

Chromatographic determination of low-molecular mass unsaturated aliphatic aldehydes with peroxyoxalate chemiluminescence detection after fluorescence labeling with 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole

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Abstract

A highly sensitive, selective and reproducible chromatographic method is described for determination of low-molecular mass unsaturated aliphatic aldehydes in human serum. The method combines fluorescent labeling using 4-(*N,N*-Dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole with peroxyoxalate-chemiluminescence. The derivatives were separated on a reversed-phase column C₈ isocratically using a mixture of acetonitrile and 90 mM imidazole-HNO₃ buffer (pH 6.4, 1:1, % v/v). The calibration ranges were: 20-420 nM for methylglyoxal, 16-320 nM for acrolein, 15-360 nM for crotonaldehyde and 20-320 nM for trans-2-hexenal. The detection limits were ranged from 4.4 to 6.5 nM (88-130 fmol/injection), the recovery results were within the range of 87.4-103.8% and intra and inter-day precision results were lower than 5.5%. The proposed validated method has been successfully applied to healthy, diabetic and rheumatic arthritis patients' sera with simple pretreatment method. In conclusion, this new method is suitable for routine analysis of large numbers of clinical samples for assessment of the oxidative stress state in patients.

Keywords: peroxyoxalate chemiluminescence (PO-CL), fluorescence labeling, oxidative stress, lipid peroxidation, serum analysis

1. Introduction

Oxidative stress is a condition associated with the imbalance between production of reactive oxygen species (ROS) and the antioxidant defense system *in vivo* [1]. ROS initiates lipid peroxidation (LPO) process by their reaction with polyunsaturated fatty acids (PUFAs) in cellular membranes forming numerous aldehydic end products [2]. From these end products, the low-molecular mass unsaturated aliphatic aldehydes (LMMUAA) are the most cytotoxic one due to the presence of both alkene and aldehydic group. These reactive groups can react rapidly with DNA and protein leading to many pathological diseases such as atherosclerosis, diabetes mellitus (DM), rheumatic arthritis (RA), chronic renal failure, cancer and neurodegenerative diseases [1,3-8].

To date, almost all the previous articles [10-22] highlighted on determination of LMMUAA individually in biological fluids. However, analysis of a broad spectrum of LMMUAA will provide a complete picture of the overall oxidation process to evaluate their role in the pathogenesis of these diseases. Therefore, a sensitive, selective and reproducible method for simultaneous determination of methylglyoxal (MG), acrolein (AC), crotonaldehyde (CR) and trans-2-hexenal (HE) in healthy and different patients' sera is developed.

However, direct determination of LMMUAA is difficult due to absence of intrinsic chromophores or fluorophores and chemical derivatization is required to convert them to a detectable and stable structures. Until now, several hydrazine based reagents such as 2,4-dinitrophenylhydrazine [9,10], dansylhydrazine [11], 2,4,6-trichlorophenylhydrazine [12,23] were used for determination of aldehydes. Beside hydrazine reagents, several reagents containing diamine group were used for

determination of MG only such as 1,2-diamine-4,5-dimethoxybenzene [13,14], 1,2-diamino-1,2-diphenylethane [15], 2,3-diamino-2,3-dimethylbutane [16], 4,5-dimethyl-1,2-phenylenediamine [17], and 5,6-diamino-2,4-hydroxypyrimidine sulfate [18] Furthermore, other reagents such as *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride [19], 1,3-cyclohexandione [20], 2-thiobarbituric acid [21], and fluorescein-5-thiosemicarbazide [22] were used for aldehyde derivatization.

Although various reagents have been used so far but some methods lack of sufficient sensitivity [10-16,23], suffer from harsh conditions [13,14,20-22], or require expensive instrumentation which may be not available in all laboratories [17,19,20].

To overcome these problems, 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H), fluorogenic labeling reagent, was used in this study. DBD-H contains hydrazine group (-NH-NH₂) which reacts specifically towards aldehydes and ketones, however its reaction with aldehydes is much faster and proceed to a greater degree of completion than its reaction with ketones [24]. Uzo *et al* [25] and Nakashima *et al* [26] reported the reaction of DBD-H with saturated aliphatic aldehydes at room temperature within 30 min. However, its reaction with ketones proceeds at 4 °C and take 4-5 hr [26,27]. Therefore, DBD-H can be considered as a selective FL labeling reagent for LMMUAA determination in biological fluid at room temperature within 30 min and without any interference from coexisting ketonic compounds. In addition, the resultant fluorescent derivatives have large quantum Φ values, and long excitation and emission wavelengths which avoid the interference derived from the bio-matrixes [28]. Beside all these advantages, to the

best of our knowledge, DBD-H wasn't used before for determination of LMMUAA which indicates the novelty of the method.

