(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 June 2016 (02.06.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/082003 A1

(51) International Patent Classification:
G01N 33/52 (2006.01) *B01L 3/00* (2006.01)

(21) International Application Number:
PCT/AU2015/050747

(22) International Filing Date:
26 November 2015 (26.11.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2014904799 26 November 2014 (26.11.2014) AU

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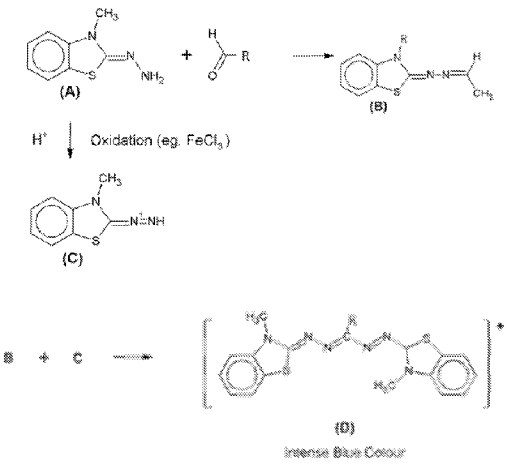
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) **Title**: METHOD AND DEVICE FOR DETERMINATION OF ALDEHYDES IN BIOLOGICAL SAMPLES



R is H or CH_3

Figure 1

(57) **Abstract**: The present invention relates to microfluidic sensor technology and more specifically microfluidic sensor technology for the determination of aldehydes in biological samples, such as saliva.

WO 2016/082003 A1

METHOD AND DEVICE FOR DETERMINATION OF ALDEHYDES IN BIOLOGICAL SAMPLES

FIELD OF THE INVENTION

[0001] The present invention relates to microfluidic sensor technology and more specifically microfluidic sensor technology for the determination of aldehydes in biological samples, such as saliva.

BACKGROUND OF THE INVENTION

[0002] The ingestion of aldehydes or their production in the human body are associated with numerous day to day activities (e.g. smoking, alcohol consumption). These compounds are known to be toxic and the body of evidence regarding their carcinogenic properties has been growing rapidly.

[0003] Oral cancer embodies approximately 5% of malignant lesions worldwide, but this proportion is increasing, with over 1000 new intra-oral squamous cell carcinomas registered annually in Australia . Chronic use of tobacco and alcohol consumption are well-documented, independent risk factors for the upper aerodigestive (UAD) tract (mouth, larynx, pharynx, oesophagus, stomach): alcohol presents a five-fold increase in risk while tobacco smoke increases risk of oral cancer by six times; both risk factors account for 67% of all incidences.

[0004] It has also been reported that smoking and alcohol consumption together exert a synergistic carcinogenic action (they act in a multiplicative rather than additive manner). Since many compounds in tobacco smoke are undoubtedly carcinogenic, the connection between smoking and cancer has been extensively documented. On the contrary, epidemiology findings have revealed only several tumour-promoting effects of alcohol. These include the enhanced solubility of carcinogens, increased oxidant exposure, or suppression of immune system. These are all systematic effects and alone may not explain the relatively high risk of UAD cancers compared to relatively low risk of malignancies at other body sites, such as liver or bowel cancer. Moreover, it has also been reported that ethanol itself is not carcinogenic. Hence, alcohol has always been considered a "risk enhancer" in smokers.

[0005] Over the years there has been accumulating evidence for tumour-producing effects of alcohol intake through acetaldehyde – the first metabolite of ethanol, a well-known

mutagenic and carcinogenic agent. Upon ingestion, ethanol is oxidized by enzymes known as alcohol dehydrogenases (ADHs) to acetaldehyde, which is further oxidized into acetate by aldehyde dehydrogenases (ALDHs).

[0006] Several *in vitro* and *in vivo* experiments in prokaryotic and eukaryotic cell cultures, and in animal models have substantiated the mutagenic and carcinogenic effects of acetaldehyde. These include: causing sister chromatid exchanges, gross chromosomal aberrations, DNA cross-links; covalently binding to DNA and forming stable adducts; interfering with DNA-repair machinery and inhibiting O6-methylguanine transferase, an enzyme important in repairing adducts; and binding to cellular proteins, subsequently impairing cell function. It has been reported that acetaldehyde levels within the range of 50-150 μM are capable of inducing mutagenic changes.

[0007] Considerable upsurge of acetaldehyde levels in saliva during normal social drinking has also been reported. As saliva is present via normal distribution or evaporation throughout the UAD tract, the long-term presence and effects of acetaldehyde on these epithelium may account for the localized higher UAD cancer risk among heavy drinkers.

[0008] There is high individual variation in acetaldehyde levels produced upon exposure to ethanol. This could potentially be explained by different individuals' genotype of isozymes of ADHs and ALDHs, subsequently different efficacy in production and clearance of acetaldehyde in different individuals. Alternatively, the variation could be explained by different microflora profiles in individuals and the resultant variation in acetaldehyde production capacities of various microorganisms. Individuals who are high producers of acetaldehyde upon exposure to ethanol may be at increased risk of developing UAD cancer.

[0009] The emergence of the concept of ethanol-induced oral cancer mediated by salivary acetaldehyde invariably led to the hypothesis that widespread use of alcohol-containing mouthrinses in modern society may contribute to the uprise of UAD cancers. Mouthrinses are used for the treatment of a wide range of oral conditions. Ethanol is used as a solvent for the active agents in many commercially available mouthrinses, with concentrations ranging from 6% to 26.9%. Because these mouthrinses are kept in direct contact with the oral mucosa for longer periods than alcohol-containing beverages, it would appear important to investigate the relationship between the use of alcohol-containing mouthrinses and oral cancer.

[0010] The development of simple and non-invasive analytical techniques to assist with biological monitoring studies is in itself a great challenge. Saliva, for instance, often contains compounds of clinical interest which are also found in blood and urine and hence has the potential to provide a cleaner and non-invasive alternative to blood and urine analysis in terms of sampling. Analytical techniques such as gas chromatography (GC) or high performance liquid chromatography (HPLC) are frequently used for the determination of aldehydes, although they are costly and require specialized personnel to operate the relevant instrumentation. Hence, alternative analytical techniques are needed to improve the efficiency, cost effectiveness and scalability of biological sampling and analysis.

SUMMARY OF THE INVENTION

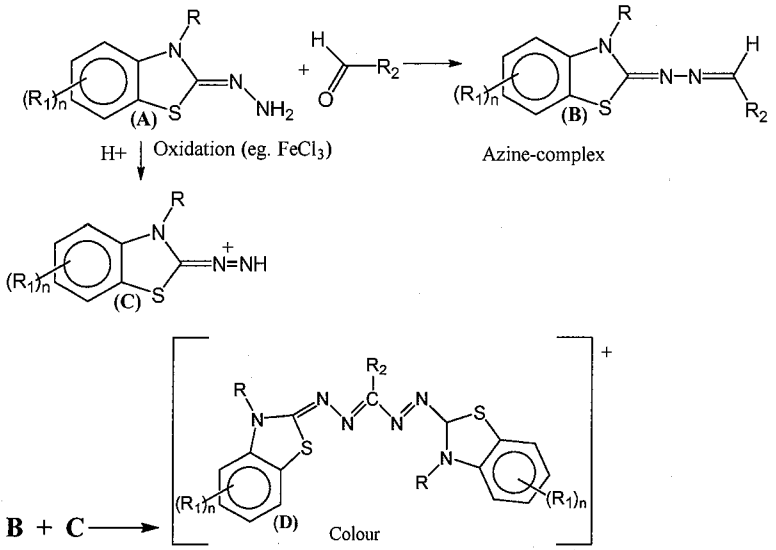
[0011] In an aspect this invention provides a low cost, disposable, portable, and easy to use paper-based microfluidic sensor device for the determination of aldehydes in a sample containing at least one aldehyde, for instance, a biological samples such as a saliva sample. This provides a simple and non-invasive determination of total aldehydes in saliva samples for use in epidemiological studies to, for instance, study the risk of oral cancer.

