APPLICATION OF RESPONSE SURFACE MODELING FOR OPTIMIZATION AND DETERMINATION OF MALONDIALDIALDEHYDE BY VORTEX-ASSISTED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND GC-FID

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ABSTRACT

An analytical method based on vortex-assisted dispersive liquid-liquid microextraction and gas chromatography-flame ionization detection is presented for the extraction and determination of malondialdehyde (MDA) in blood plasma of human. Various parameters affecting the extraction efficiency such as type and volume of extraction and dispersive solvents, vortex and centrifuge times, volume, ionic strength and pH of the sample solution were evaluated using, one-variable-at-a-time and response surface methodology. In order to optimize the MDA extraction and determination, seven factors in five- levels were used for design of experiments (DOE). Under optimum extraction condition, this method showed linear range of calibration curve between 10–1150 μg L^{-1}. The detection limit of the proposed method was found to be 0.8 μg L^{-1} with a relative standard deviation better than 5.5% (n=10) for blood serum samples. Enrichment factor was calculated to be 175 fold and the total analysis time including microextraction was about 13 min. The method was successfully applied for the analysis of MDA in blood plasma of human.

Keywords: Malondialdehyde, Vortex-assisted dispersive liquid-liquid microextraction, Response surface methodology, Human serum analysis**,** GC-FID.

INTRODUCTION

MDA is widely used as a biomarker for assessing oxidative stress in biomedical fields. Lipid peroxidation is a chain phenomenon resulting in the formation of various active compounds that result in cellular damage. Biomonitoring of malondialdehyde has been used in both biological and medical studies as a key biomarker for various disease patterns including hypertension, diabetes, atherosclerosis, neurodegenerative disorders, heart failure and cancer [1-4].

In the last decades a number of methods have been published for determination of the lipid peroxidation product, MDA, with improvements in analytical technologies and further development of HPLC [5], GC [6] and GC–MS [7]. However, because of the trace amount of MDA in body fluids, a derivatization step is universally necessary before sample injection, which is time consuming and also decreases the reproducibility. The level of MDA in biomedical samples is usually assessed by 2-thiobarbituric acid (TBA) assay. In the reaction between them, MDA forming an MDA–TBA_2 adduct, a red complex. The level of MDA–TBA_2 adduct can be determined either by spectrofluorimeter or by spectrophotometer [8]. However, the method has been criticized for low selectivity and sensitivity since several MDA-unrelated species from biomedical samples can react with TBA. High performance capillary electrophoresis does not utilize derivatization, but it is too expensive for routine use of bioanalytical laboratories [9]. Utilizing extraction before chromatographic analysis can overcome these problems because it can pre-concentrate the analyte and furthermore, eliminate the interfering elements at the same time [10-12]. Meanwhile, no derivatization step will be necessary. Classical liquid–liquid extraction (LLE) is the most universally used separation technique, but it is labor, time consuming and requires high purity organic solvents which their disposal after usage brings a major threat to the environment [13, 14]. Therefore researchers tried to extract MDA from serum media using extraction methods which consume less solvents such as solid phase extraction [15, 16] and solid phase microextraction [17, 18]. However, these methods are complicated, expensive and time consuming [19-21]. Recently, one kind of extraction based on miniaturized conventional LLE is introduced, in which, the solvent to aqueous phase ratio is greatly reduced, leading to the development of solvent microextraction methodologies.

As a novel mode of solvent microextraction, dispersive liquid-liquid microextraction (DLLME) has been distinguished as a very popular preparation technique due to its simplicity of operation, time-saving, high enrichment factor and low cost [22-25]. In the prior studies, various techniques were employed for assisting dispersion in DLLME.

Manual shaking was a convenient and traditional procedure, but usually time consuming and non-efficient. Ultrasound assisted emulsification microextraction and microwave assisted emulsification microextraction were distinguished as two efficient and rapid methods [26]. However, these methods are consuming more energy and might cause analyte decomposition with more matrix interference. In 2010, Yiantzi et al. reported vortex assisted DLLME (VADLLME), a milder emulsification procedure compared with ultrasonic and microwave assisted DLLME, in which the extraction solvent was dispersed into aqueous samples by vortex mixing [27].

In this paper, a simple and rapid method using VADLLME followed by GC was applied for the determination of MDA in blood serum. Several parameters affecting microextraction have been optimized using including one-variable-at-a-time and response surface methodology techniques, and the optimized method was successfully applied to serum samples analysis.

