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Synthesis of short-chain hydroxyaldehydes and their 2,4-dinitrophenylhydrazone derivatives, and separation of their isomers by high-performance liquid chromatography

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ABSTRACT

An easy to handle high-performance liquid chromatography (HPLC) method for the separation of structural isomers of short-chain aldehydes as their hydrazones is presented. Some aldehydes were not available as reference compounds, therefore, synthesis routes for these hydroxy-aldehydes and their dinitrophenylhydrazone derivatives are reported. The reported method has a detection limit of 2.4–16.1 μg/L for the hydrazones and shows good linearity and reproducibility for various tested aldehydes.

1. Introduction

Aldehydes and ketones of low molecular weight are quite volatile and reactive molecules which tend to form oligomers by spontaneous reactions, i.e. aldol addition/condensation. This tendency, together with further degradation by oxidation makes their quantitative determination in biological samples rather difficult. Classically, aldehydes are identified after transformation to their hydrazones by derivatization with 2,4-dinitrophenylhydrazine (DNPH). These are stable, non-volatile and often crystalline solids with a sharp melting point. Aldehydes and their DNPH derivatives can be determined by gas chromatography [1,2] or by HPLC on standard C_{18}-phases, commonly using water (or buffer)/acetonitrile (MeCN) mixtures as eluent (in some cases with additional methanol, isopropanol and/or tetrahydofurane) [3–7]. Unfortunately, these methods do not allow separation of structural isomers such as butyraldehyde DNPH and isobutyraldehyde DNPH or their hydroxy analogues [3–7]. In a non-aqueous system, separation of butyraldehyde and isobutyraldehyde was achieved with hexane as an eluent, but this protocol is not applicable to biological samples with their variable water content [8]. As possible intermediates of acetone degradation by sulfate-reducing bacteria, the determination of 2-hydroxyisobutyraldehyde, 3-hydroxybutyraldehyde and acetoacetaldehyde turned out to be essential for the elucidation of a proposed biochemical pathway [9,10]. No method for the analytical separation and quantification of these hydroxyaldehydes has been described in the literature. The same holds true for their non-hydroxylated congeners butyraldehyde and isobutyraldehyde.

In the present work, we describe the synthesis and characterization of 2-hydroxyisobutyraldehyde, 3-hydroxybutyraldehyde, and acetoacetaldehyde and their DNPH derivatives as authentic samples for the intended validation of the proposed biodegradation pathway of acetone. Furthermore, an easy to handle HPLC-method for separation and detection of butyraldehyde, isobutyraldehyde, 2-hydroxyisobutyraldehyde, 3-hydroxybutyraldehyde, and acetoacetaldehyde (in the form of its corresponding [1H]-pyrazole) as DNPH derivatives was developed. We demonstrate that this method is applicable also to the separation of other hydrazones (e.g. for formaldehyde, acetaldehyde, acetoin, acetone, crotonaldehyde, and other short-chain aldehydes/ketones). In the literature, no method is described that allows a separation of short-chain aldehyde isomers (e.g. 3-hydroxybutyraldehyde and 2-hydroxyisobutyraldehyde) as their hydrazones. The method described here provides a solution to this problem.

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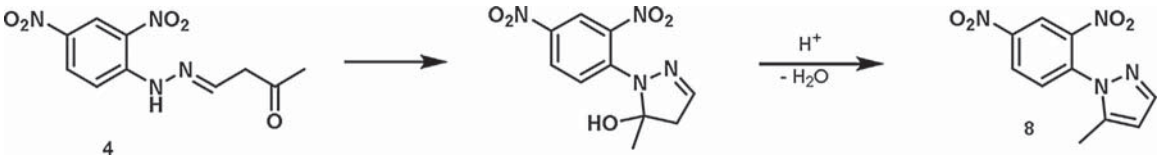

Scheme 1. Decomposition of (*E*)-4-(2-(2,4-dinitrophenyl)hydrazono)butan-2-one (**4**) to form pyrazole **8.**

2. Methods/experimental section

2.1. Reagents

For HPLC and sample preparation, ultrapure water was obtained using a Milli-Q Water System (Millipore). MeCN was HPLC grade (HiPerSolv CHROMANORM) from VWR Chemicals (Germany). For the *in situ* derivatization a mix consisting of 5 mg DNPH and 0.05% conc. H_3PO_4 (v/v) in 10 mL MeCN was employed.

All chemicals of analytical grade or better were purchased from Sigma-Aldrich (Germany), DNPH standards were purchased as certified reference materials solution in MeCN from Aldrich (formaldehyde ([order code: CRM47177), acetaldehyde (CRM47340), acrolein (CRM47342), acetone (CRM47341), crotonaldehyde (CRM47175), butyraldehyde (CRM47345) and isobutyraldehyde (CRM47886)).

2.2. HPLC system

A Shimadzu Prominence HPLC system equipped with a photodiode array (PDA) detector (SPD-M20A) and a Kinetex® PFP column (5 μm, 100 Å, 250 × 4.6 mm; [order code: 00G-4602-E0] Phenomenex, USA) was used. A binary gradient system was used for separation consisting of 50% H_2O (MilliQ water) and 50% MeCN (v/v) kept isocratic during the first 19 min of the run, followed by a rapid increase to 100% MeCN during minutes 19–20 and kept at 100% MeCN for one minute. A reversion during minutes 21–22 back to initial concentrations of 50/50 (H_2O/MeCN) was set before finally re-equilibrating the column for 8 min. The flow rate was 1 mL per min and the injection volume was 5 μL. The temperature was set to 40 °C (column oven and flow cell). The wavelength for detection was 360 nm with 550 nm set as reference wavelength.