[0012] In a further aspect the invention provides a method of determining the amount of aldehydes in a sample using the above mentioned paper-based microfluidic sensor device.

[0013] The invention provides a paper-based microfluidic sensor device for the determination of aldehydes in a sample, said device comprising a sheet of paper or plurality of sheets which has/have been separated into at least two zones wherein zone one is impregnated with a 3-alkyl-2-benzothiazolinone hydrazone (ABTH) and zone two is impregnated with a chemical oxidant of ABTH, such that in use the sheet of paper or plurality of sheets is/are arranged such that the sample can be contacted with zone one for a time and under conditions to react with ABTH, and after such a time, zone one is then contacted with zone two for a time and under conditions.

[0014] To address the need for a low-cost and user-friendly (i.e., for instance, non-invasive) diagnostic device for the determination of aldehydes in a biological sample the present inventors have developed a paper-based microfluidic aldehyde sensor device. It utilizes a colourmetric diagnostic system relying on the reaction of aldehydes in the biological sample (e.g. acetaldehyde, formaldehyde) with a 3-alkyl-2-benzothiazolinone hydrazone

(ABTH) (A, see scheme 1) to form an azine (B, scheme 1). An appropriate chemical oxidant (such as Fe^{3+} chloride) then oxidises another ABTH molecule to produce an intermediate (C, scheme 1), which then reacts with the azine to form the visibly coloured formazan dye (D, scheme 1).

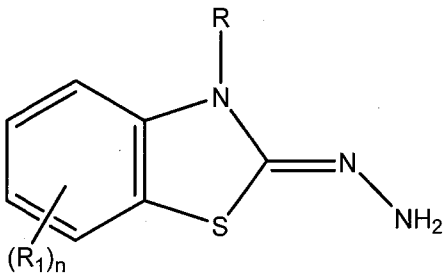


Scheme 1 Reaction mechanism.

[0015] In the above embodiment, R_2 may be hydrogen or C_{1-6} alkyl.

[0016] In the above embodiment, preferably R_2 is methyl or hydrogen.

[0017] In one embodiment the 3-alkyl-2-benzothiazolinone hydrazone is a compound of formula (I) or a salt thereof



(I)

wherein

R is C_{1-3} alkyl;

R_1 is independently selected from the group consisting of cyano, halo, nitro, optionally substituted lower alkyl, optionally substituted aryl, optionally substituted aryloxy, optionally substituted arylalkyl, optionally substituted heteroaryl, optionally substituted heterocyclyl, optionally substituted C_{3-7} cycloalkyl, $-OR^*$, $-C(O)R^*$, $-C(O)OR^*$, $-OC(O)R^*$ (where R^* is selected from hydrogen, optionally substituted alkyl, optionally substituted alkenyl, optionally

substituted alkynyl, optionally substituted C_{3-7} cycloalkyl, optionally substituted heterocyclyl, optionally substituted heteroaryl, and optionally substituted aryl), -C(O)NR'R'', -NR'C(O)R'', -$S(O)_2$-NR'R'' and –NR'R'' (where R' and R'' are independently selected from hydrogen or lower alkyl), -S(O)R''' (where R''' is lower alkyl, or cycloalkyl), -$S(O)_2$R''' (where R''' is lower alkyl, cycloalkyl or OH), or any two adjacent R_1 together form heterocyclyl, heteroaryl, or aryl; and

n is an integer from 0 to 4.

[0018] In an embodiment R is methyl.

[0019] In an embodiment n is 0.

[0020] In an embodiment the volume of the compound of formula (I) is about 2-10μL in the paper based device/sample (measured per each hydrophilic zone), such as about 2, 3, 4, 5, 6, 7, 8, 9 or about 10μL.

[0021] In an embodiment the concentration of the compound of formula (I) is about 1-21mL in the paper based device/sample (measured per each hydrophilic zone), such as about 2, 3, 4, 5,6, 7,8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, or about 21mL.

[0022] In an embodiment the compound of formula (I) is 3-methyl-2-benzothiazolinone hydrazone (MBTH).

[0023] In an embodiment the compound of formula (I) is 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate [CAS 149022-15-1].

[0024] In an embodiment the chemical oxidant is selected from hexavalent chromium, for instance, chromic and dichromic acids, chromium trioxide, pyridinium chlorochromate (PCC), etc; permanganate compounds, for instance, potassium permanganate; sodium perborate; silver oxide; or potassium nitrate. In an embodiment the amount and nature of the chemical oxidant is such that for where the paper sensor device is manufactured from a cellulose based paper, the oxidant does not, to any great extent, oxidise the cellulose forming detectable amounts of aldehyde groups.

[0025] In an embodiment, the volume of oxidant in the paper based device is between about 2-12μL (measured per each oxidant zone).

[0026] In an embodiment, the volume of oxidant in the paper based device is about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or about 12μL (measured per oxidant zone), or any range between any two of these figures.

[0027] In an embodiment the concentration of oxidant in the paper based device is between about 11-240mM (measured per each oxidant zone).

[0028] In an embodiment the concentration of oxidant in the paper based device is about 11, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, or about 240mM (measured per each oxidant zone), or any range between any two of these figures.

[0029] In an embodiment the chemical oxidant is Fe^{3+}, such as ferric chloride.

[0030] In an embodiment the compound of formula (1) is MBTH and the chemical oxidant is Fe^{3+}.

DESCRIPTION OF THE FIGURES

[0031] Figure 1 – Schematic of a reaction mechanism for the sensing of acetaldehyde and/or aldehyde of one embodiment of the invention which utilizes MBTH and $FeCl_3$.

[0032] Figure 2 – A design of an embodiment of paper-based device for the determination of aldehydes according to the invention.

[0033] Figure 3 – Study of the ratio between Fe^{3+} and MBTH reagents according to an embodiment of the present invention.

[0034] Figure 4 – Study of the optional concentrations of Fe^{3+} and MBTH according to an embodiment of the present invention.

[0035] Figure 5 – Calibration curves for acetaldehyde and formaldehyde under optimal conditions for an embodiment of the present invention.

[0036] Figure 6 - A design of an embodiment of a paper-based device for the determination of aldehydes according to the invention.

DEFINITIONS

[0037] The term "alkyl" as used alone or in combination herein refers to a straight or branched chain saturated hydrocarbon group. For instance, the term "C_{1-3} alkyl" refers to such

a group containing from one to three carbon atoms, such as methyl ("Me"), ethyl ("Et") and n-propyl.

[0038] The term "cycloalkyl" refers to non-aromatic, saturated non-aromatic carbocycles. The term "C_{4-9} cycloalkyl", for instance, refers to such a group having from 4 to 9 carbon atoms. Examples include cyclobutyl, cyclopentyl and cyclohexyl.