Furthermore, in this study a combination of FL labeling using DBD-H with peroxyoxalate chemiluminescence (PO-CL) will afford higher sensitivity for determination of trace amount of LMMUAA in human serum.

Therefore, the present study aimed to develop a PO-CL detection method for simultaneous determination of LMMUAA after labeling with DBD-H. The proposed method was optimized, validated and successfully applied to serum samples from healthy subjects, DM and RA patients and the measured biomarkers were compared to investigate their role in the pathogenesis of these diseases.

2. Experimental

2.1. Chemicals

AC, 4-ethylbenzaldehyde used as an internal standard (IS), DBD-H, bis (2,3,5-trichloro-6-((pentyloxy)carbonyl)phenyl)oxalate (CPPO), bis (2,4,6-trichlorophenyl)oxalate (TCPO) and imidazole were purchased from Tokyo Chemicals Industry (Tokyo, Japan). CR, trifluoroacetic acid (TFA), hydrogen peroxide, and bis (4-nitro-2-(3,6,9-trioxadecyloxy)carbonyl)phenyl)oxalate (TDPO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). MG was purchased from Sigma–Aldrich (Milwaukee, WI, USA). HE was purchased from Nacali Tesque (Kyoto, Japan).

Nitric acid, methanol and acetonitrile were obtained from Kanto Chemical Company (Tokyo). The water was purified by a Simpli Lab UV (Millipore, Bedford, MA, USA). All reagents used were of analytical grade. The stock solution of aldehydes and internal standard were prepared in methanol at a concentration of 5 mM and saved in

4 °C for one month. To obtain reproducible results, daily working solutions were prepared by diluting the stock solutions with methanol to obtain the required concentrations. Solutions of DBD-H and TFA were prepared in acetonitrile.

2.2. Apparatus

The HPLC-PO-CL system consisted of two Shimadzu LC-20AD pumps (Kyoto, Japan), a Rheodyne (Cotati, CA, USA) 7125 injector with a 20- μ L sample loop. The chromatographic separation was performed on a Cosmosil 5C₈-MS (150 mm \times 4.6 mm, I.D., 5 μ m) from Nacalai Tesque by an isocratic elution with a mixture of acetonitrile and 90 mM imidazole-HNO₃ buffer (pH 6.4, 1:1, % v/v). CL signal was measured using a Shimadzu CLD-10A chemiluminescence detector using a mixture of 0.7 mM CPPO and 15 mM hydrogen peroxide in acetonitrile as a post column CL reagent, and UNI-1 noise cleaner (Union, Gunma, Japan) which smoothes the baseline. The mobile phase and CL reagent were degassed by vacuum degassing with sonication and filtered through a 0.45- μ m filter prior to use. The flow rates of the mobile phase and post column CL reagent were set at 0.7 and 0.8 mL/min, respectively.

For the HPLC-FL system, a Shimadzu RF-20AXS fluorescence detector was used where the excitation and emission wavelengths were set at 445 and 553 nm, respectively. The HPLC systems were connected to an EZChrom Elite chromatography data acquisition system (Scientific software, Pleasanton, CA, USA).

2.3. Fluorescence labeling procedure

In a screw-capped vial, 100 μL of a mixture of aldehyde solution in methanol, 100 μL of 10 mM DBD-H and 100 μL of 10% TFA were added. After vortex-mixing, the reaction mixture was left at room temperature for 30 min. An aliquot of 20 μL was injected into the HPLC system after filtration through a 0.45- μm cellulose acetate membrane filter.

2.4. Sample collection

Serum samples were obtained from 12 healthy volunteers (6 females, 6 males; age range, 48-62; mean age 55.2 ± 4.8) and from 12 DM patients (5 females, 7 males; age range, 39-69; mean age 52.3 ± 10.1) and from 12 RA patients (9 females, 3 males; age range, 43-84; mean age 65.1 ± 11.9) attending Sasebo Chuo Hospital. The collected serum samples were frozen at $-80\text{ }^{\circ}\text{C}$ prior to analysis. The present experiments were approved by the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, and performed in accordance with established guidelines.

2.5. Pretreatment of serum sample

To a 100 μL of serum sample, 20 μL of IS (10.8 ng/mL), 100 μL of 10 mM DBD-H, and 100 μL of 10% TFA were added in a 2.0-mL eppendorf tube. The mixture was vortex mixed and left at room temperature for 30 min then centrifuged at $2200 \times g$ for 20 min at 4°C . A 20 μL of the clear supernatant was injected into the HPLC system after filtration through a 0.45- μm cellulose acetate membrane filter.

2.6. Data analysis

The data are presented as mean \pm standard error (SE) for the number of experiments. The current study employed the decision tree method proposed by Kobayashi [29]. Bartlett's test was used to determine if there is a difference in variance between the 3 studied groups (healthy, diabetic, and rheumatic subjects). It was found that the k sampled populations have unequal variances and Steel's multiple comparison tests was used for subsequent data analysis. All the statistical tests were two-sided at a significant level of $\alpha=0.01$.