EXPERIMENTAL

Reagents and materials

All organic solvents and NaCl salt were of analytical grade and were purchased from Merck KGaA (Germany) and used as received. Milli-Q® water (18.3 MΩ cm^{-1}) was used throughout the experiment after filtering through 0.22 mm Nylon membrane. 1,1,3,3-tetraethoxypropane (TEP) (99%) as precursor of malondialdehyde preparation was also purchased from Merck KGaA (Germany). 1,1,3,3-tetramethoxypropane (TMP) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as internal standard.

TEP was used to prepare a MDA stock solution. A volume of 10 μL of TEP was accurately diluted to 10 mL with 0.1N HCl in a screw-capped test tube and incubated in boiling water bath for 15 min and then rapidly cooled with tap water. A working solution of MDA was prepared by pipetting 1 mL of this solution into a 100 mL volumetric flask and diluted to volume with ultrapure water. This solution contains 1 mg of MDA per litter which is stable for few days at 4 ºC.

Apparatus

A Varian 450-GC gas chromatograph (Varian Inc., USA) equipped with a flame ionization detector was used for all analyses. The GC was fitted with a Varian capillary column (30 m × 0.32 mm, film thickness 0.25 μm). The gas chromatography conditions were as follows: (1) the injector port was operated in split mode with a split ratio of 50:1 and it was kept at 200 ºC; (2) the FID temperature was 250 ºC; (3) the initial oven temperature was 60 ºC for 1 min, and increased to 140 ºC at 20 ºC min^{-1} then raised to 180 ºC at 40 ºC min^{-1}, and

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remained for 1 min at this final temperature; (4) usage of high-purity nitrogen as a carrier gas (1.8 mL min^{-1}). Hydrogen and air were used as detector gases at 30 and 300 mL min^{-1}, respectively. A vortex mixer (50 Hz) from Labnet International, INC (USA) was used. Also for this work a Universal 32 R, Hettich Zentrifugen (Germany) centrifuge was employed. A Denver (Germany) model UB-10 pH meter was used for pH measurements.

VADLLME procedure

6 mL of the aqueous standard solution (1 mg L^{-1}) or sample solution is transferred into a 10-mL glass extraction vessel. A mixture of 450 µL acetic acid as dispersive solvent with 150 µL benzyl alcohol as extracting solvent is transferred into the vessel. The mixture was then vigorously shaken on vortex agitator for 1 min at max speed rate, a cloudy solution is obtained. Then, the mixture is centrifuged for 4 min at 2000 rpm. As a result of centrifugation, benzyl alcohol droplets were precipitated at the bottom of the centrifuge tube. Deposited phase was transferred to a micro-tube by a conventional sampler, from which, 0.9 µL was mixed with 0.1 µL of TMP (as internal standard) inside a GC microsyringe and injected into the GC. The microsyringe was cleaned with methanol three times before next injection to avoid formation of air bubbles and the carryover of compounds between extractions. In all cases, the analytical signal was recorded as the area ratio of the analyte peak to the internal standard peak. Calibration was performed using aqueous calibration solutions subjected to the same VADLLME procedure described above.

RESULTS AND DISCUSSION

Optimization of VADLLME

With the aim of achieving the best efficiency of the proposed method, different factors affecting extraction efficiency were investigated, including the type and volume of the extraction and dispersive solvents, vortex and centrifugation times, and volume, ionic strength and pH of the sample solution. A uni-variate approach was employed to optimize influential factors in order to simplify the optimization procedure. A series of experiments were designed for this purpose as discussed below.

Type of the extraction solvent

The selection of an appropriate extraction is a critical point for all microextraction processes. While the extraction solvent needs to be polar enough to have a large equilibrium distribution constant for MDA, it should have low solubility in aqueous, good extraction capability of the compound, low volatility and good chromatographic behavior. Based on these facts, six solvents including methyl isobutyl ketone, o- xylene, n- pentanol, n-octanol, n- heptanol and benzyl alcohol were selected. The results are shown in Figure 1. The best extraction efficiency was achieved when benzyl alcohol was utilized as an extraction solvent. This solvent is suitable because benzyl alcohol is a polar, protic solvent with relatively high dielectric constant which can easily elute highly polar MDA. Thus, in the present study this solvent was used for all extractions.