[0039] The term "alkenyl" refers to a straight or branched hydrocarbon containing one or more double bonds, preferably one or two double bonds. The term "C_{2-12} alkenyl", for instance, refers to such a group containing from two to twelve carbon atoms. Examples of alkenyl include allyl, 1-methylvinyl, butenyl, iso-butenyl, 1, 3-butadienyl, 3-methyl-2-butenyl, 1,3-butadienyl, 1,4-pentadienyl, 1-pentenyl, 1-hexenyl, 3-hexenyl, 1,3-hexadienyl, 1,4-hexadienyl and 1, 3, 5-hexatrienyl.

[0040] The term "cycloalkenyl" refers to cyclic alkenyl groups having a single cyclic ring or multiple condensed rings, and at least one point of internal unsaturation, preferably incorporating 4 to 11 carbon atoms. Examples of suitable cycloalkenyl groups include, for instance, cyclobut-2-enyl, cyclopent-3-enyl, cyclohex-4-enyl, cyclooct-3-enyl, indenyl and the like.

[0041] The term "alkynyl" refers to a straight or branched hydrocarbon containing one or more triple bonds, preferably one or two triple bonds. The term "C_{2-12} alkynyl", for instance, refers to such a group containing from two to twelve carbon atoms. Examples include 2-propynyl and 2- or 3-butynyl.

[0042] The term "alkoxy" as used alone or in combination refers to a straight or branched chain alkyl group covalently bound via an oxygen linkage (-O-) and the terms "C_{1-6} alkoxy" and "lower alkoxy" refer to such groups containing from one to six carbon atoms, such as methoxy, ethoxy, propoxy, isopropoxy, butoxy, t-butoxy and the like.

[0043] The term "aryl" refers to carbocyclic (non-heterocyclic) aromatic rings or ring systems. The aromatic rings may be mono- or bi-cyclic ring systems. The aromatic rings or ring systems are generally composed of 5 to 10 carbon atoms. Examples of suitable aryl groups include but are not limited to phenyl, biphenyl, naphthyl, tetrahydronaphthyl, and the like.

[0044] Preferred aryl groups include phenyl, naphthyl, indenyl, azulenyl, fluorenyl or anthracenyl.

[0045] The term "heteroaryl" refers to a monovalent aromatic carbocyclic group, preferably of from 2 to 10 carbon atoms and 1 to 4 heteroatoms selected from oxygen, nitrogen and sulfur within the ring. Preferably the heteroatom is nitrogen. Such heteroaryl groups can have a single ring (e.g., pyridyl, pyrrolyl or furyl) or multiple condensed rings (e.g., indolizinyl or benzothienyl).

[0046] The term "heterocyclyl" refers to a monovalent saturated or unsaturated group having a single ring or multiple condensed rings, preferably from 1 to 8 carbon atoms and from 1 to 4 hetero atoms selected from nitrogen, sulfur, oxygen, selenium or phosphorous within the ring.

[0047] Examples of 5-membered monocyclic heterocyclyl and heteroaryl groups include furyl, thienyl, pyrrolyl, H-pyrrolyl, pyrrolinyl, pyrrolidinyl, oxazolyl, oxadiazolyl, (including 1,2,3 and 1,2,4 oxadiazolyls) thiazolyl, isoxazolyl, furazanyl, isothiazolyl, pyrazolyl, pyrazolinyl, pyrazolidinyl, imidazolyl, imidazolinyl, triazolyl (including 1,2,3 and 1,3, 4 triazolyls), tetrazolyl, thiadiazolyl (including 1,2,3 and 1,3,4 thiadiazolyls).

[0048] Examples of 6-membered monocyclic heterocyclyl and heteroaryl groups include pyridyl, pyrimidinyl, pyridazinyl, pyranyl, pyrazinyl, piperidinyl, 1,4-dioxanyl, morpholinyl, 1,4-dithianyl, thiomorpholinyl, piperazinyl, 1,3,5-trithianyl and triazinyl.

[0049] Examples of 8, 9 and 10-membered bicyclic heterocyclyl and heteroaryl groups include 1H thieno[2,3-c]pyrazolyl, thieno[2,3-b]furyl, indolyl, isoindolyl, benzofuranyl, benzothienyl, benzoxazolyl, benzothiazolyl, benzisoxazolyl, benzisothiazolyl, benzimidazolyl, indazolyl, isoquinolinyl, quinolinyl, quinoxalinyl, uridinyl, purinyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, benzotriazinyl, naphthyridinyl, pteridinyl and the like.

[0050] The term "arylalkyl" refers to carbocyclic aromatic rings or ring systems as previously described and substituted by an alkyl group, also as previously described. Unless otherwise indicated the aryl substituent is attached by the alkyl part of the substituent. Likewise the terms "aryl C_{1-12} alkyl", "aryl C_{2-12} alkenyl" and "aryl C_{2-12} alkynyl" refer to carbocyclic aromatic rings or ring systems as previously described and substituted by a C_{1-12} alkyl, C_{2-12} alkenyl or C_{2-12} alkynyl group, as previously described.

[0051] The terms "halo" and "halogen" refers to fluoro, chloro, bromo and iodo groups.

[0052] The term "halo alkyl" group has one or more of the hydrogen atoms on an alkyl group replaced with halogens. A notable example is $-CF_3$.

[0053] The term "aryloxy" refers to an aryl group as earlier described linked to the parent structure via an oxygen linkage (-O-). A notable example is phenoxy. Similarly the term "heteroaryloxy" refers to a heteroaryl group as earlier described linked to the parent structure via an oxygen group. A notable example is a 4, 6 or 7-benzo[b]furanyloxy group.

[0054] The term "optionally substituted" means that a group may include one or more substituents.

[0055] A list of preferred optional substituents includes: halogen (in particular, Cl, Br or F), C_{1-6} alkyl, C_{1-6} alkoxy, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-6} haloalkyl (in particular $-CF_3$), C_{1-6} haloalkoxy (such as $-OCF_3$), -OH, phenyl, benzyl, phenoxy, benzyloxy, benzoyl, $-NH_2$, $-NHC_{1-4}$ alkyl, $-N(C_{1-4}$ alkyl$)_2$, -CN, $-NO_2$, mercapto, $-S(O)_2NH_2$, $-S(O)_2NHC_{1-4}$ alkyl, $-S(O)_2N(C_{1-4}$ alkyl$)_2$, C_{1-6} alkylcarbonyl, C_{1-6} alkoxycarbonyl, CO_2H, -S(O)R''' (where R''' is lower alkyl or cycloalkyl) and $-S(O)_2$R''' (where R''' is lower alkyl, cycloalkyl or OH).

[0056] The term "biological sample" as used herein refers to a fluid sample which comprises a biological material such as blood, urine, saliva, etc. The sample may be presented as a diluted sample with, for instance, an aqueous medium such as water, a buffering solution or salt solution. In an embodiment the sample may be a saliva swab sample.

[0057] In an embodiment the biological sample is a saliva sample.

[0058] In an embodiment the aldehyde to be detected is formaldehyde and/or acetaldehyde.

PREPARATION OF THE DEVICE

[0059] The paper used in the device can be any cellulose based or non-cellulose fibrous paper material which can form a hydrophilic pathway for liquid transport using only capillary action without the need for any other driving force. In an embodiment preferably the paper used is paper which does not contain amounts of formaldehyde or is cellulose free (i.e., composed of glass microfibers and silica gel). Examples of paper which may be employed include: untreated filter paper (e.g. Grade 4), treated filter paper (hot water treatment) chromatographic paper (iTLC), formaldehyde-free filter paper, unbleached natural filter paper, recycled paper (not absorbent), and untreated Grade 4 filter paper printed with AKD.