3. Results and discussion

3.1. Optimization of fluorescence labelling conditions

In the present study, the target aldehydes were first labeled with DBD-H to form fluorescence hydrazone derivative as shown in Fig.1. In order to obtain higher reaction yields, labeling conditions including reagent concentrations, reaction temperature and time were optimized by using a standard mixture of the studied aldehydes (2 μ M each). The effect of different concentrations of DBD-H (1-14 mM) and TFA (2-12%) on the fluorescence intensities of the labeled aldehydes were tested. It was found that the maximum peak areas were obtained in the presence of 10 mM of DBD-H (Fig. S1a, supplementary data) and 10% of TFA (Fig. S1b, supplementary data), respectively. The reaction temperature and time were also investigated. The reaction proceeded at room temperature (25 ± 5) °C and the highest and stable peak areas were obtained after 20 min; so 30 min was selected as reaction time.

3.2. Optimization of chemiluminescence reaction variables

Previously, it was reported that diaryloxalate esters with electron withdrawing groups are known to provide the highest quantum yield [30]. Therefore, the effects of CPPO, TDPO, and TCPO on CL intensity were tested using Sirius Luminometer (Berthold Technologies, Bad Wildbad, Germany). Among the tested diaryloxalate esters, CPPO gave the highest CL intensities (310) compared to TDPO (304) and TCPO (298) using AC as representative aldehyde. Therefore, CPPO was selected as the optimum diaryloxalate ester and its concentration was examined from 0.4 to 1.4 mM using HPLC-PO-CL system and the optimum concentration was 0.8 mM by monitoring the signal-to-noise ratio (S/N) for aldehydes peaks (Fig. 2Sa, supplementary data). The concentration of hydrogen peroxide was examined from 5 to 50 mM and 15 mM was selected as optimum concentration (Fig. 2Sb, supplementary data). Imidazole was identified as the best catalyst for PO-CL reaction and nitric acid is the best acid used in combination with imidazole to form buffer solutions as reported before [30,31]. The pH of the mobile phase has a great effect on CL intensities; therefore different buffer solutions from 5.8 to 7.2 were studied and the optimum pH was 6.4, as shown in Fig. S3a (supplementary data). Finally, the flow rate of the CL reagent was varied from 0.2 to 1.5 mL/min, increase in signal intensities were observed by increasing the flow rate. However, flow rate above 0.8 mL/min led to an increase in the noise; therefore 0.8 mL/min was thus selected as the optimum flow rate as shown in Fig. S3b (supplementary data). A chromatogram of the studied LMMUAA after optimization of both FL labelling and PO-CL conditions was shown in Fig. 2.

3.3. Method Validation

A standard mixture of the studied aldehydes with different concentrations was analyzed under the optimized reaction conditions. The calibration curves showed good linearity between concentration and peak area ratio over concentration range from 15 to 420 nM and the detection limits were in the range from 4.4 to 6.5 nM (88-130 fmol/injection) at (S/N=3), as shown in Table 1 (supplementary data). On the other hand, the detection limits of the studied aldehydes obtained by FL detection were in the range from 43 to 46 nM. These results were 7-10 times less sensitive than PO-CL detection which indicated that the application of PO-CL for the detection of DBD-labelled aldehydes would improve the sensitivity. The higher sensitivity of the proposed HPLC-PO-CL method was brought by the significant reduction in baseline noise due to lack of external excitation source.

The sensitivity of the HPLC-PO-CL method was 4 and 10 times more sensitive compared with HPLC-UV [10,13], 14 times compared with CE-LIF [11], 4-34 times compared with GC-MS [12,23], 12 times compared with MEKC [15], 20 times compared with GC-FID [16], 13 and 76 times compared with HPLC-FL [14,19] and 34 times compared with LC-MS [20].

The reproducibility of the proposed method was examined using three different concentrations of the studied aldehydes. The precision was expressed by relative standard deviation (RSD). It was found that RSD values for intra-day (n=5) and inter-day (n=5) precision were 1.0-3.2 and 1.2-3.8%, respectively as shown in Table 2 (supplementary data). It was confirmed that the proposed method have sufficient reproducibility.

The proposed method showed high sensitivity and good reproducibility without requiring expensive instrumentation which may be not available in all

laboratories. In addition, the pretreatment method was simple, fast and doesn't requires further clean up methods to remove excess reagent. These advantages decreased the analysis time and made our proposed method more suitable for routine analysis of large numbers of clinical samples other than previous methods [14,20,21].

