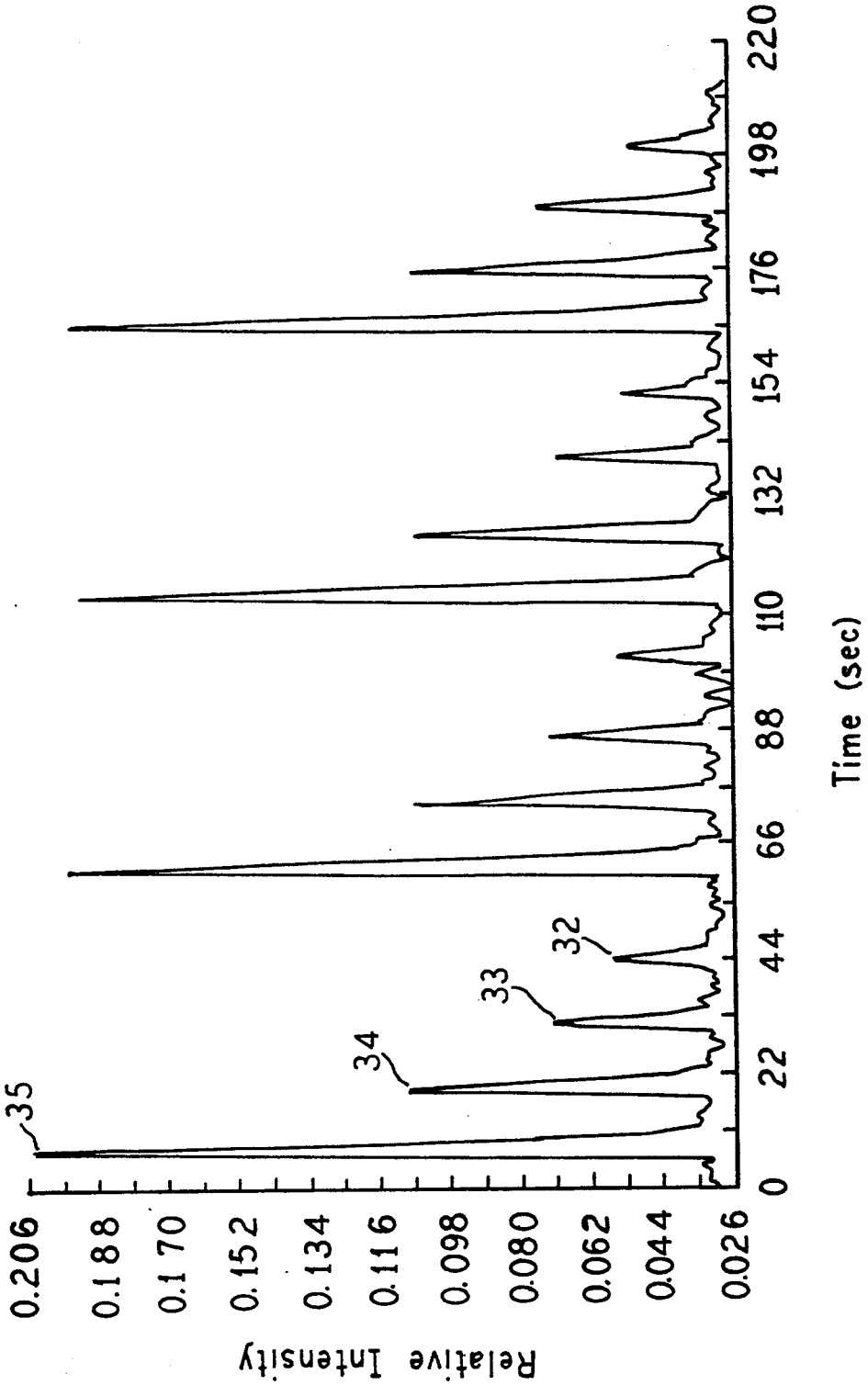

36.0
32.4
28.8
25.2
21.6
18.0
14.4
10.8
7.2
3.6
0.0
N/S
8.0
8.3
8.6
8.9
9.2
9.5
9.8
10.1
10.4
10.7
11.0
pH
31
30