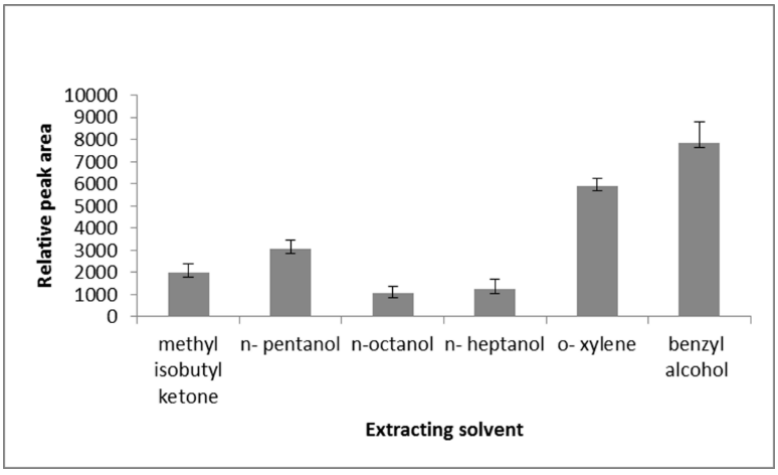

Figure 1. Effect of dispersive solvent on the extraction efficiency. Conditions: extraction solvent volume, 150 µL; dispersive solvent, acetic acid; dispersive solvent volume, 450 µL; vortex time, 60 s; centrifuge time, 4 min; sample volume, 4 mL; pH, 3.

Type of the dispersive solvent

Methanol, ethanol, acetone, acetonitrile, and acetic acid were selected and examined as dispersive solvent. Also, extraction without the dispersive solvent was evaluated. From the five dispersive solvents tested, the acetic acid showed the best chromatographic behavior; also the highest efficiency was achieved with this solvent. Therefore, subsequent experiments were accomplished by using it. In vortex assisted DLLME, micro volumes of a low-density organic solvent are dispersed into an aqueous sample using vortex mixing, a mild emulsification procedure. The well micro droplets formed ensure fast partitioning rates, i.e. short equilibration times, due to the shorter diffusion distance and larger specific surface area.

Response surface methodology for optimization of the extraction of MDA

Response surface methodology (RSM) is an affordable and reliable technique to optimizing certain processes. This method leads to a reduction of designed experiments to study the effect of operation factors. To study the parameters affecting the pre-concentration of MDA, the seven factors in five-levels were used for design of experiments (DOE) (Table 1). The input variables were benzyl alcohol volume (A) (50- 200 µL), acetic acid volume (B) (150-450), vortex time (C) (20- 100 s), centrifugation time (D) (2- 10 min), volume of sample solution (E) (3- 7 mL), amounts of NaCl (F) (10- 40 g L^{-1}) and pH (G) (2-6). The factor levels were coded as -2 (-α, low), -1, 0 (central point), +1, +2 (+α, high).

Table 1. Design- Expert parameters and experimental.

Factors	Level				
	-2(-α, low)	-1	0	+1	+2 (-α, high)
Extraction solvent volume (µL)	50	100	150	200	250
Dispersive solvent volume (µL)	150	250	350	450	550
Vortex time(s)	20	40	60	80	100
Centrifuge time (min)	2	4	6	8	10
Volume of sample solution (mL)	3	4	5	6	7
Concentration of NaCl (g L^{-1})	0	10	20	30	40
pH	2	3	4	5	6

Table 2 indicates the analyses of the experimental results of the second- order polynomial model for the MDA yield. The behavior of the system is described using the following quadratic equation.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_{ii} + \sum \beta_{ij} X_i X_j + e \quad (1)$$

Where Y is the natural logarithm of predicted response (Y=Ln*(Response)*), β_0 is the constant, X_1, X_2, …, X_k are the coded independent variables, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} demonstrates the coefficient of the interaction factor, e is the random error or allows for description or uncertainties between predicted and determined value [28].

Table 2. Matrix for the experiments of Design-Expert.