[0060] For instance, the paper industry often uses formaldehyde to improve, for instance, the wet-strength of paper and paper products. Accordingly, in another embodiment the paper

used to form the hydrophilic zone is paper which is aldehyde free, or formaldehyde free or at least substantially aldehyde free.

[0061] In this context this hydrophilic pathway is referred to as a "zone" or "hydrophilic zone". This aforementioned hydrophilic pathway or zone is defined by patterning the paper with a hydrophobic agent (such as polystyrene, polydimethylsiloxane (PDMS) or a wax), or a hydrophobic paper-sizing agent such as an alkenyl ketene dimer (AKD) (for instance, as a 4% v/v solution in n-heptane) or alkyl succinic anhydride. The patterning may involve printing of the hydrophobic agent or paper-sizing agent onto the paper with, for instance, a printer such as an ink-jet printer. The hydrophilic pathways or zones can be punched or cut out by removing predetermined sections of the hydrophobic material to expose a hydrophilic zone(s). The paper may be, for instance, laboratory grade filter paper. The resulting hydrophilic zones provide the means for transport of the sample to the reagent (i.e., the first contact between the sample and ABTH, and then the subsequent reaction with the oxidant, as shown, for instance, in Scheme 1 and Figure 1).

[0062] Accordingly, the paper may be arranged in anyway which allows for fluid contact with the sample and ABTH and then subsequently to the oxidant. As such it will be appreciated that zone one is a hydrophilic zone which is impregnated with ABTH. As discussed above formation of the zones may be facilitated by exposing the hydrophilic zone using, for instance, a biopsy punch to remove the hydrophobic paper-sizing agent or hydrophobic agent. This may produce zones of many different shapes such as channels, circles, squares etc. It may also be advantageous to have a plurality of zone one and a plurality of zone two hydrophilic zones.

[0063] In an embodiment, the number, size and shape of the zone one and zone two hydrophilic zones are the same. In an embodiment, sheet of paper or plurality of sheets of paper, which have been separated into the at least two zones, is arranged such that when the paper-base device is folded, this action superimposes the respective zone one and zone two hydrophilic zones.

[0064] The impregnation of the analytical reagents maybe achieved by depositing an aqueous solution of the reagents in the respective hydrophilic zones. The device may or may not be dried.

[0065] In the embodiment depicted in Figure 2 the device comprises a single sheet of paper (1) which is folded in a concertina manner to form three panels. Panel 1 (2) is the sample panel where a biological sample is applied to one or more hydrophilic circular zones (3). In this embodiment there are six sample zones. It would be appreciated that the sample panel layer may be optional, that is, the sample could also be applied directly to the MBTH layer in use. For this embodiment however, the sample is initially applied to any one or more of (3), (5) is removed and (3) and (4) are pressed together to facilitate the reaction between the sample and MBTH. Panel 3 (6) is the chemical oxidant layer where the chemical oxidant (in this embodiment Fe^{3+}) is impregnated into circular zones which are superimposable unto the circular zones of panel 2 (4), which are in turn superimposable unto the circular zones of panel 1 (2). The transparency sheet (5) (preferably PTFE) may be placed on the backside of panel 2 or the front side of panel 3 in order to physically separate the MBTH zones from the Fe^{3+} zones prior to pressing Panel 2 to Panel 3 in order to facilitate the colourmetric analysis of the sample by facilitating the reaction between the MBTH zones containing the sample and Fe^{3+} zones.

[0066] In an embodiment depicted in Figure 6 the device comprises a single sheet of paper (10) which is folded in half to form two panels. Panel 1 (11) is the sample panel where a biological sample is applied to one or more hydrophilic circular zones comprising MBTH (13). In this embodiment there are 15 sample zones. For this embodiment the sample is initially applied to any one or more of (13), the transparency sheet (14) is removed and (1) and (12) are pressed together to facilitate the reaction between the sample, MBTH and chemical oxidant layer (Fe^{3+}) (12). In this embodiment, the colourmetric detection of any aldehydes in the sample is visualized on the MBTH layer (11).

[0067] In an embodiment the first reaction time (reacting MBTH and sample) takes place from about 1 min- about 10minutes, such as 2mins, 3mins, 4mins, 5mins, 6mins, 7mins, 8mins, 9mins, or about 10minutes.

[0068] In another embodiment the second subsequent reaction time (reacting oxidant to MBTH/sample product) takes place from about 1minute -30mins, such as about 2 minutes, 3minutes, 4minutes, 5minutes, 6minutes, 7mins, 8mins, 9mins, 10mins, 11mins, 12mins, 13mins, 14mins, 15mins, 16mins, 17mins, 18mins, 19mins, or about 20mins.

USE OF THE DEVICE

[0069] Analysis of the sample is intended to be carried out by visual inspection of colour changes in the zone two (or chemical oxidant zone) (see for instance the arrangement provided for in Figure 1). In another embodiment the visual inspection of the colour changes is evident in the hydrophilic zone (see for instance the arrangement provide for in Figure 6). This can be done with a disc comparator or a colour card, or the use of a dedicated photometer such as a flat bed scanner. In an embodiment, the device may further comprise a separate sheet of paper which allows the user or physician to physically compare the changes in colour to standardized colour changes which are indicative of specific ranges of total aldehyde within the sample.

[0070] In one embodiment the analytical procedure may involve first depositing 5-100 µL, for instance, 20 µL, of a sample or standard aldehyde solution into the hydrophilic sample zone containing MBTH. The sample is then left to react with MBTH for a time before the interleaving PTFE sheet separating the MBTH layer and the oxidant layer is removed by simply snipping off one end of the laminated card and drawing it out. This may take from 30 seconds to 10 minutes depending on the concentration of the reagents. For instance, the reaction time may be 1-4 minutes, such as 1.5 minutes, such as 2 minutes, such as 2.5 minutes, such as 3 minutes, such as 3.5 minutes, or such as 4 minutes. The card is then pressed together briefly to facilitate the lateral migration of the MBTH-sample azine to the Fe^{3+} (or oxidant). The reaction is then allowed to further react for another time, upon which the colour intensity of the dye produced (on the Fe^{3+} zone side of the devices) is measured. This may take from 30 seconds to 10 minutes depending on the concentration of the reagents. For instance, the reaction time may be 1-4 minutes, such as 1.5 minutes, such as 2 minutes, such as 2.5 minutes, such as 3 minutes, such as 3.5 minutes, or such as 4 minutes.

[0071] Optimization of the paper-based device will depend on the reagents used and the amount of reagent used in each zone however this is within the ambit of the skilled addressee. The present inventors have studied the optimal MBTH: Fe^{3+} reagent ratio, the reagent concentration and the colour development time. The colour absorbance of the formazan dye was first examined as a function of MBTH: Fe^{3+} ratio. Fig. 3 shows that the absorbance of the dye increases when the concentration of Fe is in considerable excess. Based on this study, an MBTH: Fe^{3+} ratio of 3:5 was found to achieve optimal formation of the formazan dye.

[0072] The effect of the MBTH and Fe^{3+} concentrations at 3:5 ratio was studied next. Fig. 4 shows that the absorbance of the formazan dye reaches its maximum value when the MBTH and Fe^{3+} concentrations were 30 and 50 mM, respectively before decreasing with further increasing these concentrations.