FIG. 4


Relative Intensity
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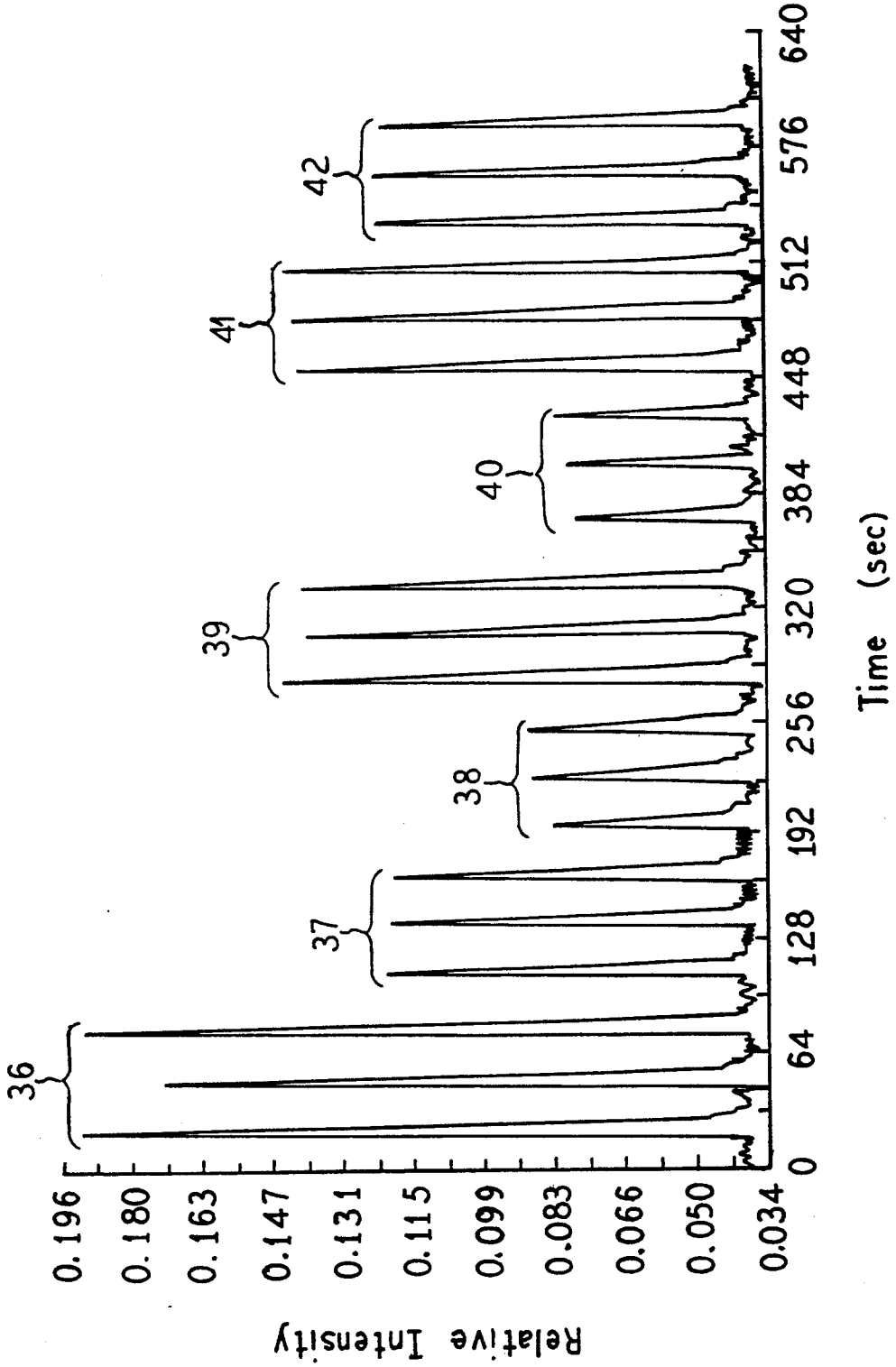
0.188
0.170
0.152
0.134
0.116
0.098
0.080
0.062
0.044
0.026
0
22
44
66
88
110
132
154
176
198
220
Time (sec)
35
34
33
32

FIG. 5


Relative Intensity
0.196
0.180
0.163
0.147
0.131
0.115
0.099
0.083
0.066
0.050
0.034
0
64
128
192
256
320
384
448
512
576
640
Time (sec)
36
37
38
39
40
41
42

CHEMILUMINESCENT DETECTION OF AMINO ACIDS

This is a continuation of application Ser. No. 07/513,753, filed Apr. 24, 1990 now abandoned.

FIELD OF THE INVENTION

The present invention relates to a chemiluminescent detection system of the type suitable for generating luminosity indicative of the quantity of amino acid in a sample stream.

BACKGROUND OF THE INVENTION

A sample stream containing amino acid may be the effluent of a high performance liquid chromatography system or a flow injection analysis system. Once a sample stream is obtained, the amino acid may be converted by derivitization. Detecting the quantity of amino acid present has then been achieved by either spectrophotometric absorption or fluorescence spectroscopy.