Number	Factor 1 (A): Extraction solvent volume (µL)	Factor 2 (B): Dispersive solvent volume (µL)	Factor 3 (C): Vortex time (s)	Factor 4 (D): Centrifuge time (min)	Factor 5 (E): Volume of sample solution (mL)	Factor 6 (F): Concentration of NaCl (g L^{-1})	Factor 7 (G): pH	Response (relative peak area)	predicted
1	200	250	80	8	4	10	3	38.1	38.0772
2	100	450	40	4	4	30	5	0.4	0.400488
3	200	450	80	8	6	30	5	8.8	8.765089
4	200	250	40	4	4	30	5	2.4	2.411551
5	100	250	40	4	6	30	3	65.7	65.70344
6	100	250	80	4	4	30	3	45.9	45.76249
7	200	250	40	8	6	10	3	48.8	48.61024
8	150	350	60	6	5	20	4	2.9	2.89312
9	150	350	20	6	5	20	4	4.1	4.098048
10	50	350	60	6	5	20	4	2.9	2.892918
11	100	450	40	8	6	30	3	40.8	40.73553
12	150	350	60	6	7	20	4	4.5	4.534434
13	200	450	40	4	6	30	5	8.4	8.365468
14	200	450	80	8	6	10	3	59.6	59.00009
15	100	450	80	4	6	10	5	8.8	8.748922
16	200	450	40	4	4	30	3	40.3	40.08068
17	200	250	80	4	6	10	3	70.9	70.28432
18	150	350	60	6	5	20	4	3.0	2.89312
19	150	350	60	6	5	20	4	2.9	2.89312
20	150	350	60	10	5	20	4	8.1	8.058268
21	200	250	40	4	6	10	5	12.4	12.37713
22	100	250	80	4	4	10	5	9.2	9.170646
23	200	250	80	8	6	30	3	51.5	51.53698
24	150	350	60	6	5	40	4	2.7	2.65607
25	150	350	60	2	5	20	4	3.2	3.200531
26	100	450	40	4	6	10	3	59.5	58.99041
27	250	350	60	6	5	20	4	4.0	3.989818
28	150	350	60	6	5	20	2	32.5	32.76319
29	150	350	60	6	5	20	4	2.85	2.89312
30	100	250	80	8	4	30	5	4.8	4.818307
31	150	350	60	6	5	20	4	2.95	2.89312
32	100	250	40	8	6	30	5	9.0	8.986247
33	150	350	60	6	5	0	4	13.2	13.35143
34	100	450	80	4	6	30	3	61.1	60.86098
35	200	450	40	4	4	10	5	7.4	7.359508
36	200	450	80	4	4	30	5	4.2	4.194015
37	200	450	40	8	4	10	3	37.2	37.17129
38	200	250	80	8	6	10	5	12.7	12.70623
39	100	450	80	4	4	10	3	51.6	51.41452
40	150	350	60	6	3	20	4	3.7	3.653607
41	100	450	80	8	4	10	5	2.5	2.48668
42	150	350	100	6	5	20	4	1.1	1.095043
43	150	550	60	6	5	20	4	3.7	3.775524
44	150	350	60	6	5	20	6	0.5	0.49346
45	100	250	80	8	6	10	3	60.5	60.14196
46	100	250	40	8	4	30	3	27	27.01786
47	100	250	40	8	4	10	5	8.1	8.09104
48	150	150	60	6	5	20	4	2.2	2.216949
49	150	350	60	6	5	20	4	2.9	2.89312
50	200	450	40	8	4	30	5	3.0	3.011632

The most commonly selected technique in the RSM method is central composite design (CCD). Design Expert software was applied for RSM regression analysis and optimization of input parameters by observed response (relative peak area). The statistical testing of the model, which inclusive linear, quadratic and interaction coefficient, was carried out with ANOVA analysis by *F*-test to achieve the empirical correlation between input and output parameters. To evaluate the goodness of fit of the model, each term of model was tested statistically which confirmed the significance of *F*- values by $p \leq 0.05$. The values of R^2, adjusted R^2 and predicted R^2, lack of fit and adequate precision of models were obtained to check the quality of the proposed polynomial. To visualize the input-output relationships, the response surface plot and contour plot were drawn.

The quadratic model in terms of actual value variables is indicated in equation (2).