[0073] Preliminary kinetic experiments indicated that about 2.5 minutes were required for the completion of the first step of the analytical reaction (coupling of MBTH to aldehyde in the sample to form an azine) and 2.5 minutes were also required for the completion of the second step (formation of the fomazan dye) so that reliable aldehyde quantification could be performed. Hence, under the optimal conditions outlined above, the paper-based device was calibrated for the two dominant aldehydes in saliva, i.e. acetaldehyde and formaldehyde. The corresponding calibration curves are shown in Fig. 5 and indicate that similar sensitivity for the two aldehydes which are appropriate for their determination in saliva. This result indicates that the proposed paper-based sensor is suitable for determining the total aldehyde concentration in saliva.

[0074] Table 1 below shows a summary of the optimal paper based parameters for a MBTH/Fe(III) based device.

Table 1: Summary of the optimized paper device parameters for saliva sampling..

Parameter	**Range**	**Optimal Value**
MBTH and sample volume [μL]	2 – 10	8
[Fe(III)] : [MBTH] ratio in the detection zone	1.0 – 4.0	2.9
MBTH concentration [mmol L^{-1}]	1.0 – 21	14
Fe(III) concentration [mmol L^{-1}]	11.4 – 240	160
Reaction timing – (azine formation) Step 1 [min]	2 – 7	5
Reaction timing – (formazan formation) Step 2 [min]	1.5 – 10	5

[0075] Reference will now be made to experiments that embody the above general principles of the present invention. However, it is to be understood that the following description is not to limit the generality of the above description.

EXAMPLES

[0076] 1.0 Reagents and solutions

All reagents were of analytical grade and all solutions were prepared in deionized water (18 MΩ cm, Millipore Synergy 185). Working acetaldehyde solutions in the concentration range from 15.9 – 114 μM were prepared daily from a standardised stock solution of 22.7 mM acetaldehyde (Sigma-Aldrich). To ascertain the sensitivity of the paper device to other aldehydes, such as formaldehyde, formaldehyde solutions in the same concentration range were tested and prepared from a standardised stock solution of 47.5 mM formaldehyde (Chem-Supply).

Both aldehyde solutions were standardised by potentiometric titration as recommended by the Internal Organisation for Standardisation. The acetaldehyde stock solution was stored at 4 °C to minimise evaporative losses. A 14 mM MBTH solution was prepared daily by dissolving 15.1 mg of MBTH (Sigma-Aldrich) in 5 mL of deionized water. The oxidizing agent, which consisted of 160 mM $FeCl_3$ was prepared by dissolving 216 mg $FeCl_3$ (Sigma-Aldrich) in 5 mL of 0.005 M HCl (Ajax FineChem).

A 1.85 mM MBTH solution in 0.1 M hydrochloric acid and a 3.70 mM $FeCl_3$ solution were used in the standard reference method.

[0077] 1.1 Design and fabrication of the paper device

The proposed paper device was credit card size (75 x 58 mm) and consisted of 15 pairs of reagent-detection zones. The template (as seen in Fig. 6) was first created in Microsoft Office Word (2007) and then printed on hydrophilic paper (e.g. filter paper) using a wax printer (Xerox ColorQube 8870). The printed template was then placed in an oven (Binder) at 150 °C for 120 s to allow the wax to melt and penetrate the cellulose fibres. The printed area became hydrophobic leaving the unprinted circular zones (e.g. 13) hydrophilic.

The 3D design paper device consisted of two paper layers. Due to the 2-step nature of the reaction, a cellulose acetate interleaving sheet (70 x 40 mm) was placed in between Zones 1 and 2 (14). Each layer contained 15 circular hydrophilic zones, 4 mm (Fig. 6, Zone 1) or 8 mm (Fig. 6, Zone 2) in diameter, with capacity to accommodate 2 μL of Fe(III) solution and 8 μL of MBTH or sample solution, respectively. After the addition of 8 μL MBTH solution to all 15 detection zones (Fig. 6, Zone 2) the paper device was oven dried at 50 °C for 5 min.

The paper device was laminated (Lowell) with a pre-punched plastic cover to preserve the alignment of the sample/reagent zones. A Japanese screw punch was used to punch a 2 mm sample/reagent insertion hole in the plastic cover: one hole was punched over the corner of Zone 2 for sample insertion and an additional hole was punched in the centre of Zone 1 to allow easy addition of the Fe(III) solution.

[0078] Example based on paper based device depicted in Figure 6

A) Parameters — Conditions

Addition of Fe(III) as $FeCl_3$ to the paper	Wet
Volume of Fe (III)	2.9µL
Volume of MBTH/sample	8 µL
Concentration of Fe (III)	160mM
Concentration of MBTH	14mM
First and second reaction time	5 min
Zone sizes	8mm + 4mm
Zones per panel	15
Panels	2
Final reaction time	5 min

B) Analytic figures for device

Linear concentration range	up to 10mg L^{-1}
Limit of detection	0.404mg L^{-1} (9.2 µM)
Limit of quantification	1.35mg L^{-1} (31 µM)
Repeatability (2.0 mg L^{-1}, n=11)	4.2%
Recoveries (saliva samples)	83.1 – 102%

[0079] 1.2 Aldehydes measurement procedure

The analytical procedure resulted in measuring the combined concentration of acetaldehyde and formaldehyde in saliva which is referred to as total aldehyde concentration and for convenience is expressed as acetaldehyde concentration. The analytical procedure for the proposed paper device was conducted as follows: (1) Standard solutions or saliva samples containing aldehydes were first deposited in Zones 2 (Fig. 6) through the corresponding

sample introduction holes to allow the reaction between aldehydes (acetaldehyde and formaldehyde) and MBTH to take place and produce an azine; (2) This was followed by the addition of 2 μL of $FeCl_3$ to Zone 1 (Fig. 6); (3) The interleaving sheet was then pulled out 5 minutes after sample addition to allow physical contact between the two paper layers of the paper device and this led to the oxidation of the unreacted with aldehydes MBTH in Zone 2 by Fe(III) transported to Zone 2 from Zone 1. The oxidized form of MBTH reacted with the azine to produce a blue coloured formazan dye which changed the colour of Zone 2 (Fig. 6) accordingly; (4) The paper device was then scanned by means of a flatbed scanner (Canoscan™ Lide 700f) and the image was processed using Image J (National Institutes of Health, USA, http://imagej.nih.gov./ij). The red, green, blue (RGB) colour intensity profile plots were obtained for a chord passing through the centre of each detection zone (Fig.6, Zone 2). The highest sensitivity was obtained using the red colour intensity. Using approach proposed by Birch and Stickle, the measured colour intensities were converted into absorbance values, defined as: Absorbance = - $\log_{10}$ (I_S/I_0), where I_s is the mean measured colour intensity of the blank, standard or sample, and I_0 is the mean measured colour intensity of the original white filter paper.

[0080] 1.3 Saliva samples

Saliva samples were obtained from healthy volunteers from the School of Chemistry at the University of Melbourne. Participants were required to rinse their mouth with 10 mL of distilled water for 30 sec. The collected samples were then filtered using a syringe filter (0.45 μm Millex-HP) and spiked with the appropriate concentration of acetaldehyde.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A paper-based microfluidic sensor device for the determination of aldehydes in a sample, said device comprising a sheet of paper or plurality of sheets which have been separated into at least two zones wherein zone one is impregnated with a 3-alkyl-2-benzothiazolinone hydrazone (ABTH) and zone two is impregnated with a chemical oxidant of ABTH, such that in use the sheet of paper or plurality of sheets is/are arranged such that the sample can be contacted with zone one for a time and under conditions to react with ABTH, and after such a time zone, one is then contacted with zone two for a time and under conditions.

