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Development of a Selective ESI-MS Derivatization Reagent: Synthesis and Optimization for the Analysis of Aldehydes in Biological Mixtures

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In LC−MS, derivatization is primarily used to improve ionization characteristics, especially for analytes that are not (efficiently) ionized by ESI or APCI such as aldehydes, sugars, and steroids. Derivatization strategies are then directed at the incorporation of a group with a permanent charge. A compound class that typically requires derivatization prior to LC−MS is the group of small aliphatic aldehydes that are, for instance, analyzed as the key biomarkers for lipid peroxidation in organisms. Here we report the development of a new tailor-made, highly sensitive, and selective derivatization agent 4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC) for the quantification of aldehydes in biological matrixes with positive ESI-MS/MS without additional extraction procedures. 4-APC possesses an aniline moiety for a fast selective reaction with aliphatic aldehydes as well as a quaternary ammonium group for improved MS sensitivity. The derivatization reaction is a convenient one-pot reaction at a mild pH (5.7) and temperature (10 °C). As a result, an in-vial derivatization can be performed before analysis with an LC−MS/MS system. All aldehydes are derivatized within 30 min to a plateau, except malondialdehyde, which requires 300 min to reach a plateau. All derivatized aldehydes are stable for at least 35 h. Linearity was established between 10 and 500 nM and the limits of detection were in the 3−33 nM range for the aldehyde derivatives. Furthermore, the chosen design of these structures allows tandem MS to be used to monitor the typical losses of 59 and 87 from aldehyde derivatives, thereby enabling screening for aldehydes. Finally, of all aldehydes, pentanal and hexanal were detected at elevated levels in pooled healthy human urine samples.

Analyte derivatization has played an important role in analysis using combined gas chromatography−mass spectrometry (GC/MS). In GC/MS, derivatization is performed to enhance the volatility of the analyte, to alter its ionization characteristics, or to influence its fragmentation behavior. In combined liquid chromatography−MS (LC−MS), however, where soft ionization techniques like electrospray (ESI) and atmospheric pressure chemical ionization (APCI) are applied, derivatization is generally not needed and avoided as much as possible. In LC−MS, derivatization is primarily used to improve ionization characteristics, especially for analytes that are not (efficiently) ionized by ESI or APCI such as aldehydes, sugars, and steroids.[1−4] Derivatization strategies are then directed at the incorporation of a group with a permanent charge (cationic groups for positive-ion mode and strong acidic functionalities for negative-ion mode) or other groups that enhance ionization (secondary or tertiary amine for positive-ion mode or aromatic nitro groups in negative-ion mode). In addition, derivatization may be directed at improving the fragmentation characteristics in tandem MS (MS/MS). One of the drawbacks of derivatization is that the reaction may need harsh conditions, which may not only derivatize the target analyte but can also affect other constituents of the complex samples to be analyzed. Such side products may effect the derivatization reaction or may require additional sample pretreatment steps or advanced chromatographic separation to be removed.

The most efficient derivatization agents directed at achieving higher ionization efficiency in ESI contain a group with a permanent charge in addition to a reactive functional group. A variety of such derivatization agents are available for different classes of analytes, such as the commercially available Girard T and P reagents, containing a quaternary ammonium and a pyridinium moiety, respectively.[5−8] Girard T and P reagents have been reported as reagents for steroids, peptides, and nucleotides. New synthesized derivatization agents for these molecules featur-

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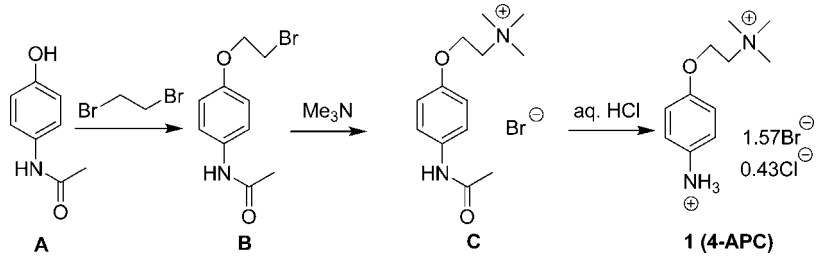
Scheme 1. Reaction Sequence for the Three-Step Synthesis of 4-APC



ing a permanent charge have been reported, providing selectivity toward specific analytes.[4,9−11] A compound class that requires derivatization prior to LC−MS analysis is the group of small aliphatic aldehydes that are, for instance, analyzed as the key biomarkers for lipid peroxidation in organisms.[12] Lipid peroxidation plays an important role in a wide variety of diseases such as heart disease, diabetes mellitus, and atherosclerosis.[13−15] As biomarkers, these aldehydes indicate the extent of free radical damage to different polyunsaturated fatty acids. They are excreted and thus analyzed in biological matrixes, for example, plasma, urine, or exhaled breath condensate. The most widely used derivatization agent for aldehydes is 2,4-dinitrophenylhydrazine (DNPH).[16−20] Alternatively, pentafluorophenyl-, 2-chloro-, and 2,4-dichlorophenylhydrazine and cyclohexanedione[21,22] are applied as derivatization reagents. These reagents were typically designed for UV−vis and fluorescence detection but are currently also used in combination with MS detection, for example, for the detection of aldehyde-DNPH derivatives in the nanomolar range by means of negative-ion APCI.[18,19] Derivatization of aldehydes is mostly based on the fast reaction of hydrazines under relatively harsh conditions, e.g., at high temperatures and in acidic medium. An additional liquid−liquid extraction step, frequently with *n*-hexane, is implemented prior to reversed-phase LC separation and MS detection. DNPH derivatization is fast, and stable derivatives are formed under acidic conditions. A drawback is that stereoisomeric hydrazone derivatives are formed,[23] which may be hydrolyzed under nonacidic conditions. Furthermore, because hydrazines not only react with aldehydes but also with ketones and small carboxylic acids, this derivatization lacks selectivity.