$$Y= 97.38644 - 0.34173 \times A - 0.41575 B - 2.92176 C + 6.41213 D + 3.11689 E + 0.36924 F + 17.64255 \times G + 1.10425 \times 10^{-3} \times A \times B + 0.022379 \times A \times C - 0.16075 A \times D - 9.71520 \times 10^{-3} \times A \times E - 7.57030 \times 10^{-4} \times F + 0.010507 \times B \times C - 0.051758 \times B \times D + 0.020778 \times B \times E + 2.39083 \times 10^{-3} \times B \times F - 7.72269 \times 10^{-3} \times G + 0.042713 \times C \times D - 0.13840 \times C \times E + 7.78166 \times 10^{-3} - 0.069701 \times C \times G + 2.43061 \times D \times E + 0.026222 \times D \times F - 0.25383 \times D \times G - 0.21088 \times E \times F - 2.93199 \times E \times G - 0.14032 \times F \times G + 1.6067 \times 10\text{-}5 (A)^2 - 1.94802 \times 10 \text{-}4 (C)^2 + 0.035167 (D)^2 + 0.085343 (E)^2 + 1.80475 \times 10^{-3} (F)^2 - 0.82365 (G)^2 - 6.43265 \times 10^{-5} \times A \times B \times C + 4.70649 \times 10^{-4} \times A \times B \times D - 2.55848 \times 10^{-4} \times B \times C \times E + 5.21769 \times 10^{-6} \times B \times C \times F - 6.49897 \times 10^{-4} \times B \times D \times E - 1.53885 \times 10^{-4} B \times D \times F - 8.64585 \times 10^{-4} \times B \times D \times G + 4.76582 \times 10^{-3} \times B \times E \times G + 0.015099 \times C \times (G)^2 \quad (2)$$

The obtained values for the critical points are as follows: benzyl alcohol volume = 100 µL, acetic acid volume = 251 µL, vortex time = 79.85 s, centrifugation time 4.02 min, volume of sample solution= 5.98 mL, amounts of NaCl= 10 g L^{-1} and pH= 3.03.

Using the sum of squares (SS) values of the corresponding term (Eq. 3), the percent contribution (PC %) of each of the individual term in final model were computed (Table 3) [29].

$$\% PC = (SS / \sum SS) \times 100 \quad (3)$$

The results of the response surface model fitting in the form of analysis of variance (ANOVA) are showed in Table 3. The ANOVA results determine which the model was significant, as evident from the Fisher's *F* test (F_{model} = 4282.818) by a very low probability value (p_{model}= < 0.0001).

Table 3. ANOVA analysis for multiple response function.

Source	Sum of Squares	df	Mean Square	*F* Value	p-value Prob > F	(PC%)[a]
Model	90.90467	42	2.164397	4282.818	< 0.0001	-
A-Extraction solvent volume	0.051708	1	0.051708	102.3177	< 0.0001	0.047
B- dispersive solvent volume	0.153722	1	0.153722	304.1786	< 0.0001	0.14
C-Vortex time	0.865503	1	0.865503	1712.621	< 0.0001	0.79
D-Centrifuge time	0.432948	1	0.432948	856.6985	< 0.0001	0.40
E-Volume of sample solution	0.028058	1	0.028058	55.5203	0.0001	0.026
F-Concentration of NaCl	1.851616	1	1.851616	3663.9	< 0.0001	1.69
G-pH	10.55827	1	10.55827	20892.26	< 0.0001	9.67
AB	0.738218	1	0.738218	1460.755	< 0.0001	0.67
AC	0.053346	1	0.053346	105.5579	< 0.0001	0.049
AD	0.387922	1	0.387922	767.6048	< 0.0001	0.35
AE	0.566311	1	0.566311	1120.592	< 0.0001	0.52
AF	0.278881	1	0.278881	551.8389	< 0.0001	0.25
BC	1.273357	1	1.273357	2519.665	< 0.0001	1.16
BD	5.627325	1	5.627325	11135.12	< 0.0001	5.15
BE	6.693145	1	6.693145	13244.12	< 0.0001	6.13
BF	6.254488	1	6.254488	12376.12	< 0.0001	5.73
BG	3.241148	1	3.241148	6413.448	< 0.0001	2.97
CD	5.758564	1	5.758564	11394.81	< 0.0001	5.27
CE	8.135653	1	8.135653	16098.49	< 0.0001	7.45
CF	6.336956	1	6.336956	12539.31	< 0.0001	5.80
CG	2.791283	1	2.791283	5523.274	< 0.0001	2.55
DE	7.103907	1	7.103907	14056.92	< 0.0001	6.50
DF	0.969101	1	0.969101	1917.617	< 0.0001	0.89
DG	3.019493	1	3.019493	5974.848	< 0.0001	2.76
EF	6.996587	1	6.996587	13844.55	< 0.0001	6.41
EG	5.680252	1	5.680252	11239.85	< 0.0001	5.2
FG	5.32179	1	5.32179	10530.54	< 0.0001	4.87
A^2	0.041304	1	0.041304	81.73035	< 0.0001	0.038
C^2	0.155435	1	0.155435	307.568	< 0.0001	0.14
D^2	0.506565	1	0.506565	1002.37	< 0.0001	0.46
E^2	0.186457	1	0.186457	368.9525	< 0.0001	0.17
F^2	0.833828	1	0.833828	1649.944	< 0.0001	0.76
G^2	0.173411	1	0.173411	343.1386	< 0.0001	0.16
ABC	7.173609	1	7.173609	14194.84	< 0.0001	6.57
ABD	6.52802	1	6.52802	12917.37	< 0.0001	5.98
BCE	0.510086	1	0.510086	1009.337	< 0.0001	0.48
BCF	0.042987	1	0.042987	85.06027	< 0.0001	0.04
BDE	0.067618	1	0.067618	133.7991	< 0.0001	0.062
BDF	0.290729	1	0.290729	575.2831	< 0.0001	0.27
BDG	0.11967	1	0.11967	236.7988	< 0.0001	0.11
BEG	1.148444	1	1.148444	2272.492	< 0.0001	1.05
CG^2	0.337441	1	0.337441	667.7148	< 0.0001	0.31
Residual	0.003538	7	0.000505			
Lack of Fit	0.001982	2	0.000991	3.18449	0.1283	
Pure Error	0.001556	5	0.000311			