2. A device according to claim 1 wherein the device comprises a single sheet of paper folded in half to form two panels, the first panel comprising zone one and the second panel comprising zone two.

3. A device according to claim 2 wherein zone one and zone two are super imposable when the panels come into contact during use.

4. A device according to any one of claims 1 to 4 wherein zone one comprises one or more sample zones which are super imposable with respective zone two oxidant zones when the panels come into contact during use.

5. A device according to any one of claims 1 to 3 wherein prior to use, zone one and zone two are physically separated by a transparency sheet.

6. A device according to any one of claims 1 to 5 wherein the ABTH is MBTH.

7. A device according to any one of claims 1 to 6 wherein the chemical oxidant is Fe^{3+}.

8. A device according to any one of claims 1 to 7 wherein the chemical oxidant is $FeCl_3$.

9. A method of determining the amount of aldehydes in a sample using the paper-based microfluidic sensor device of claim 1.

10. A method according to claim 9 wherein the method is used for determining the amount of aldehydes in a saliva sample.

11. A method according to claim 9 or claim 10 wherein the sample is contacted with zone one for about 5 minutes.

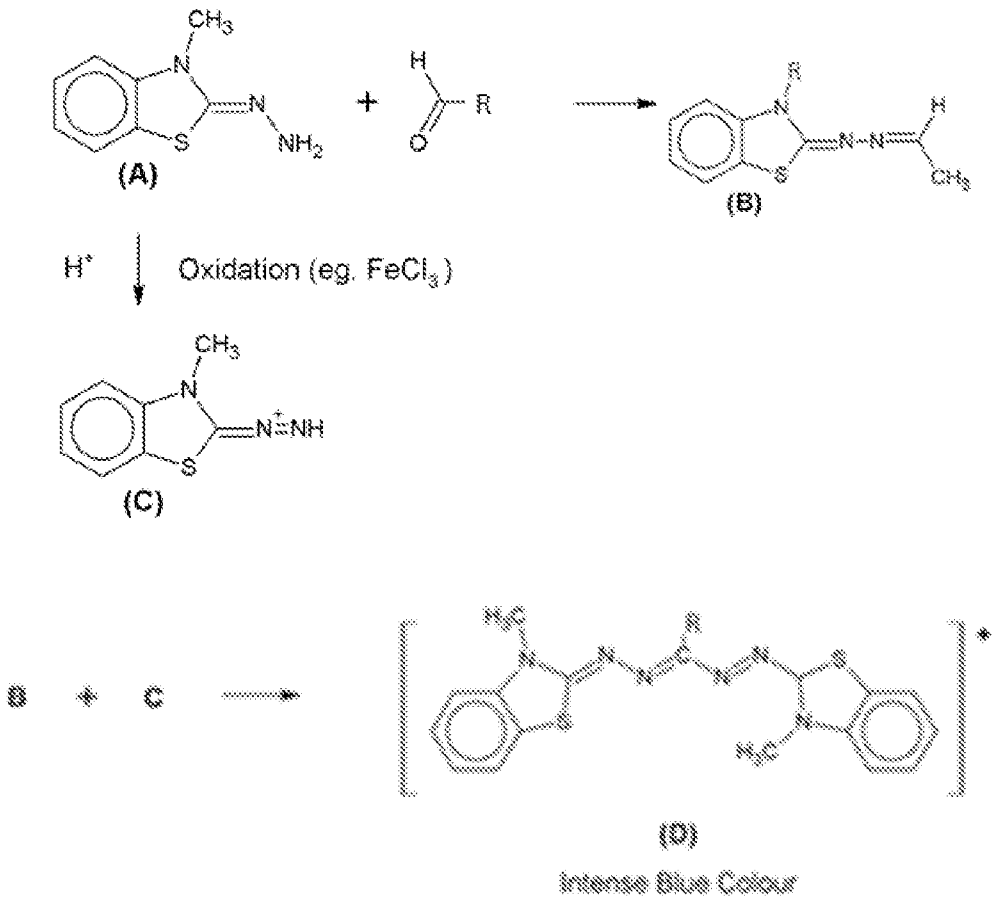
12. A method according to any one of claims 1 to 11 wherein zone one is contacted with zone two for about 5 minutes.

13. A method according t any one of claims 9 to 12 wherein the determination of amount of aldehyde in the sample is conducted by colourmetric analysis in zone one.

14. A method according to any one of claims 9 to 13 wherein the ABTH is MBTH.

15. A method according to any one of claims 9 to 14 wherein the claimed oxidant is Fe^{3+}.

16. A device according to claim 15 wherein the chemical oxidant is $FeCl_3$.



R is H or CH_3

Figure 1

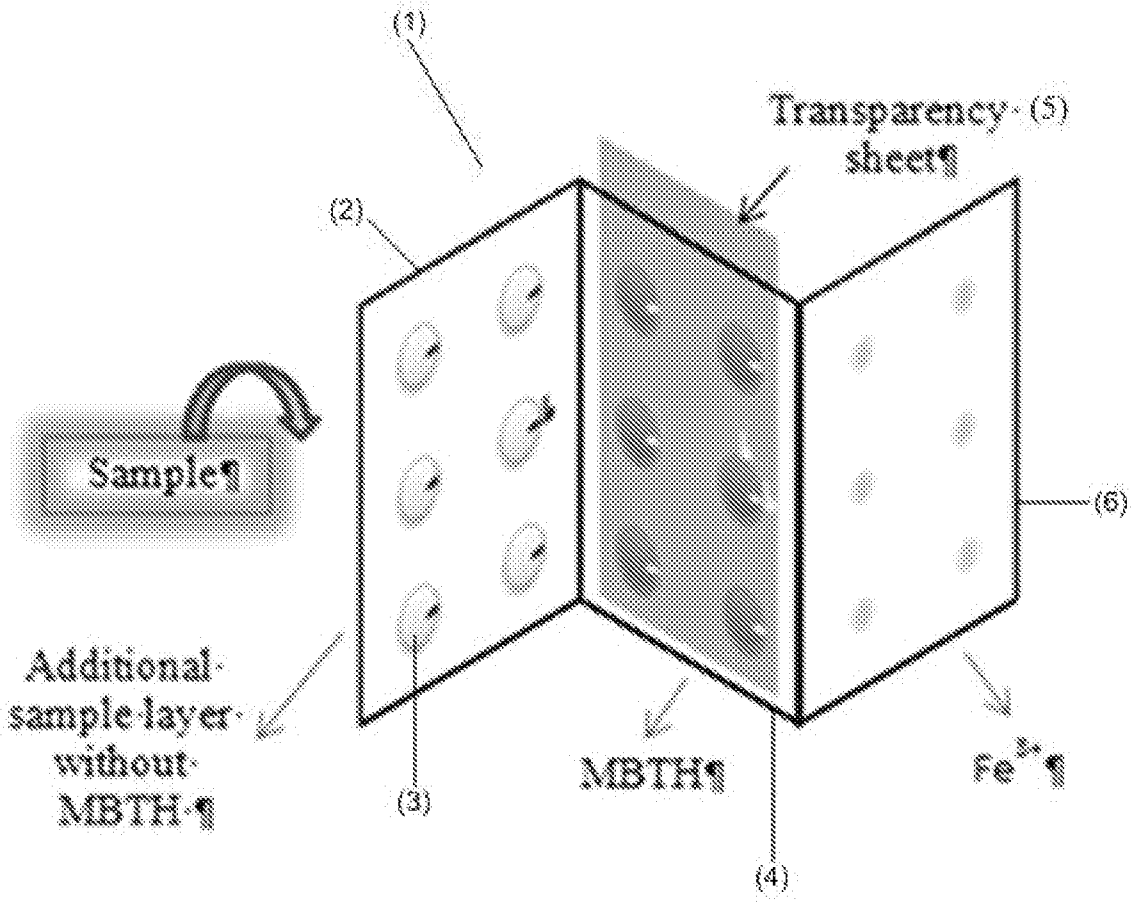

(1)
Transparency (5) sheet
(2)
Sample
(6)
Additional sample layer without MBTH
(3)
MBTH
(4)
Fe^{3+}