2.3. Synthesis of aldehydes and hydrazones

2.3.1. General remarks

NMR spectra were measured on Bruker Avance 400 and Bruker Avance DRX 600 spectrometers. Chemical shifts are referenced with respect to the chemical shift of the residual protons present in the deuterated solvents. The following reference values were used: $CDCl_3$: δ = 7.26 ppm (^{1}H NMR), δ = 77.16 ppm (^{13}C NMR); d_6-DMSO: δ = 2.50 ppm (^{1}H NMR), δ = 39.51 ppm (^{13}C NMR); d_8-THF: δ = 1.73, 3.58 ppm (^{1}H NMR), δ = 25.37, 67.57 ppm (^{13}C NMR). Data were processed using the software MestReNova (v. 10.0). The following abbreviations were used for NMR data: s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet. Structure assignments were done based on 2D-NMR (COSY, HMBC, HSQC) experiments.

2.3.2. 4,4-Dimethoxybutan-2-ol (1)

4,4-Dimethoxybutan-2-one (5.00 g, 37.8 mmol) was dissolved in dichloromethane (DCM) (100 mL) and $NaBH_4$ (1.43 g, 37.8 mmol) in methanol (MeOH) (20 mL) was added in portions of ~3 mL each. The reaction mixture was stirred at 20–23 °C for 3 h. Water (100 mL) was added, phases were separated and the aqueous phase was extracted with DCM (2 × 50 mL). The combined organic phases were washed with saturated aqueous NaCl brine (50 mL), dried over $MgSO_4$, and the solvent was evaporated. **1** was obtained as colorless oil (4.29 g, 32.00 mmol, 84%).

^{1}H NMR (400 MHz, Chloroform-d) δ 4.57 (dd, J = 6.0, 5.1 Hz, 1H, $C(OCH_3)_2$), 4.13 – 3.87 (m, 1H, COH), 3.38 (s, 3H, OCH_3), 3.35 (s, 3H, OCH_3), 1.91 – 1.60 (m, 2H, CH_2), 1.20 (d, *J* = 6.3 Hz, 3H, CH_3).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 104.1 ($\underline{C}(OCH_3)_2$), 64.8 (COH), 53.9 (OCH_3), 53.0 (OCH_3), 41.1 (CH_2), 23.5 (CH_3).

2.3.3. (E)-1-(2-(2,4-Dinitrophenyl)hydrazono)-2-methylpropan-2-ol (2)

1,1-Dimethoxy-2-methylpropan-2-ol (1.00 g, 7.46 mmol) was dissolved in MeCN (200 mL), then H_2SO_4 (1.0 g, 10.2 mmol) and water (750 mg, 38.9 mmol) were added and the mixture stirred at rt for 3 h. Na_2SO_4 (5 g) and (2,4-dinitrophenyl)hydrazine (stabilized with 50 wt% water, 2.95 g, 7.46 mmol) were added and the mixture was stirred for 3 h, then adjusted to pH 7.0 using solid $KHCO_3$ and filtered. The filtrate was evaporated, the product was isolated by column chromatography (DCM, then DCM:MeOH 20:1) and recrystallized from ethanol to obtain **2** as red needle-shaped crystals (327 mg, 1.22 mmol, 16%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.31 (s, 1H, NH), 8.84 (d, *J* = 2.7 Hz, 1H, H_{ar}), 8.35 (dd, *J* = 9.6, 2.7 Hz, 1H, H_{ar}), 8.01 (s, 1H, N=CH), 7.90 (d, *J* = 9.6 Hz, 1H, H_{ar}), 5.15 (s, 1H, OH), 1.33 (s, 6H, CH_3).

^{13}C NMR (101 MHz, DMSO-d_6) δ 158.8 (C=N), 145.0 (C_{ar}), 136.7 (C_{ar}), 129.7 (C_{ar}), 129.1 (C_{ar}), 122.9 (C_{ar}), 116.4 (C_{ar}), 69.7 (COH), 27.4 (CH_3).

2.3.4. (E)-4-(2-(2,4-Dinitrophenyl)hydrazono)butan-2-ol (3)

The compound was prepared following the same procedure as for (*E*)-1-(2-(2,4- dinitrophenyl)hydrazono)-2-methylpropan-2-ol **2** using 4,4-dimethoxybutan-2-ol (**1)** (500 mg, 3.73 mmol), H_2SO_4 (500 mg, 5.10 mmol), water (350 mg, 19.43 mmol), (2,4-dinitrophenyl)hydrazine (stabilized with 50 wt% water, 1.48 g, 3.73 mmol) and anhydrous Na_2SO_4 (2.5 g). The product was obtained in form of yellow crystals (220 mg, 0.82 mmol, 22%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.34 (s, 1H, NH), 8.83 (d, *J* = 2.7 Hz, 1H, H_{ar}), 8.32(dd, *J* = 9.7, 2.7 Hz, 1H, H_{ar}), 8.02 (t, *J* = 5.9 Hz, 1H, N=CH), 7.85 (d, J = 9.7 Hz, 1H, H_{ar}), 4.77 (d, J = 4.7 Hz, 1H, OH), 4.03 – 3.79 (m, 1H, $\underline{H}$COH), 2.41 (t, J = 5.9 Hz, 2H, CH_2), 1.15 (d, J = 6.2 Hz, 2H, CH_3).

^{13}C NMR (101 MHz, DMSO-d_6) δ 153.6 (N=CH), 144.7 (C_{ar}), 136.5 (C_{ar}), 129.7 (C_{ar}), 128.6 (C_{ar}), 123.0 (C_{ar}), 116.3 (C_{ar}), 64.3 (COH), 42.0 (CH_2), 23.4 (CH_3).