Design- Expert software generated two dimensional response surface plots indicated in Figure 2. With the correlation coefficient (R^2) the goodness of the model was checked. The adjusted R^2 value of 0.999728 indicated which only 0.0272 % of the total variation was not described using the model. Hence, the graph in Figure 3 shows a good correlation the value of between the experimental and predicted data of the response (R^2 =0.999). The lack-of-fit determine the failure of the model to represent value in the experimental domain at point that is not included in the regression [29]. The not significant data of lack-of-fit (>0.05) revealed which the quadratic model is statistically significant for the response.

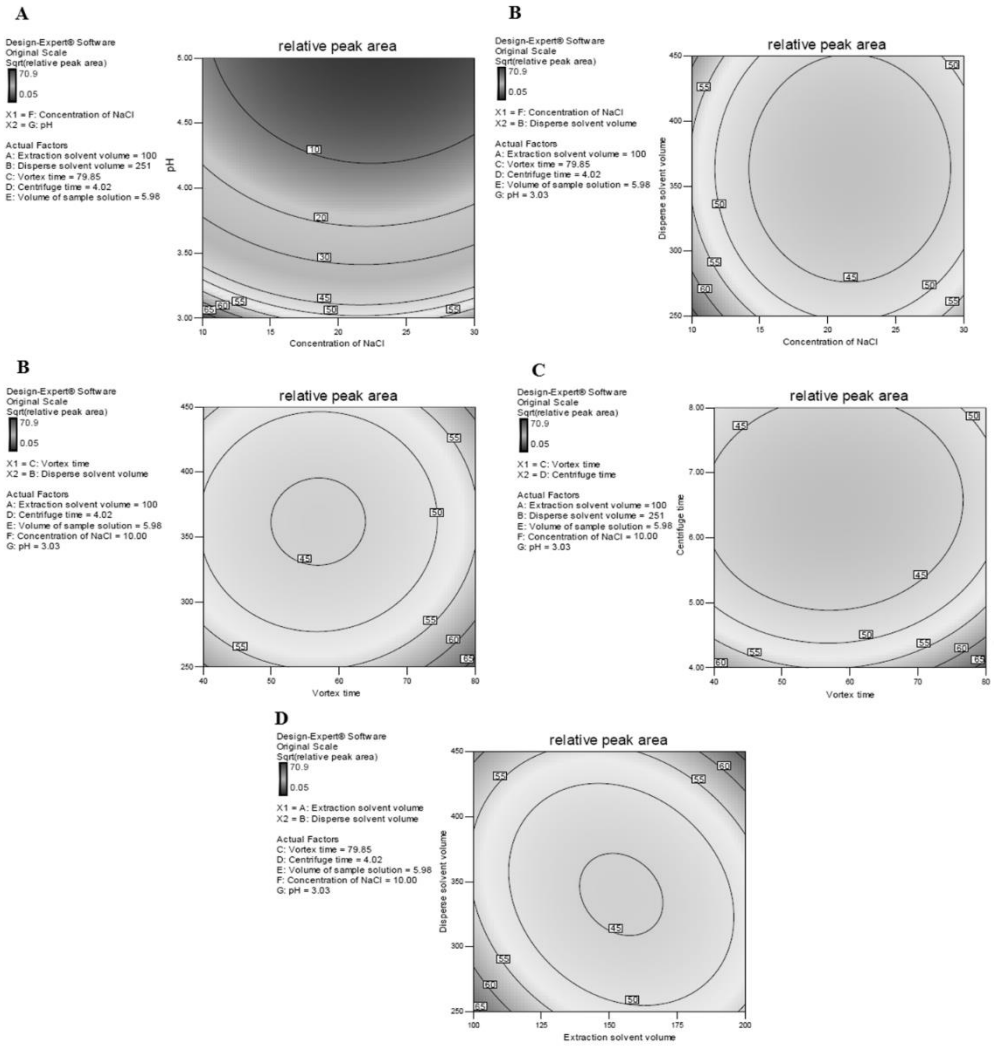

Figure 2. Response surface-2D/ contours showing the effect of independent variable on the relative peak area of MDA.

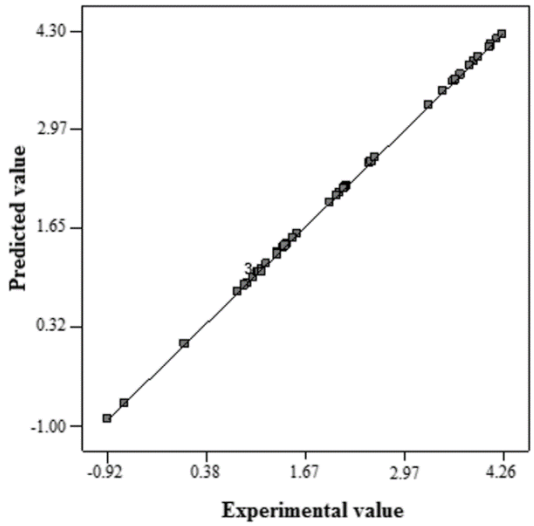

Figure 3. Correlation plots between predicted and experimental values.

Analytical performance for determination of MDA by VADLLME

Linear range, limit of detection and precision

Under the optimized conditions, the linearity of the VADLLME method was examined by extracting the aqueous MDA samples. The calibration curve was linear for the concentrations ranging 10 to 1150 μg L^{-1}. The calibration equation was Y = 0.001C + 0.072 with a correlation coefficient of 0.998, where Y is the ratio of the area of MDA peak to the internal standard peak in the chromatogram, and C is the concentration of MDA in the sample solution (μg L^{-1}). The limits of detection and quantification of the VADLLME method defined as $3S_b$ m^{-1} and $10S_b$ m^{-1} (where S_b is the standard deviation of ten times extraction and measuring the blank and m is the slope of the calibration graph) were found to be 0.798 and 2.66 μg L^{-1}, respectively. The relative standard deviation (RSD) for ten replicate measurements of 5 and 750 μg L^{-1} of MDA was found to be 7.65% and 5.48%, respectively. An enrichment factor