3.4. Determination of unsaturated aliphatic aldehydes in human serum

The validation studies of biological fluid were carried out using pooled serum sample pre-analyzed for aldehyde contents before spiking with standard aldehydic solutions. Calibration curves showed good linearity between concentration and peak area ratio over concentration range from 16 to 420 nM and the detection limits were in the range from 4.9 to 6.9 nM (98-138 fmol/injection) at (S/N=3), as shown in Table 3 (supplementary data).

To evaluate intra and inter-day precision, five replicates of three sets of serum samples spiked with three different concentration levels of standard aldehyde solutions were performed. It was found that RSD values for intra-day (n=5) and inter-day (n=5) precision were 1.4-4.0% and 2.7-5.4%, respectively. The recovery of the proposed method was determined by using serum samples spiked with standard mixture of aldehyde solution at three concentrations before derivatization. Recoveries were expressed as [(found amount/ spiked amount) x100%] and found to be higher than 87%, which is higher than previous methods [10,12,19,25] and the results are listed in Table 1.

3.5. Data analysis of aldehydes in health, DM and RA sera

The proposed method was applied for determination of the studied aldehydes in human sera. Fig. 3 shows typical chromatograms of spiked serum sample and sera samples from healthy, DM and RA patients determined by the proposed method. The peaks of the studied LMMUAA were detected clearly in the chromatogram without any interference from other biological components. However, the peaks appeared in the chromatograms before 5 min were attributed to shorter chain aliphatic aldehydes which may be produced *in vivo* either from LPO or from other biological process.

The concentration of the studied aldehydes were compared between healthy subjects, DM and RA patients using Steel's multiple test [29,32] the statistical tests were two-sided at a significant level of $\alpha=0.01$. The results were summarized in Table 2 and healthy subjects' results were in good agreement with other previous reports [5,9,12,21].

In DM patients, serum MG and AC level were found to be significantly higher than those in controls ($p<0.01$). The simultaneous elevated level of both MG and AC can be used as a distinguishable marker to measure the severity of oxidative stress in DM and its contribution to many diabetic complications. The endogenous formation of MG may be either from autoxidation of sugars and glycation reaction [3], LPO process [13] or from lipid and protein catabolism [33]. These processes are elevated in DM patients and consequently MG level is elevated too. In addition, Meszaros *et al* [34] and Garpenstrand *et al* [35] reported the elevated activity of sensitive semicarbazide aminoxidase (SSAO) enzyme, which catalyzes the deamination of endogenous primary amines to MG, in DM patients than healthy human. As a result, it is considered that monitoring of MG biogenesis in diabetic patients might help to assess the risk of progression of diabetic complications. In addition, the elevated level

of AC in DM patients may be due to the elevated glucose level which consequently stimulates the production of AC from PUFAs in LPO process [36].

For the first time, we found a significantly elevated level of AC in RA patients than those in controls ($p < 0.01$). This elevated level may be due to the elevated level of oxidative stress in RA patients [37]. Beside oxidative stress, Stevens *et al* [38] found that the oxidative deamination of polyamine compounds is another significant source of AC *in vivo*. These polyamine compounds were detected in high concentrations in RA patients' urine samples as reported by Furumitsu *et al* [39]. The pathophysiology of DM and RA diseases is mostly related to oxidative stress, so MG and AC can be used as oxidative stress biomarkers. However, CR and HE concentrations were found to be insignificantly higher in DM and RA sera than those in controls and they can't be considered as oxidative stress biomarkers for either DM or RA.

4. Conclusion

In this study, the feasibility of PO-CL detection method for simultaneous determination of LMMUAA after labeling with DBD-H was demonstrated. The proposed method was successfully applied to determine LMMUAA in healthy, DM and RA human sera without any interference from the biological components after simple pretreatment method. For the first time, we found a significant elevation in AC concentration in serum of RA patients compared to healthy humans. In addition, a significant elevation of both MG and AC were found in DM patients compared to healthy humans that confirms suitability of using MG and AC as biomarkers for DM and RA. Since the proposed method is sensitive, selective, reproducible and doesn't

require expensive instrumentation the method should be suitable for routine analysis of large numbers of clinical samples for the evaluation of oxidative stress process.

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Figure Caption

Fig. 1. Reaction scheme of aldehyde derivatization with DBD-H and the structure of the studied aldehydes .

Fig. 2. Representative chromatograms of (a) reagent blank, (b) standard solution of a mixture of the studied aldehydes (120 nM each) with peroxyoxalate chemiluminescence detection, and (c) standard solution of a mixture of the studied aldehydes (120 nM each) with fluorescence detection, peaks: 1-MG, 2-AC, 3-CR, and 4-HE.

Fig. 3. Chromatograms of (a) human serum spiked with a mixture of the studied aldehydes (200 nM each), (b) healthy human serum, (c) DM patient serum, and (d) RA patient serum; peaks 1-4 as in Fig. 2.