2.3.5. (E)-4-(2-(2,4-Dinitrophenyl)hydrazono)butan-2-one (4)

4,4-Dimethoxybutan-2-one (985 mg, 7.46 mmol) was dissolved in MeCN (200 mL) and H_2SO_4 (1.00 g, 10.20 mmol) and water (38.9 mmol) were added and stirred at rt for 2.5 h. The pH was adjusted to 5.0 by addition of $KHCO_3$, then Na_2SO_4 (5 g) and (2,4-dinitrophenyl)hydrazine (stabilized with 50 wt% water, 2.95 g, 7.46 mmol) were added and the mixture was stirred for 1 h, then neutralized with $KHCO_3$ (pH = 7.0), filtered and evaporated. An analytically pure sample (30 mg, 0.11 mmol) was obtained by semi-preparative HPLC (Acetonitrile).

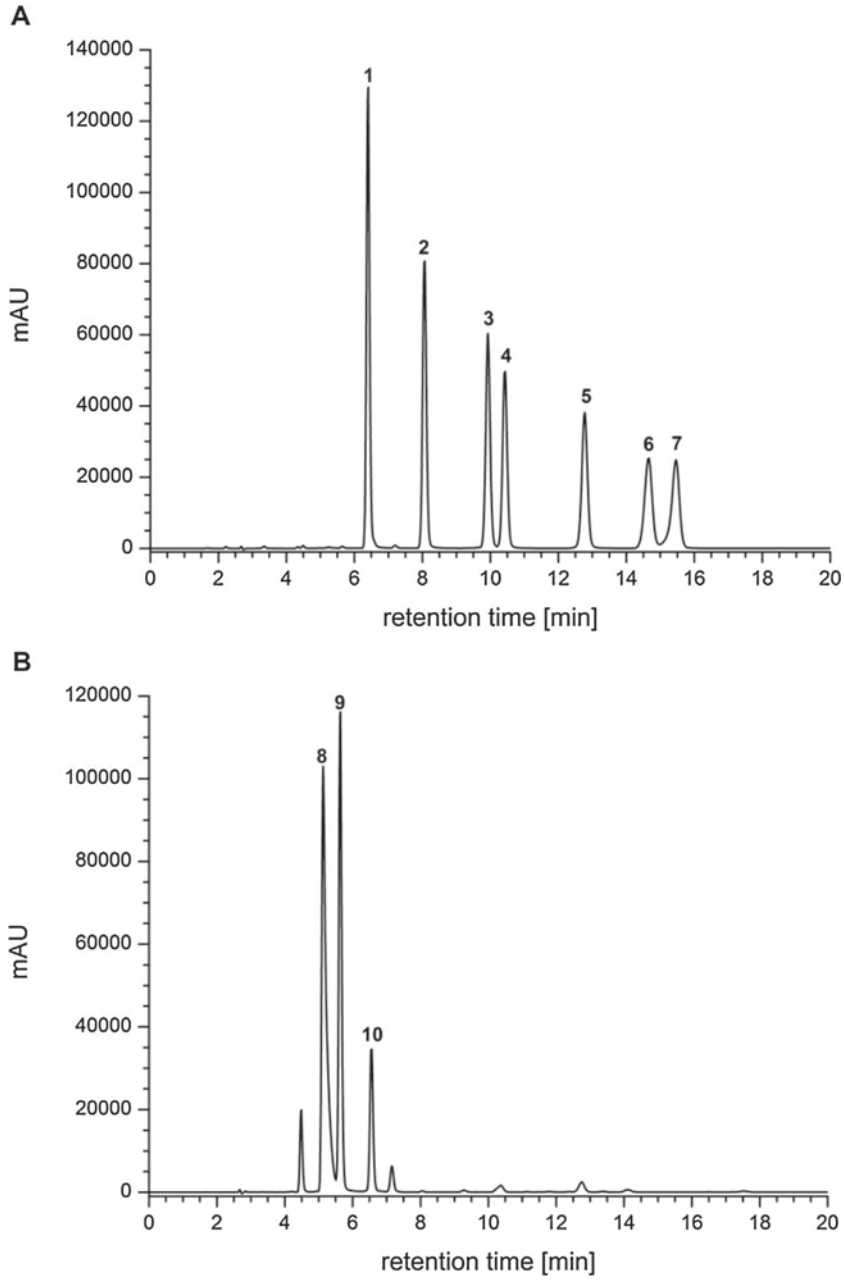

Fig. 1. Chromatograms of DNPH mixtures at 360 nm. A) aldehydes and ketones (**mix 1**) and B) hydroxy-aldehydes and pyrazole (**mix 2**); **1**: Formaldehyde-DNPH (t_R: 6.40 min), **2**: Acetaldehyde-DNPH (t_R: 8.06 min), **3**: Acrolein-DNPH (t_R: 9.93 min), **4**: Acetone-DNPH (t_R: 10.43 min), **5**: Crotonaldehyde-DNPH (t_R: 12.77 min), **6**: Butyraldehyde-DNPH (t_R: 14.65 min), **7**: Isobutyraldehyde-DNPH (t_R: 15.46 min), **8**: 3-Hydroxybutyraldehyde-DNPH (t_R: 5.13 min), **9**: 2-Hydroxyisobutyraldehyde-DNPH (t_R: 5.63 min), **10**: Acetoacetaldehyde-DNPH (pyrazole) (t_R: 6.55 min).

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.46 (s, 1H, NH), 8.83 (d, J = 2.7 Hz, 1H, CH_{ar}), 8.34 (dd, J = 9.6, 2.7 Hz, 1H, CH_{ar}), 8.08 (t, J = 5.7, 1H, N=CH), 7.83 (d, J = 9.6 Hz, 1H, CH_{ar}), 3.62 (d, J = 5.7 Hz, 2H, CH_2), 2.21 (s, 3H, CH_3).