of 175 was obtained when analysis of a MDA standard solution with a concentration of 1.0 mg L^{-1} was performed by the proposed method and compared to the direct injection of 1.0 μL of the same standard to GC [30]. The analytical figures of merit for the proposed method obtained under optimal conditions are summarized in Table 4.

Table 4. Analytical figures of merit for VADLLME extraction of MDA.

Parameter	Analytical feature
Equation of calibration curve	S = 0.001CMDA + 0.072
Dynamic range (μg L^{-1})	10 - 1150
R^2 (determination coefficient)	0.998
Repeatability (RSD* %, n =10, 5 μg L^{-1})	7.65
Repeatability (RSD %, n =10, 750 μg L^{-1})	5.48
Limit of detection (μg L^{-1})	0.798
Limit of Quantification (μg L^{-1})	2.66
Enrichment factor (fold)	175

*RSD, relative standard deviation.

Analysis of human blood plasma

To evaluate the applicability of the VADLLME-GC method, it was applied to the analysis of blood plasma sample of humans without any pretreatment. In the first step, 250 μL of blood plasma was transferred into 100 μL of 6 M NaOH, and then heated in a hot water bath at 60 °C for 30 minutes. After cooling to 2 °C, 500 μL of 20% w/v trichloroacetic acid was added. The mixture was vortexed for 1 min and the sample was left for 10 min at ambient temperature for completion of the reaction. The mixture was then centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting deproteinized supernatant solution was transferred into a 6 mL vial and diluted to volume with ultrapure water and then analyzed [31], in which MDA was detected at the concentration of 284.7 μg L^{-1} (RSD=6.0%, n=3). The GC chromatogram obtained from VADLLME for MDA extraction without sample spiking is depicted in Figure 4. For validation of this analysis, the most common method of MDA analysis, which is spectrophotometry [31, 32] was used. This gave the MDA concentration 297.3 μg L^{-1}. As can be seen, the measured value is in good agreement with the standard method of MDA analysis at 95% confidence interval.