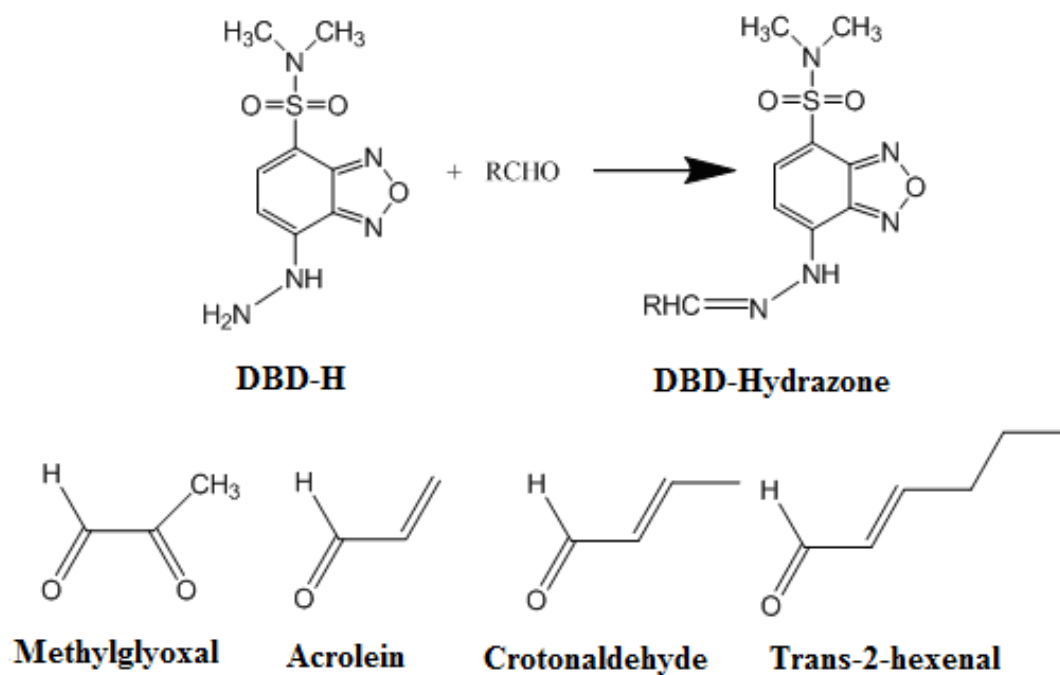


Fig. 1

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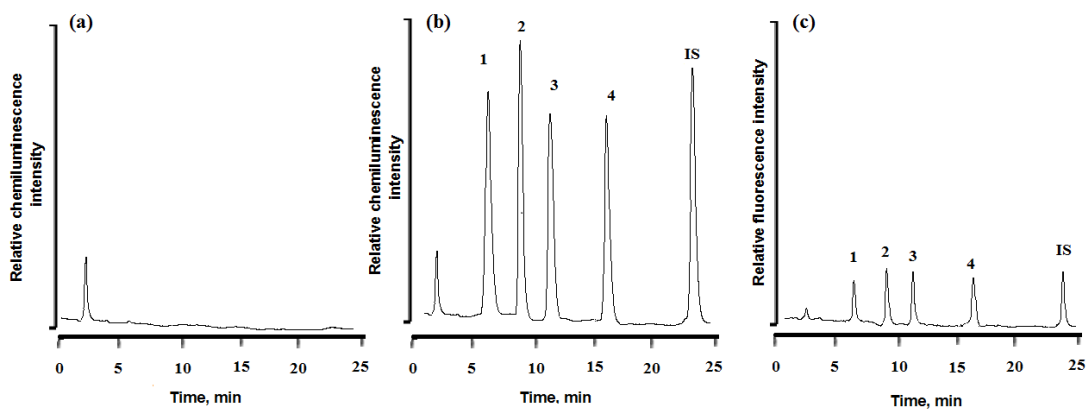


Fig. 2

Fig. 2. Representative chromatograms of (a) reagent blank, (b) standard solution of a mixture of the studied aldehydes (120 nM each) with peroxyoxalate chemiluminescence detection, and (c) standard solution of a mixture of the studied aldehydes (120 nM each) with fluorescence detection, peaks: 1-MG, 2-AC, 3-CR, and 4-HE