Remarks. Isolation of (*E*)-4-(2-(2,4-dinitrophenyl)hydrazono)butan-2-one (4) was found difficult; **4** degrades on silica gel, mostly by formation of 1-(2,4-dinitrophenyl)-5-methyl-1H-pyrazole (Scheme 1). An attempted recrystallization failed due to the compound's sensitivity to increased temperatures (>40 °C).

Compound **4** dissolved in d_6-DMSO (neutral, water free conditions) was found to decompose slowly with $t_{1/2}$ = 6 h (based on ^{1}H NMR).

2.3.6. Acetoacetaldehyde (3-Oxobutyraldehyde) (5)

4,4-Dimethoxybutan-2-one (3.00 g, 22.70 mmol) was dissolved in water (100 mL) and conc. HCl (10 mL) was added. The mixture was stirred for 20 min, then extracted with DCM (3 × 30 mL). The combined organic phases were dried over $MgSO_4$ and the solvent was evaporated. The product was isolated by evaporation directly from the crude at rt into a cold trap kept at −196 °C and subsequently stored at −80 °C. The product is a colorless oil at −20 °C (712 mg, 8.27 mmol, 36%). Its NMR shows signals of both, keto and enol-form in a ratio of 1:6 at 20 °C in chloroform-*d*.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.82 (s, 1H, CHO (ketone)), 7.89 (d, J = 4.2 Hz, 1H, CHO (enol)), 5.54 (d, J = 4.2 Hz, 1H, C=CH (enol)), 3.54 (d, J = 2.3 Hz, 2H, CH_2 (ketone)), 2.26 (s, 3H, CH_3 (ketone)), 2.11 (s, 3H, CH_3 (enol)).

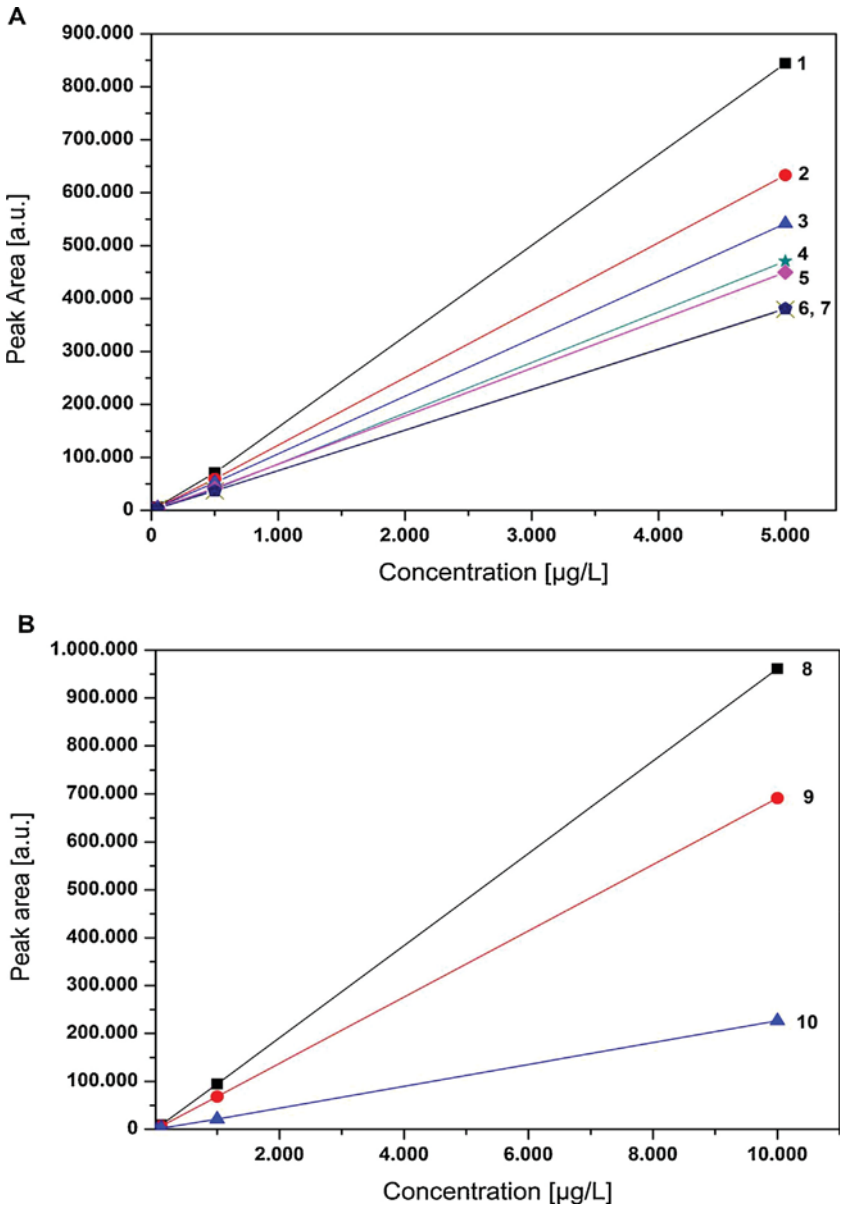

Fig. 2. Linearity plot of DNPH mixtures at 360 nm. A) Aldehydes and ketones (**mix 1**) at 10, 50, 500 and 5000 µg/L. B) Hydroxy-aldehydes and pyrazole (**mix 2**) at 10, 100, 1000 and 10000 µg/L. Enlarged representations of both graph showing the lower concentration regime are to be found in the SI. **1**: Formaldehyde-DNPH ($y = 168.56x$; $R^2 = 0.9997$), **2**: Acetaldehyde-DNPH ($y = 126.58x$; $R^2 = 1$), **3**: Acrolein-DNPH ($y = 108.33x$; $R^2 = 1$), **4**: Acetone-DNPH ($y = 93.992x$; $R^2 = 0.9997$), **5**: Crotonaldehyde-DNPH ($y = 89.841$; $R^2 = 0.9999$), **6**: Butyraldehyde-DNPH ($y = 75.983x$; $R^2 = 1$), **7**: Isobutyraldehyde-DNPH ($y = 76.239x$; $R^2 = 1$), **8**: 3-Hydroxybutyraldehyde-DNPH ($y = 96.108x$; $R^2 = 1$), **9**: 2-Hydroxyisobutyraldehyde-DNPH ($y = 69.131x$; $R^2 = 1$), **10**: Acetoacetaldehyde-DNPH (pyrazole) ($y = 22.624x$; $R^2 = 0.9999$).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 196.9 (C=O (enol)), 175.6 (HOC=C (enol)), 102.5 (HOC=C (enol)), 26.4 (CH_3 (enol)); Signals of the keto-tautomer are omitted because of low signal-to-noise ratio.