The derivitization process is both time consuming and complex. Efforts to eliminate the need for conversion of the amino acid have resulted in the use of the reagent Tris(2,2'-bipyridyl)ruthenium(III) (sometimes referred to herein as $Ru(bpy)_3^{3+}$), which may be generated on site, resulting in immediate luminescence when merged with amino acids in a buffered solution of a specified pH range.

SUMMARY OF THE INVENTION

The present invention provides a detection system for amino acids in volumes >1 microliter by the use of an electrochemically generated reagent to produce immediate luminescence when merged with amino acids in a buffered solution of a specified pH range.

The above object is achieved by merging the electrochemically generated $Ru(bpy)_3^{3+}$ with a sample stream of amino acid, buffered at a pH in the range of 10–11. As the reaction occurs with facility above pKa of the amino acid, the presence of amino acid in the sample stream when merged with the reagent $Ru(bpy)_3^{3+}$ results in luminescence, which may be detected by a photomultiplier tube. The measured emissions are indicative of the quantity of amino acid present in the sample stream.

Thus, a detection system appropriates for volumes <1 microliter which allows for on-site electrochemical generation of $Ru(bpy)_3^{3+}$, and which results in spontaneous luminescence in the presence of amino acid, has been achieved.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic representation of the detection system provided in accordance with this invention.

FIG. **2** illustrates the relative intensity of the luminescence for the $Ru(bpy)_3^{3+}$ reaction with an amino acid at various pH levels.

FIG. **3** is a graphical representation of the change in magnitude of the resulting signal for reactions where the pH levels are >pKa for valine and glutamate.

FIG. **4** illustrates the variation of the output response of the system for varied concentrations of methionine.

FIG. **5** illustrates the output response of six different amino acids at 0.1 mM concentration as merged with 1.5 mM $Ru(bpy)_3^{3+}$.

FIG. **6** is a schematic representation of the electrochemical generating means.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

FIG. **1** is a schematic representation of the detection system. A pump **10** is used at a first point in the circuit to establish a flow rate for the buffer. At a second point in the circuit, a valve **11** is switched, introducing a sample to the buffer which proceeds through a high performance liquid chromatography system, in which the amino acids are separated out into a column **12**, the end of which is connected to a glass or plastic tube **13**. Therein, the buffer and a reagent **14**, which has been electrochemically generated by a generating means **15** identified in FIG. **6** below, are merged, and flow into a concentric coil **16** from which the luminescent output is focused by a lens **17**, causing photoemissions in a photomultiplier tube **18**, where the potential across the dynode string is supplied by a high voltage power supply **19** at 1 KV. Any suitable buffer, such as a buffer consisting of K_2HPO_4, H_3BO_3, or KH_2PO_4, can be used in conjunction with the reagent **14**. The resulting signal is displayed on a CRT **20**. After passing through the concentric coil **16**, the mixture flows to a waste container **21**.

An illustration of the relative intensity of the output signal with respect to time is shown in FIG. **2**. At **22**, **24**, **26**, and **28**, the relative intensity of the output signal with respect to time is demonstrated for the reaction of the reagent with a buffer, which does not contain amino acid, at pH levels of 8, 9, 10, and 11 respectively. At **23**, **25**, **27**, and **29**, the relative intensity of the output signal with respect to time is demonstrated for the reaction of the reagent with a buffer containing the amino acid glutamate at pH levels of 8, 9, 10, and 11 respectively.

FIG. **3** is a plot of the experimental results of the detection system for the amino acids glutamate **30** and valine **31**, demonstrating the increase in the detected signal at pH levels >pKa of the amino acid.

The system response to concentrations of 0.05 mM **32**, 0.1 mM **33**, 0.2 mM **34**, and 0.5 mM **35**, of methionine is illustrated in FIG. **4**, where the $Ru(bpy)_3^{3+}$ concentration is 1.5 mM.

FIG. **5** illustrates the system response for six amino acids. Valine **36**, glutamic acid **37**, tryptophan **38**, methionine **39**, arginine **40**, phenylalanine **41**, and glutamic acid (control) **42**, where tested at a concentration of 0.1 mM with the reagent $Ru(bpy)_3^{3+}$ at a concentration of 1.5 mM.

The following table gives the relative luminescence efficiency for the eight amino acids tested with the $Ru(bpy)_3^{3+}$ detection system.