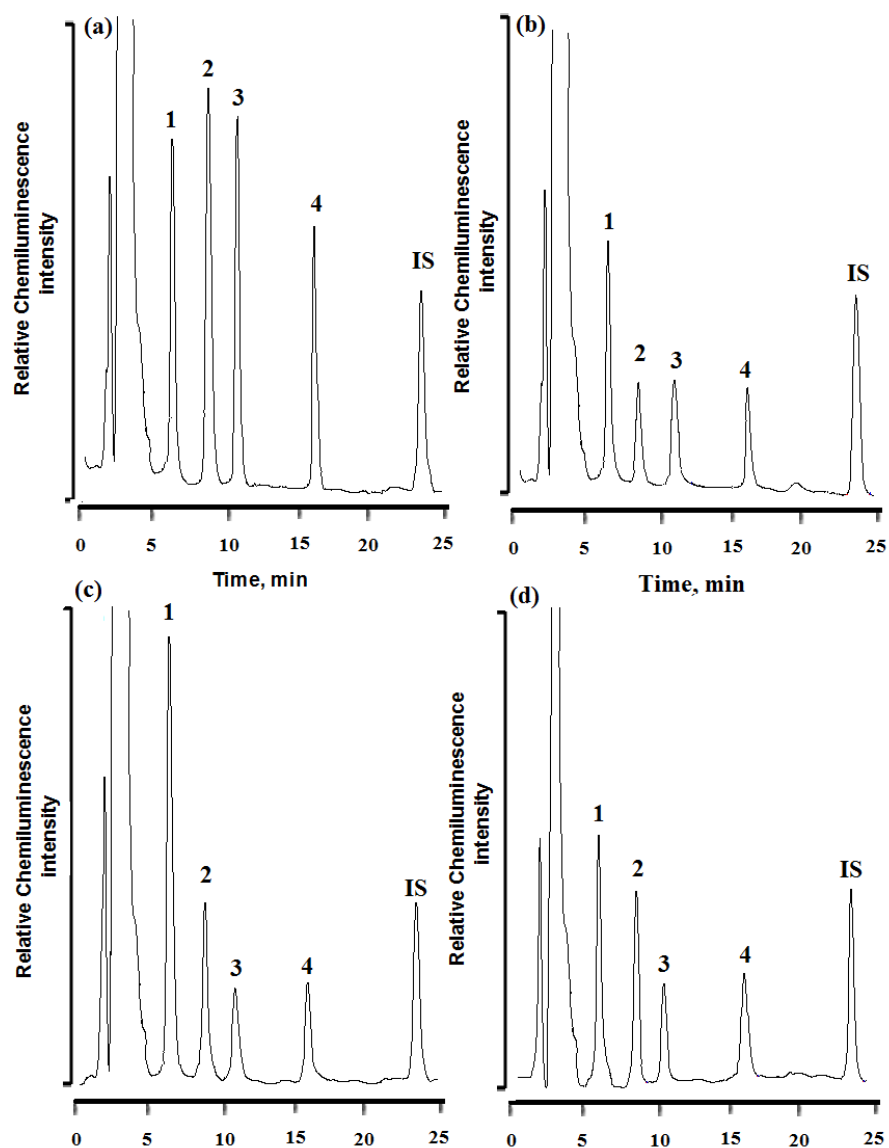


Fig. 3.

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Table 1 Recovery and precision of the proposed method for aldehydes in serum samples

Aldehyde	Spiked amount (nM)	Intra-day (n=5)			Inter-day (n=5)		
		Found \pm SD	RSD%	Recovery%	Found \pm SD	RSD%	Recovery%
MG	20	18.2 \pm 0.73	4.0	91.0	17.5 \pm 0.78	4.5	87.5
	200	195.4 \pm 3.3	1.7	97.7	197.2 \pm 7.3	3.7	98.6
	420	389.6 \pm 8.57	2.2	92.8	402 \pm 15.7	3.9	95.7
AC	16	14.5 \pm 0.48	3.3	90.6	14.2 \pm 0.77	5.4	88.8
	120	121.4 \pm 3.3	2.7	101.1	123.5 \pm 4.4	3.6	102.9
	320	305 \pm 10.1	3.3	95.3	300.4 \pm 10.5	3.5	93.9
CR	16	14.9 \pm 0.3	1.9	93.1	14.5 \pm 0.5	3.4	90.6
	120	124.6 \pm 1.7	1.4	103.8	121.3 \pm 3.3	2.7	101.1
	360	362.8 \pm 6.2	1.7	100.8	360.1 \pm 10.4	2.9	100
HE	20	19.3 \pm 0.54	2.8	96.5	18.8 \pm 0.7	3.7	94.0
	120	109.1 \pm 1.6	1.5	90.9	104.9 \pm 4.1	3.9	87.4
	320	286.4 \pm 9.5	3.3	89.5	297.6 \pm 13.1	4.4	93.0

Table 2 Aldehydes concentration in healthy, diabetic and rheumatic arthritis patients sera (n=12 each)