2.3.7. 2-Hydroxyisobutyraldehyde (2-Hydroxy-2-methylpropanal) (6)

Following the method reported in the literature [13], 1,1-dimethoxy-2-methylpropan-2-ol (537 mg, 4.00 mmol) was dissolved in deionized water (40 mL) and acidic ion exchange resin (Merck Ion exchanger type I) was added. The suspension was stirred at rt for 5 h, then the resin was filtered off. The solution was concentrated at reduced pressure at 40 °C to remove methanol. Finally, the concentration of the product in aqueous solution was adjusted to 100 mM by addition of deionized water.

^{1}H NMR (400 MHz, Deuterium oxide) δ 9.58 (s, 1H, CHO), 1.38 (s, 6H, CH_3).

2.3.8. 3-Hydroxybutyraldehyde (7)

The compound was prepared following the same procedure as for 2-hydroxyisobutyraldehyde (**6**) using 4,4-dimethoxybutan-2-ol **1** (537 mg, 4 mmol) in deionized water (40 mL) and a reaction time of 26 h.

^{1}H NMR (400 MHz, Deuterium Oxide) δ 9.75 (t, $J = 2.2$ Hz, 1H, CHO), 4.49 – 4.39 (m, 1H, CH(OH)), 2.79 – 2.65 (m, 2H, CH_2), 1.30 (d, $J = 6.2$ Hz, 3H, CH_3).

Stock solutions of **6** and **7** were stored at −80 °C and used for all subsequent experiments.

2.3.9. 1-(2,4-Dinitrophenyl)-5-methyl-1H-pyrazole (8)

2,4-Dinitrophenylhydrazine (stabilized with 50 wt% water, 1.50 g, 3.78 mmol) was suspended in ethanol (10 mL) and H_2SO_4 (2 mL) was added until DNPH was completely dissolved. 4,4-Dimethoxybutan-2-one (500 mg, 3.78 mmol) in ethanol (12 mL)