In order to investigate the validity of the proposed method for different concentrations, blood plasma samples were also spiked at three levels of 5, 25 and 50 μg L^{-1} with MDA. All the steps mentioned above were performed to remove proteins. Results are presented in Table 5. As can be seen, good recoveries between 95.8% to 106.0% were achieved which indicate that different levels of MDA can be measured successfully by using VADLLME-GC method. Relative standard deviation (n=3) better than 7.15% was obtained.

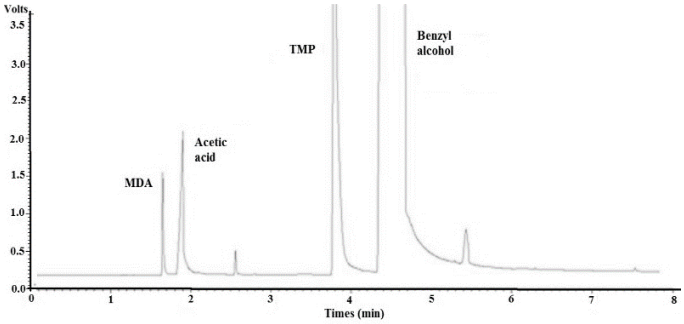

Figure 4. GC chromatogram obtained from VADLLME extraction. Extraction conditions: extraction solvent, benzyl alcohol; extraction solvent volume, 150 μL; dispersive solvent, acetic acid; dispersive solvent volume, 450 μL; vortex time, 60 s; centrifuge time, 4 min; sample volume, 6 mL; pH, 3; MDA = malondialdehyde; TMP = 1,1,3,3-tetramethoxypropane (internal standard).

Table 5. Analysis of MDA in a spiked plasma sample by the VADLLME extraction.

MDA added (μg L^{-1})	MDA found (μg L^{-1})	Recovery (%)	RSD (%), n=3
- 5	284.7 290.0	- 106.0	6.05 7.15
25	309.1	97.6	5.87
50	332.6	95.8	6.70

Comparison of the suggested method with related techniques

Table 6 compares the characteristic data of the present method with those using gas chromatography for determination of MDA, reported in the literature recently. The limit of detection and enrichment factor obtained by the present method in most cases is in the same order of magnitude of the other methods, while it has the advantages such as a higher accuracy, wider linear dynamic range and faster analysis time. In addition, simplicity of operation, low cost and low sample volume are some other advantages of the proposed methods which can be used for preconcentration of malondialdehyde in human serum samples.

Table 6. Comparison of the proposed method with other reported methods involving GC detection for determination of MDA.

Sample	Extraction Technique	Detection Technique	EF	LOD (μg L^{-1})	Linear Range (μg L^{-1})	Repeatability (RSD, %)	Estimated Analysis Time (min)	Ref.
Human Plasma	HS-SPME	GC/MS	NM	0.4	5 - 100	<8%	~50	[14]
Complex Lipid Matrix	HS-SPME*	GC	NM	0.742	NM***	NM	NM	[15]
Human Plasma	SS-SDME**	GC	204.2	0.760	10 - 1000	8.37	≤16	[30]
Human Plasma	VADLLME	GC	175	0.798	10 - 1150	5.48	≤13	This work

* Headspace solid phase microextraction.

** Salt saturated single-drop microextraction.

*** Not mentioned.

CONCLUSION

In the present study, a rapid and efficient analytical method based on vortex-assisted dispersive liquid-liquid microextraction was studied and optimized for the determination of trace amounts of malondialdehyde in human serum plasma using one-variable-at-a-time and response surface methodology. For optimization of the malondialdehyde pre-concentration, seven factors in five-levels were employed for design of experiments. The results showed that the proposed method exhibits good linearity, precision, enrichment factor and detection limit for the extraction of this analyte. This method is fast, simple, sensitive, and inexpensive and allows sample extraction and pre-concentration to be done in a single step. This technique is environmentally friendly, since only less than a mililiter of organic solvent is required for extraction. More than 170 fold pre-concentration makes this method of choice for trace analysis of MDA and potentially other similar compounds without the need for a derivatization step. The total analysis time is 13 min.

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