		Aldehyde data			Statistical test ^{c,*}
		Range (nM)	Mean \pm SE ^a	95% CI ^b	
MG	Healthy	141.4-152.6	146.5 \pm 1.0	144.6-148.4	-----
	DM	281.7-314.8	299.1 \pm 2.8	293.7-304.5	< DM Healthy
	RA	140.1-150.5	145.3 \pm 1.1	143.1-147.5	-----
AC	Healthy	38.4-57.0	42.2 \pm 1.6	39.1-45.3	-----
	DM	61.0-75.6	68.7 \pm 1.4	65.9-71.5	< DM Healthy
	RA	73.1-81.5	76.3 \pm 0.7	74.9-77.7	< RA Healthy
CR	Healthy	57.2-64.9	61.3 \pm 0.8	59.8-62.8	-----
	DM	59.8-68.4	63.5 \pm 0.3	61.7-65.3	-----
	RA	58.6-64.1	60.9 \pm 0.4	60.1-61.7	-----
HE	Healthy	55.1-66.3	59.5 \pm 1.2	57.2-61.8	-----
	DM	54.4-69.9	59.2 \pm 1.2	56.9-61.5	-----
	RA	58.5-70.7	61.7 \pm 1.0	59.8-63.6	-----

^a Standard error

^b Confidence interval

^c Steel's test

* $p < 0.01$

Supplementary Files

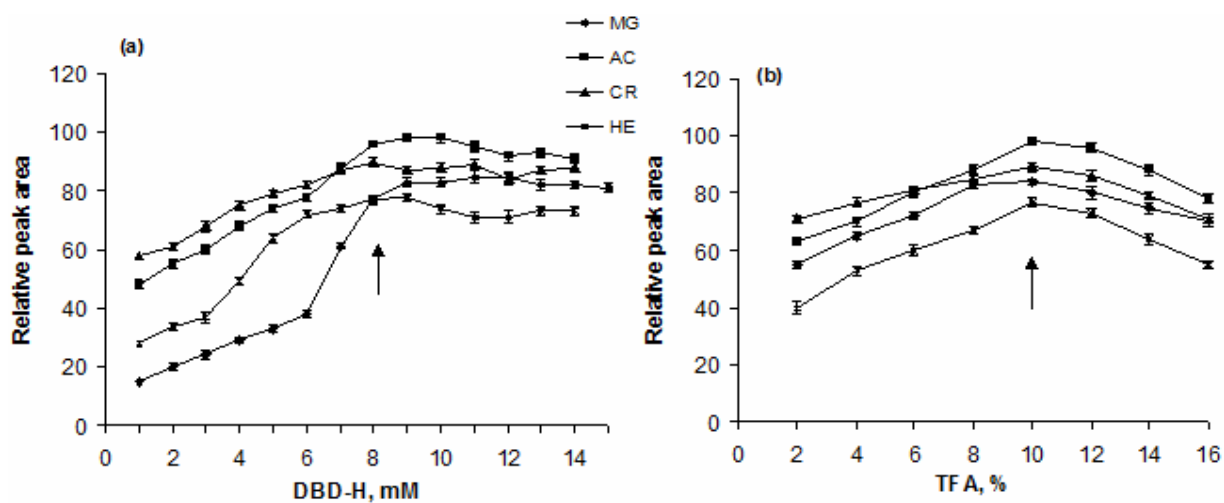


Fig.S1a,b

Fig. S1. Effects of (a) DBD-H and (b) TFA concentrations on relative peak areas of a mixture of the studied aldehydes ($2 \mu\text{M}$ each), the data represent error bars of three determinations