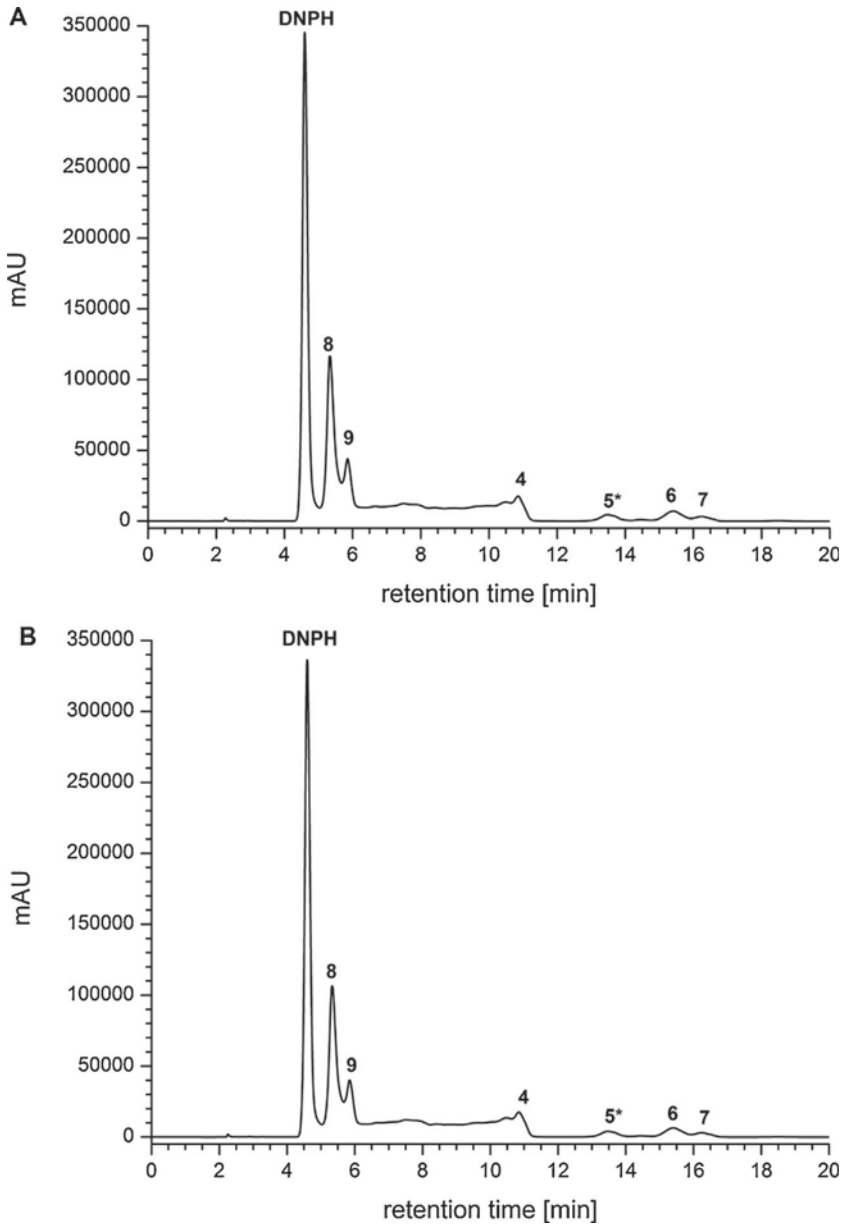

Fig. 3. Chromatograms of **sample A** and **sample B** at 360 nm after DNPH derivatization at rt for 30 min **Sample A** (equimolar mixture of: acetone, butyraldehyde, isobutyraldehyde, hydroxybutyraldehyde and 2-hydroxyisobutyraldehyde) in 20 mM Tris/HCl at pH 7.5; **sample B** contained additionally 56 μg protein from bacterial cell-free extract. Formation of crotonaldehyde-DNPH (**5***) are due to minor elimination of 3-hydroxbutyraldehyde (**8**).

was added and the mixture was stirred at rt for 45 min. The reaction was quenched with $NaHCO_3$, and water (50 mL) and DCM (50 mL) were added. The phases were separated, the water phase was extracted with DCM (3×30 mL) and the combined organic phases were dried over $MgSO_4$. The solvent was evaporated, the product was isolated by column chromatography (gradient PE:EE 10:1–5:1) in form of a yellow solid (505 mg, 2.03 mmol, 54%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.64 (d, *J* = 2.5 Hz, 1H, H_{phenyl}), 8.47 (dd, *J* = 8.9, 2.5 Hz, 1H, H_{phenyl}), 7.83 (d, *J* = 8.9 Hz, 1H, H_{phenyl}), 7.66 (d, *J* = 2.6 Hz, 1H, H_{pyr}), 6.37 (d, *J* = 2.6 Hz, 1H, H_{pyr}), 2.34 (s, 3H, CH_3).

^{13}C NMR (101 MHz, Chloroform-*d*) δ 153.97 (C_{ar}), 137.40 (C_{ar}), 130.28 (C_{ar}), 127.49 (C_ar), 125.41 (C_{ar}), 121.23 (C_{ar}), 110.60 (C_{ar}), 13.76 (CH_3).

2.4. Preparation of standards and samples

Commercial standards of DNPH derivatives were acquired as solutions in MeCN. The initial concentration of standards was 100 μg/mL aldehyde (formaldehyde, crotonaldehyde and isobutyraldehyde) and 1000 μg/mL aldehyde/ketone (acetaldehyde, acrolein, acetone, and butyraldehyde). Commercial standards with a concentration of 1000 μg/mL were diluted with MeCN to a uniform concentration of 100 μg/mL. From these 100 μL each were joint and filled up with MeCN to a total volume of 1 mL to give **mix 1** with a final concentration of 10 μg/mL. 0.5 mL of this standard was diluted with water to give 1 mL solution of **mix 1** in 50% MeCN/H_2O and a concentration of 5 μg/mL. The consecutive serial dilution was done using 50% MeCN/H_2O to prepare 0.5 and

0.05 μg/mL standards. The latter was finally diluted 1:5 to yield the 0.01 μg/mL standard of **mix 1**. Concentrations of 5 μg/mL; 0.5 μg/mL; 0.05 μg/mL down to 0.01 μg/mL were used for the calibration line of mix 1 (Fig. 2A and Fig. S1).

Mix 2 consisting of our hydrazones of 3-hydroxybutyraldehyde, 2-hydroxyisobutyraldehyde and acetoacetaldehyde (as pyrazole) was prepared in a similar fashion, i.e. 1000 μg/mL solutions of each hydrazone were prepared in MeCN. From these, 100 μL each were pooled and filled up to a total of 1 mL with MeCN to give the initial standard of **mix 2**. 100 μL of this standard was dilutedwith 400 μL MeCN and 500 μL water. From this 10 μg/mL solution, the lower concentrations were prepared by serial dilution with 50% MeCN/H_2O in a 1:10 fashion (i.e., 1, 0.1 and 0.01 μg/mL). Concentrations of 10 μg/mL; 1 μg/mL; 0.1 μg/mL down to 0.01 μg/mL were used for the calibration line of mix 2 (Fig. 2B and Fig. S2).

Samples in biological medium: All initial samples (sample A and B) (volume: 1 mL) contained an equimolar mixture of 3-hydroxybutyraldehyde, 2-hydroxyisobutyraldehyde, acetone, butyraldehyde and isobutyraldehyde (0.5 mM each) in Tris/HCl buffer (20 mM) at pH 7.5. **Sample B** contained additionally cell-free extract (56 μg protein) of *Desulfococcus biacutus* [10]. For derivatization, 100 μL of **sample A** or **sample B** were mixed with 100 μL of derivatization reagent each, and the derivatization was conducted at 20–23 °C for 30 min. From the supernatant a sample was taken, centrifuged, and injected into the HPLC.

3. Results and discussion

3.1. Method optimization

Initially, we used a C_{18} column (Eurospher II 100-5 C18 KNAUR, 125 × 3 mm) with variable MeCN/H_2O concentrations as eluent, but no separation of the hydrazones of butyraldehyde and isobutyraldehyde was achieved. Lateron, we used the Kinetex® PFP column (5 μm, 100 Å, 250 × 4.6 mm; Phenomenex, USA) with 60/40 MeCN/H_2O as eluent as provided by the Certificate of Analysis from SUPELCO hydrazone standards. This column allowed a separation of hydrazones of butyraldehyde and isobutyraldehyde with a peak resolution (R_S) of 1.4. Best separation of both hydrazones was achieved when changing the eluent from above 60/40 ratio to 50/50 (v/v) MeCN/H_2O raising R_s to 1.95. For the hydrazones of 3-hydroxybutyraldehyde and 2-hydroxyisobutyraldehyde, R_s was determined to be 2.6 using 50/50 (v/v) MeCN/H_2O as eluent. Consequently, these conditions were used for all subsequent samples.