Figure 2

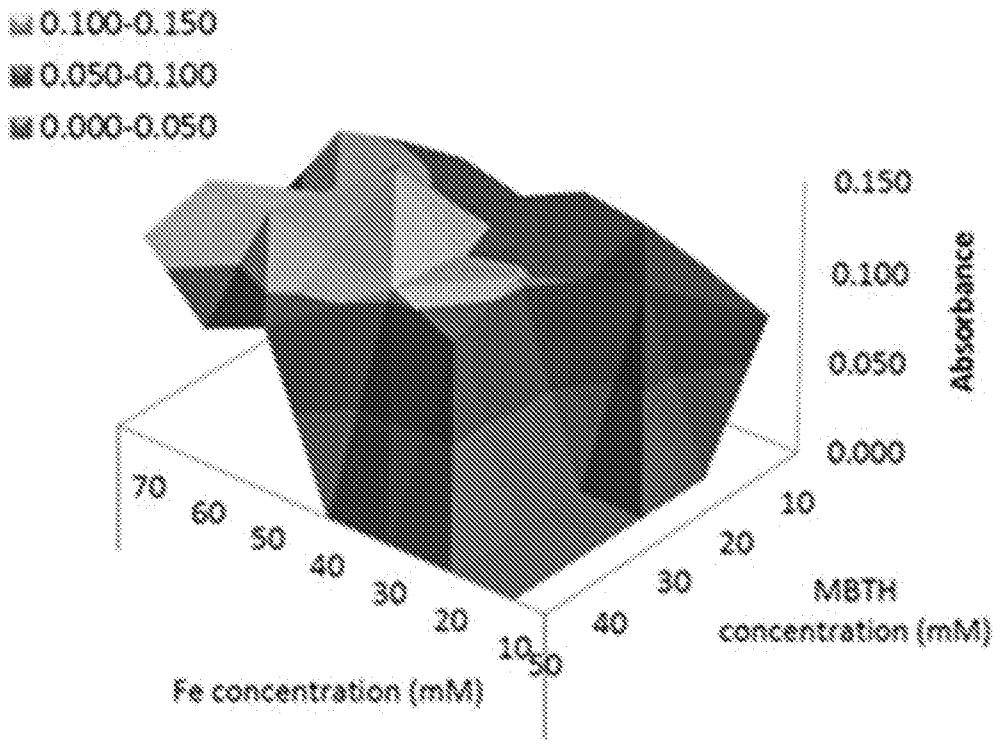

0.100-0.150
0.050-0.100
0.000-0.050
0.150
0.100
0.050
0.000
Absorbance
70
60
50
40
30
20
10
Fe concentration (mM)
10
20
30
40
50
MBTH
concentration (mM)

Figure 3

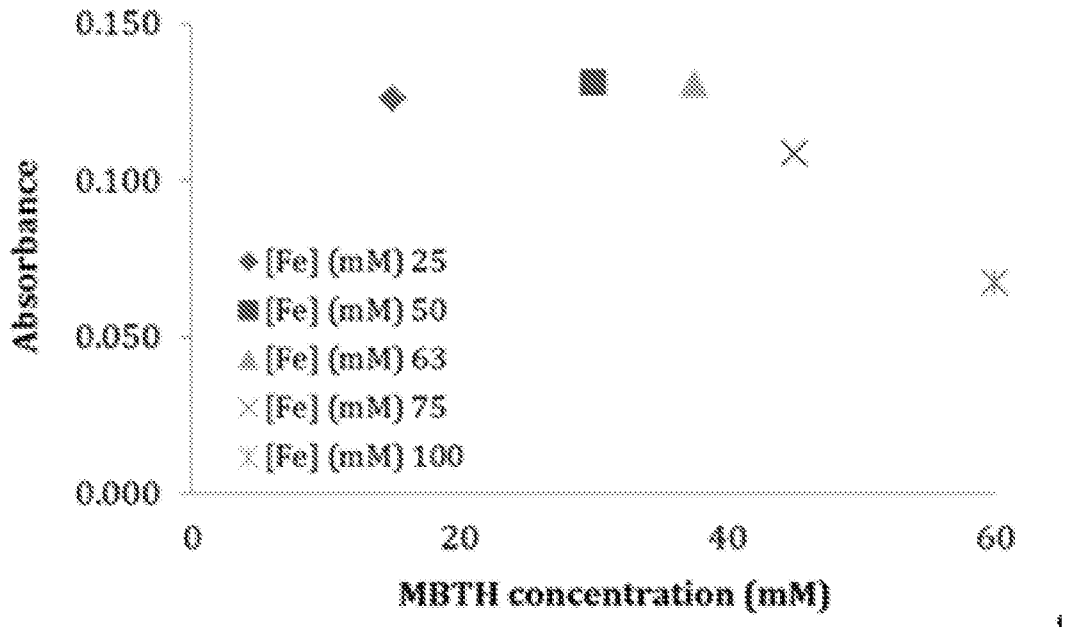

0.150
0.100
0.050
0.000
Absorbance
[Fe] (mM) 25
[Fe] (mM) 50
[Fe] (mM) 63
[Fe] (mM) 75
[Fe] (mM) 100
0
20
40
60
MBTH concentration (mM)