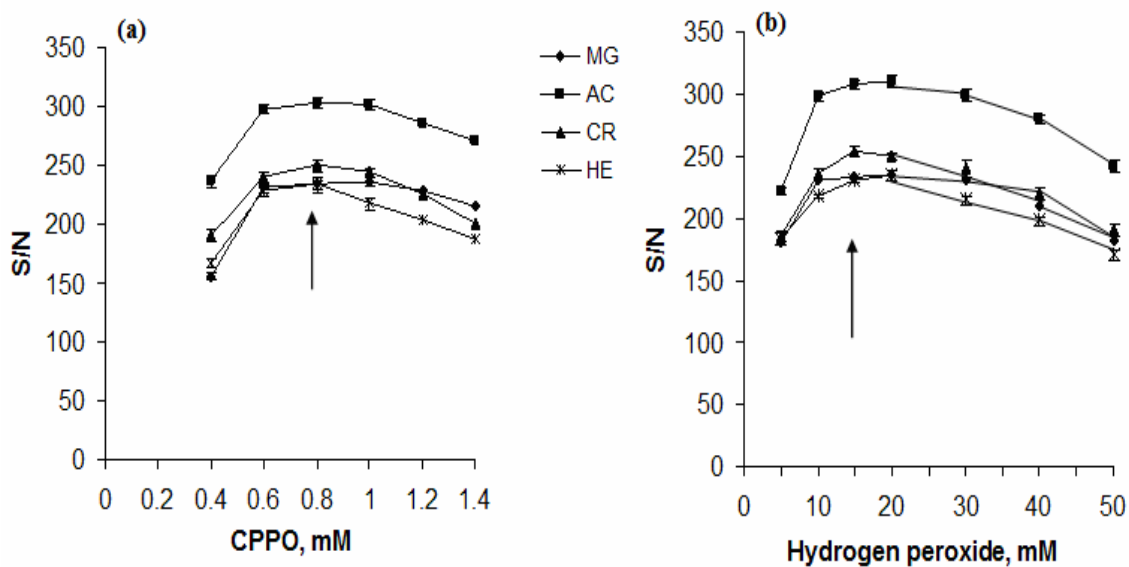


Fig.S2a,b

Fig. S2. Effects of (a) CPPO and (b) hydrogen peroxide concentrations on signal-to-noise ratio of a mixture of the studied aldehydes (120 nM each), the data represent error bars of three determinations

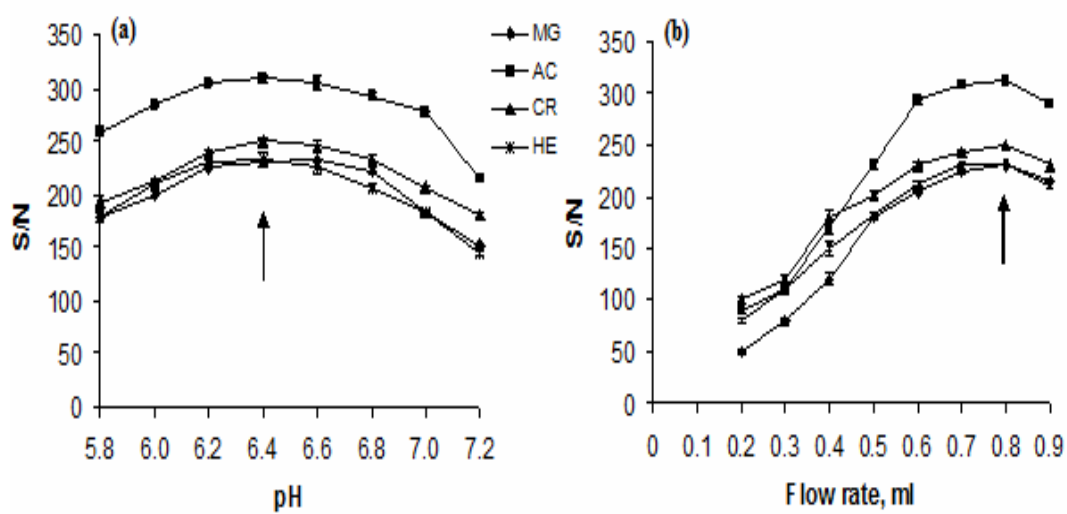


Fig. S3 a,b

Fig. S3. Effects of (a) pH and (b) flow rate on signal-to-noise ratio of a mixture of the studied aldehydes (120 nM each), the data represent error bars of three determinations

Table S1 Retention times, calibration curves and detection limits for standard aldehyde solutions

Compound	Range, nM	Calibration curve ^a , n=3	r ²	LOD ^b , nM (fmol/injection)
MG	20-420	Y= 0.034 + 0.011 X	0.9988	6.5 (131)
AC	16-320	Y= 0.010 + 0.016 X	0.9992	4.9 (98)
CR	15-360	Y= 0.010 + 0.011 X	0.9992	4.4 (88)
HE	20-320	Y= 0.016 + 0.012 X	0.9988	5.8 (115)

^a Y= peak area ratio; X= sample concentration (nM).

^b S/N= 3.

Table S2 Precision results for determination of standard aldehydic solution using the proposed method

Aldehyde	Concentration (nM)	Intra-day assay (n=5) RSD %	Inter-day assay (n=5) RSD %
MG	20	2.5	3.8
	200	1.0	1.9
	420	1.0	1.2
AC	16	1.4	3.2
	200	3.2	3.4
	320	1.6	1.7
CR	15	2.6	3.1
	200	2.4	2.5
	360	1.9	2.0
HE	20	1.7	2.7
	200	1.6	2.6
	320	1.4	1.5

Table S3 Retention times, calibration curves and detection limits for spiked aldehyde solutions

Compound	Range, nM	Calibration curve ^a , n=3	r ²	LOD ^b , nM (fmol/injection)
MG	20-420	Y= 0.012 + 0.010 X	0.9986	6.9 (138)
AC	16-320	Y= 0.016 + 0.016 X	0.9990	5.0 (100)
CR	16-360	Y= 0.015 + 0.011 X	0.9990	4.9 (98)
HE	20-320	Y= 0.012 + 0.012 X	0.9986	6.0 (120)

^a Y= peak area ratio; X= sample concentration (nM).

^b S/N= 3.