3.2. Synthesis

1,1-Dimethoxybutan-3-ol (**1**) was accessible by sodium borohydride reduction of 1,1-dimethoxybutan-3-one while the two dimethyl acetals of aldehydes **5** and **6** were commercially available. Generation of the pure aldehydes from their corresponding acetals proved to be more complicated than originally anticipated. We attribute this mainly to their high reactivity, resulting in fast decomposition of the isolated compounds at room temperature. Cleavage of acetal **1** proceeds smoothly in aqueous HCl to yield acetoacetaldehyde **5**. Isolation of instable acetoacetaldehyde **5** from oligomers formed by rapid decomposition was achieved by careful vacuum transfer into a liquid-nitrogen-cooled cold trap. Acetoacetaldehyde **5** was shown to be stable for few minutes in diluted aqueous solution, whereas pure **5** decomposes rapidly when stored above −20 °C; long-term storage demands temperatures at or below -80 °C. Hydroxy(iso)butyraldehydes **6** and **7** were not stable in pure state but were shown to be fairly stable in dilute aqueous solution. Therefore, direct synthesis of these compounds in the media used to obtain aqueous stock solutions was necessary. The corresponding diethoxy acetals were hydrolyzed with the aid of acidic anion-exchange resin utilizing the easy removability of the proton source from the medium and omitting further neutralization steps that could lead to impurities such as inorganic salts.

For DNPH derivatization of ketones/aldehydes, commonly Brady's reagent is employed. However, DNPH derivatives **2** and **3** are not accessible with this method due to dehydration to the corresponding α,β-.unsaturated hydrazones. In case of compound **2,** additionally significant amounts of its corresponding ethyl ether were obtained upon treatment with Brady's reagent. Thus, a two-step method was employed: Acetals were hydrolyzed in a separate process. In a second step, DNPH derivatization was achieved with only 0.5% H_2SO_4 (compared to the 50% H_2SO_4 of the original Brady protocol) to prevent side reactions. Acetonitrile was chosen as reaction medium due to good solubility of all reagents, whereas the solubility of DNPH in EtOH is drastically reduced if lower concentrations of H_2SO_4 are employed. To shift the equilibrium and to compensate for the water binding ability of H_2SO_4, a water binding agent had to be added as evident by NMR spectra recorded from the reaction mixture. Yields after isolation are generally high, but multiple recrystallizations in favor of high purity led to diminished yields.

The hydrazone **4** of keto-aldehyde **5** is prone to form the corresponding [1H]-pyrazole **8** already at room temperature. Under reaction conditions employed in the HPLC method, the direct formation of pyrazole **8** can be expected. Consequently, the pyrazole was prepared and chosen as reference substance for the validation of the HPLC method. The hydrazone **4** as the initial product of the derivatization was obtained by quenching the hydrolysis of the acetal with solid $KHCO_3$ and adjusting the pH to 5 before addition of DNPH. Condensation of the so formed hydrazone was thus slowed down tremendously and allowed the isolation of **4**. Subsequent tests on the stability of **4** confirmed the expected instability towards reaction to the [1H]-pyrazole.

3.3. Compound identification during HPLC separation

Every compound was tested first as a single substance to determine its specific retention time (data not shown). Afterwards, a mixture of different hydrazones (**mix 1**: formaldehyde, acetaldehyde, acrolein, acetone, crotonaldehyde, butyraldehyde and isobutyraldehyde; **mix 2**: 3-hydroxybutyraldehyde, 2-hydroxyisobutyraldehyde and acetoacetaldehyde (as pyrazole)) was measured. Chromatograms of mix 1 and mix 2 are depicted in Fig. 1A and 1B. Hydrazones of hydroxyaldehydes eluted clearly earlier than hydrazones of the non-hydroxyaldehydes. Hydrazones of unsaturated aldehydes eluted slightly earlier than hydrazones of saturated compounds. Finally, hydrazones of branched aldehydes eluted later than their linear congeners. Generally, the retention time increased with increasing chain length. Measurement of the hydrazone of formaldehyde and acetoacetaldehyde as pyrazole together in one sample was not performed, as they were not expected to occur together in our samples. Calibration lines of the different hydrazones are depicted in Fig. 2AB (A = **mix 1**; B = **mix 2**) and exhibit excellent linearity ($R^2 = 0.9997 - 1$), even for higher concentrations (5 μg/mL for mix 1; 10 μg/mL for mix 2).

The described HPLC separation method was initially developed to verify our working hypothesis of biological degradation of acetone by sulfate-reducing bacteria in an enzyme assay. In the meantime, it turned out that the bacteria do not produce 2-hydroxyisobutyraldehyde, 3-hydroxybutyraldehyde or acetoacetaldehyde as anticipated by our initial working hypothesis. We thus decided to show the feasibility of our method for the detection of different aldehydes in biological samples by comparing derivatization experiments in two different setups. In both setups an equimolar mixture of four aldehydes and acetone was analyzed.

Table 1
Detection limits and quantification limits of several DNPH derivatives.