Figure 4

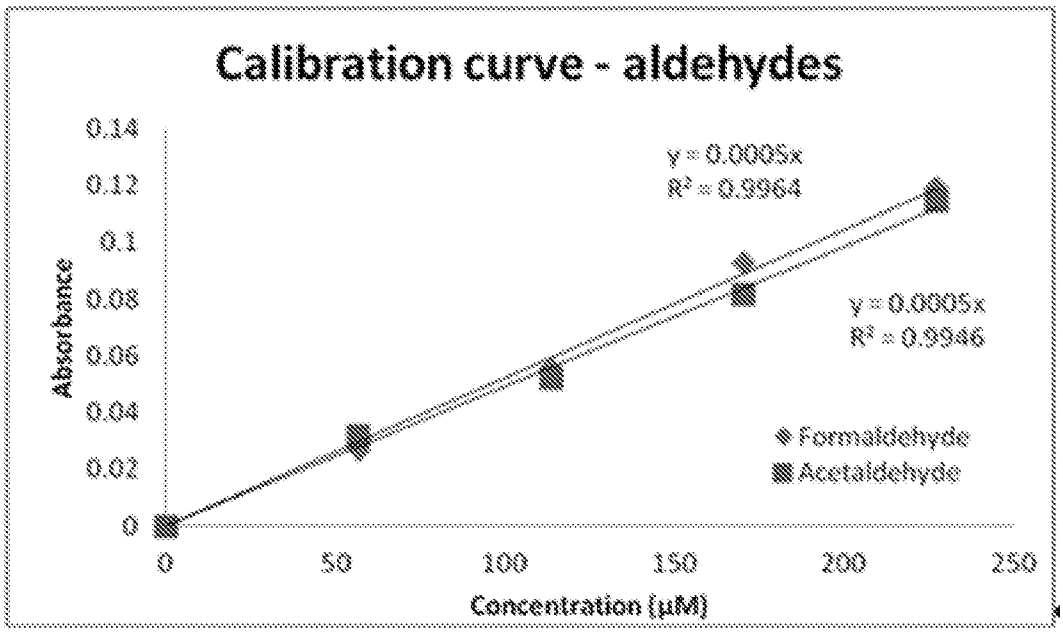

Calibration curve - aldehydes
y = 0.0005x
R² = 0.9964
y = 0.0005x
R² = 0.9946
Formaldehyde
Acetaldehyde
Absorbance
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
50
100
150
200
250
Concentration (µM)

Figure 5

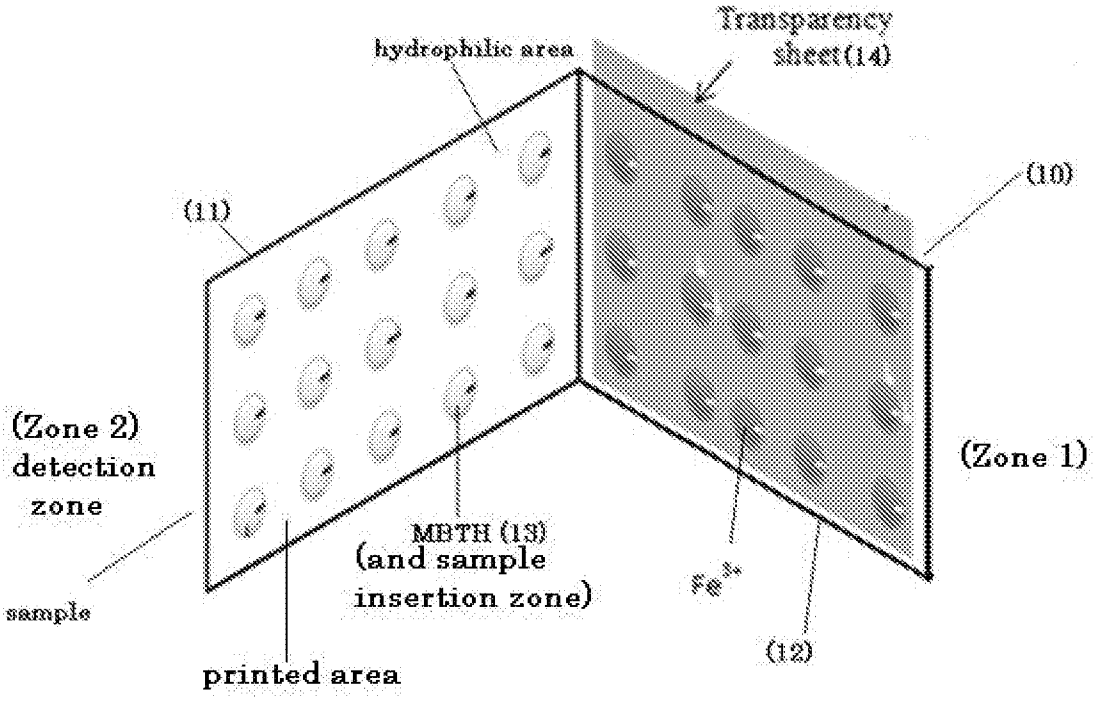

Transparency sheet(14)
hydrophilic area
(10)
(11)
(Zone 2) detection zone
(Zone 1)
MBTH (13) (and sample insertion zone)
sample
printed area
(12)

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/050747

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/52 (2006.01) B01L 3/00 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPIAP, TXTE (TXTUS5, TXTUS4, TXTUS3, TXTUS2, TXTUS1, TXTUS0, TXTEP1, TXTGB1, TXTWO1, TXTAU1, TXTCA1, TXTSG1): IPC/CPC: B01L2300/126/LOW/CN, G01N33/98/IC/CN, Y10S436/80/IC/CN/LOW, G01N2800/CN, G01N33/52/LOW/I/CN, G01N2800/50/LOW/CN G01N33/LOW/IC/CN, B01L3/00/LOW/IC/CN; Keywords: Aldehyde, Determine, Paper, Microfluidic, Colour, Oxidant, Zone, BENZOTHIAZOLINONE, HYDRAZONE, Saliva, Cancer, Diagnosis, MBTH, ABTH, and like terms. Google Scholar, Google Patents and Espacenet: Keywords: Aldehyde, Determine, Paper, Microfluidic, Colour, Oxidant, Zone, BENZOTHIAZOLINONE, HYDRAZONE, Saliva, Cancer, Diagnosis, MBTH, ABTH, and like terms. Google Scholar, Google Patents and Espacenet: Applicant/Inventor Search: "THE UNIVERSITY OF MELBOURNE", "KOLEV, SPAS D.", "MUCCULLOUGH, MICHAEL JOHN", "RAMDZAN, ADLIN N.", "ALMEIDA, MARIA INES GAMEIRO SA"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2016	23 February 2016

Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Aaron Giles AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832302

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2015/050747**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2002/0168777 A1 (ZHU et al.) 14 November 2002 Paragraph [0004], [0006]-[0012], [0014], [0015]; Figure 7A-C Paragraph [0004], [0006]-[0012], [0014], [0015]; Figure 7A-C	1, 5-9, 11-16 2-4, 10
Y	US 2011/0318227 A1 (SHEN et al.) 29 December 2011 Figure 2B; Paragraph [0048]	2-4
Y	JAYAWARDANE B.M. et al., 'Microfluidic Paper-Based Analytical Device for the Determination of Nitrite and Nitrate', Analytical Chemistry, Published 23 June 2014, Vol. 86, Pages 7274-7279 Figure 1A-D; Abstract	2-4
Y	RAMDZAN A.N. et al., 'Determination of Acetaldehyde in Saliva by Gas-Diffusion Flow Injection Analysis', Analytica Chimica Acta, Published 19 May 2013, Volume 786, Pages 70-77 Abstract; Subheading 2.2. Saliva Samples	10
A	US 2014/0220696 A1 (UNIVERSITY OF CHICAGO et al.) 07 August 2014 Paragraph [0007], [0017]	1-16

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2015/050747**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2002/0168777 A1	14 November 2002	US 2002168777 A1	14 Nov 2002
		US 7112448 B2	26 Sep 2006
		AU 2030702 A	19 Sep 2002
		CA 2376413 A1	16 Sep 2002
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		JP 2002328122 A	15 Nov 2002
		KR 20020073444 A	26 Sep 2002
		US 2006270052 A1	30 Nov 2006
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		WO 2010017578 A1	18 Feb 2010
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		EP 2334814 A1	22 Jun 2011
		EP 2604702 A1	19 Jun 2013
		EP 2604702 B1	01 Jul 2015
		US 2010216132 A1	26 Aug 2010
		US 8580516 B2	12 Nov 2013
		WO 2010028388 A1	11 Mar 2010

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.