		Relative Luminosity/mole	
Name	Sidechain Type	# in Category	Relative Luminescence/mole
THR	Alcohol	3*	1
GLY	Hydrogen	1	1.4
ARG	Amine	4	4.8
TRP	Aromatic Amine	2	6.4
GLU	Acid	2	9.8
PHE	Aromatic	2*	11.5
MET	Sulfer	2	14.6
VAL	Hydrocarbon	5	17.1

*TYR overlap

Synthesis of $Ru(bpy)_3^{3+}$

The apparatus which makes electrochemical generation of the reagent possible is schematically shown in FIG. 6. A working electrode, composed of a 6.25 cm^2 piece of platinum gauze **46**, a pseudo-reference electrode, consisting of a silver wire **45**, and an auxiliary electrode, consisting of a platinum wire **44**, are immersed in a 0.4M sulfate electrolyte **43**. The auxiliary electrode **44** is separated from the pseudo-reference **45** and working **46** electrodes by a porous glass frit **47**. The working electrode **46** is maintained at a potential of 1.3 V with respect to the pseudo-reference electrode **45**.

Synthesis of the $Ru(bpy)_3^{3+}$ is achieved by introducing 1.5 mM of Tris(2,2'-bipyridyl)ruthenium(II) (herein referred to as $Ru(bpy)_2^{2+}$ to the sulfate electrolyte **43**. The ensuing reaction in the presence of a 1.3 V potential difference across the working **46** and pseudo-reference **45** electrodes results in the electrochemical oxidation of $Ru(bpy)_2^{2+}$, which yields $Ru(bpy)_3^{3+}$. The reagent can now be extracted employing a pump **14** for introduction to the sample stream containing amino acid.

Chemicals and Compositions Utilized

Amino acid samples were obtained from Sigma Chemical Co., with a stated purity of 99+%. Tris(2,2'-bipyridyl)ruthenium(II)chloride hexahydrate (#22,475-8) was purchased from Aldrich Chemical Co. All remaining buffers and solvents were purchased from Fischer Scientific, and were listed as Reagent Grade.

It will be understood that we wish to embody within the scope of the patent warranted hereon all such modifications as might be suggested by those versed in the art.

We claim as our invention:

1. The method of detecting amino acid in volumes >1 microliter in a stream which includes the steps of:

(a) introducing a liquid sample in the form of a stream flowing through a circuit,

(b) selectively establishing the flow rate of said stream by the use of a pump,

(c) separating the amino acid from other constituents in said stream to form an amino acid stream,

(d) buffering said amino acid stream to achieve a selected pH level, wherein said selected pH level ranges from pH 10 to pH 11,

(e) electrochemically generating $Ru(bpy)_3^{3+}$ on site,

(f) merging the $Ru(bpy)_3^{3+}$ with said buffered amino acid stream to produce immediate spontaneous luminescence in the presence of various concentrations of amino acid, and

(g) photometrically measuring the quantity of amino acid present as a function of the luminescence.

2. A method as defined in claim **1**, wherein said stream is a buffer consisting of K_2HPO_4.

3. A method as defined in claim **1**, wherein said stream is a buffer consisting of H_3BO_3.

4. A method as defined in claim **1**, wherein said stream is a buffer consisting of KH_2PO_4.

5. The method of detecting amino acids in volumes >1 microliter in a stream, which includes the steps of:

(a) introducing a liquid sample in the form of a stream flowing through a circuit,

(b) selectively establishing the flow rate of said stream by the use of a pump,

(c) buffering said stream to form a buffered stream,

(d) separating buffered amino acid from other constituents in said buffered stream to form a buffered amino acid stream, wherein said buffered amino acid stream is buffered to achieve a selected pH level, and wherein said selected pH level ranges from pH 10 to pH 11,

(e) electrochemically generating $Ru(bpy)_3^{3+}$ on site,

(f) merging the $Ru(bpy)_3^{3+}$ with said buffered amino acid stream to produce immediate spontaneous luminescence in the presence of various concentrations of amino acid, and

(g) photometrically measuring the quantity of amino acid present as a function of the luminescence.

6. The method for chemiluminescent detection of amino acids via reaction with $Ru(bpy)_3^{3+}$ wherein light is produced as a product of the reaction in proportion to an amount of amino acid, which includes the steps of:

(a) adjusting the pH level of a sample to be tested to a level of approximately pH 10,

(b) introducing $Ru(bpy)_3^{3+}$ to said sample to produce a mixture, wherein introducing said $Ru(bpy)_3^{3+}$ to said sample results in the immediate luminescence of said mixture at said level, and

(c) measuring the luminescence as a function of the amount of amino acid present.

* * * * *