Standard	Detection limit (μg/L) (S/N = 3)	Quantification limit (μg/L) (S/N = 10)	Coefficients of calibration lines (Fig. 2AB)
Formaldehyde-DNPH **1**[a]	2.4	8.2	y = 168.56x; R^2 = 0.9997
Acetaldehyde-DNPH **2**[a]	3.3	10.9	y = 126.58x; R^2 = 1
Acrolein-DNPH **3**[a]	4.4	14.6	y = 108.33; R^2 = 1
Acetone-DNPH **4**[a]	5.8	19.4	y = 93.992x; R^2 = 0.9997
Crotonaldehyde-DNPH **5**[a]	7.1	23.6	y = 89.841; R^2 = 0.9999
Butyraldehyde-DNPH **6**[a]	10.3	34.3	y = 75.983; R^2 = 1
Isobutyraldehyde-DNPH **7**[a]	11.1	37.0	y = 76.239x; R^2 = 1
3-Hydroxybutyraldehyde-DNPH **8**[b]	5.1	16.9	y = 96.108x; R^2 = 1
2-Hydroxyisobutyraldehyde-DNPH **9**[b]	4.4	14.8	y = 69.131x; R^2 = 1
Acetoacetaldehyde-DNPH (as pyrazole) **10**[b]	16.1	53.7	y = 22.624x; R^2 = 0.9999

[a] Quantification as aldehyde or ketone.
[b] quantification as DNPH-derivative.

Sample B contained additionally protein from bacterial cell-free extract, while **sample A** did not contain any biological constituents. Fig. 3A and B show chromatograms of both derivatized samples. Except for a slightly enhanced background (Fig. 3AB; min 5–11) which stems from the used buffer system (Tris buffer in sample) and the additional signal of free DNPH-derivatization reagent, both chromatograms show the successful derivatization of the added aldehydes with almost no variation in intensity. Even though an equimolar mixture of aldehydes was employed, it becomes apparent from the chromatograms that both hydroxyaldehydes (**8**, **9**) were derivatized in higher yield compared to acetone (**4**), butyraldehyde, (**6**) and isobutyraldehyde (**7**). Of the latter two, the unbranched butyraldehyde is formed preferentially compared to the branched isomer.

Using lower reaction temperature and a low concentration of phosphoric acid instead of concentrated sulfuric acid as compared to Brady's original reagent, a derivatization under very mild conditions became possible, thus efficiently suppressing the dehydration of 3-hydroxybutyraldehyde with concomitant formation of only minor quantities of crotonaldehyde (**5*** in Fig. 3A and B). This elimination reaction becomes otherwise the main reaction at temperatures above 40 °C or employing Brady's reagent. Furthermore, the added MeCN results in a fast precipitation of proteins which are usually included in enzyme assay samples, thus facilitating the preparation of HPLC samples.

Quantification of derivatization products (hydrazones) of **sample A** revealed a broad variation in recovery rates. Only 0.143 ± 0.11 mM of 3-hydroxybutyraldehyde (0.081 ± 0.009 mM of 2-hydroxyisobutyraldehyde; 0.120 ± 0.025 mM of acetone; 0.088 ± 0.009 mM of butyraldehyde and 0.036 ± 0.003 mM of isoburyraldehyde) could be recovered from samples containing 0.5 mM of each reagent. This corresponds to recovery rates of 8–30% of the used substrates. However, even though this mild derivatization procedure is not quantitative, it allows derivatization of these hydroxy aldehydes with very little side reactions (e.g. dehydration). Moreover, we were able to derivatize and detect aldehydes (shown exemplary for 3-hydroxybutyraldehyde) down to 10 μM concentration in a sample (recovery of 2.97 ± 0.45 μM).

3.4. Detection limit, reproducibility and linearity

The developed HPLC method was analyzed for detection limit, quantification limit, reproducibility, and linearity.

Detection limit and quantification limit for the different hydrazones were calculated based on the ratio of peak height of the lowest concentration and noise of each chromatogram in the vicinity of the peak. Normalization of the signal with respect to noise to a factor of three and calculation of the corresponding concentration gave the detection limit. Normalization to a factor of 10 and calculation of the corresponding concentration resulted in the quantification limit. All standards were analyzed five times on different days. The data are collected in Table 1. The detection and quantification limits are quite low, in the concentration range of μg/L. The detection limits are comparable to those reported by Koivusalmi et al. [5].

Table 2
Retention times (t_R) with RSD% of several DNPH derivatives.

Compound	t_R	t_R RSD%
Formaldehyde-DNPH **1**	6.43	1.4
Acetaldehyde-DNPH **2**	8.09	1.4
Acrolein-DNPH **3**	9.98	1.6
Acetone-DNPH **4**	10.48	1.5
Crotonaldehyde-DNPH **5**	12.85	1.8
Butyraldehyde-DNPH **6**	14.74	1.8
Isobutyraldehyde-DNPH **7**	15.55	1.9
3-Hydroxybutyraldehyde-DNPH **8**	5.13	0.3
2-Hydroxyisobutyraldehyde-DNPH **9**	5.63	0.4
Acetoacetaldehyde-DNPH (as pyrazole) **10**	6.55	0.3

Linearity of the calibration lines (Fig. 2AB A = **mix 1**; B = **mix 2**) was excellent (R^2 = 0.9997 − 1) up to concentrations of 5 μg/mL for mix 1; or 10 μg/mL for mix 2.

The retention times were quite stable for all tested substances, showing also a high reproducibility (t_R RSD below 2%) as displayed in Table 2. All concentrations were measured with a minimum of five replicates.

4. Conclusions

This work describes the synthesis of 2-hydroxyisobutyraldehyde, 3-hydroxybutyraldehyde and 3-ketobutyraldehyde (acetoacetaldehyde), as well as the synthesis of their respective hydrazones. Furthermore, an HPLC method is described that allows proper separation of the synthesized hydrazones, as well as separation of hydrazones of other aldehydes and ketones. Of special interest is the option that structural isomers (e.g. butyraldehyde and isobutyraldehyde; 2-hydroxyisobutyraldehyde and 3-hydroxybutyraldehyde) can be separated by this method, which was not possible before.

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Authors' contributions

JF, TH and BS conceived and designed the study. FS and TH carried out the chemical syntheses. JF carried out the HPLC method and its analyses. JF wrote a first version of the manuscript, and

all authors improved it. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2017.11.046.

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