In this paper, we report on a novel derivatization agent, 4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC), that allows highly sensitive and selective MS detection of aldehydes. The design of the compound was based on a set of strict criteria and relies on a unique pH profile of anilines in $NaBH_3CN$-based reductive aminations. A convenient gram-scale synthesis route is given, allowing easy access to the material. Excellent quantification of aldehydes in biological matrixes with positive ESI-MS/MS can be achieved. Using 4-APC as derivatization agent, aldehydes can be detected in the low-nanomolar range. One of the key features of 4-APC, a permanent positive charge, will significantly reduce ion suppression, provide gain in ionization efficiency, and is ideally suited for structure-specific MS-MS screening strategies in biological samples.

EXPERIMENTAL SECTION

Chemicals. Pentanal, 2-pentanone, *trans*-2-pentenal, hexanal, heptanal, octanal, nonanal, decanal, cyclohexylcarboxaldehyde, 1,2-dibromoethane, trimethylamine in ethanol (4.2 M), phenethylamine, 1,1,3,3-tetramethoxypropane (TMP), hydrochloric acid, *p*-hydroxyacetanilide, tetrabutylammonium perchlorate, sodium cyanoborohydride ($NaBH_3CN$), benzoylcholine chloride, chloroform, pyridoxamine, biotin hydrazide, aniline, sodium carbonate, ammonium acetate, and methylethylketone were all purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Methanol (MeOH), formic acid were purchased from Biosolve (Valkenswaard, The Netherlands), ethanol was purchased from Mallinckrodt-Baker (Deventer, The Netherlands), and amine PEO_2 biotin was purchased from Thermo Fisher Scientific Inc. Pooled urine was obtained from five healthy human volunteers.

Synthesis of 4-(2-(trimethylammonio)ethoxy)benzenaminium Chloride Bromide (1). *General Note about the Synthesis.* The synthetic pathway is shown in Scheme 1. It should be mentioned that, in our latest efforts to further optimize the synthesis of the derivatization reagent, we found that the last step (i.e., from **C** to **1**) can be more conveniently carried out with aqueous 5 N HBr instead of aqueous 5 N HCl in an otherwise identical synthetic procedure. This protocol is higher yielding and gives 4-(2-(trimethylammonio)ethoxy)benzenaminium dibromide. As expected, in the derivatization protocols, this dibromide salt behaves identically in all respects to the current Cl,Br salt.

N-*(4-(2-Bromoethoxy)phenyl)acetamide (***B***).* A procedure adapted from Murakami et al. with minor modifications was used.[24] *p*-Hydroxyacetanilide **A** (10.0 g, 66 mmol) and 1,2-dibromoethane (57.2 mL, 124.7 g, 664 mmol) were added to saturated sodium carbonate solution (60 mL). Tetrabutylammonium perchlorate

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(0.45 g, 1.3 mmol) was added as a catalyst. The mixture was vigorously stirred for 28 h at 80 °C after which water and the excess of 1,2-dibromoethane were evaporated under high vacuum. The residue was extracted with chloroform (250 mL) to remove inorganic salts and amounts of disubstituted products. The chloroform extract was washed with a saturated sodium carbonate solution (2×). The chloroform layer was dried over $MgSO_4$, filtered, and concentrated. Recrystallization from ethanol/water yielded a white solid (5.3 g, 20.6 mmol, 31%). Spectral data were in agreement with the literature.[24] ^{1}H NMR ($CDCl_3$/drop DMSO-d_6, 200 MHz) δ 7.5 (d, 2H, C$\underline{H}$CNHAc), 6.8 (d, 2H, C$\underline{H}$COCH$_2$), 4.2 (t, 2H, OC$\underline{H}_2$), 3.6 (t, 2H, C$\underline{H}_2$Br), 2.0 (s, 3H, COC$\underline{H}_3$).

*(2-(4-Acetamidophenoxy)ethyl)trimethylammonium Bromide (***C***).* A procedure adapted from Marlow et al. with major modifications was used.[25] To prevent working with gaseous trimethylamine, a commercial solution of Me_3N in EtOH was implemented in the existing procedure. Thus, compound **B** (5.2 g, 20.2 mmol) was mixed with a solution of Me_3N in EtOH (4.2 M, 47.6 mL, 199.9 mmol). Cosolvent methylethylketone (40 mL) was added, and the mixture was stirred at room temperature for 4 days, during which time the product precipitated. It was filtered and recrystallized from hot EtOH/water to afford **C** as pinkish/beige transparent crystals (4.1 g, 12.9 mmol, 64%). ^{1}H NMR (D_2O, 200 MHz) δ 7.3 (d, 2H, C$\underline{H}$CNHAc), 7.0 (d, 2H, C$\underline{H}$COCH$_2$), 4.4 (m, 2H, OC$\underline{H}_2$), 3.7 (m, 2H, C$\underline{H}_2$NMe$_3$), 3.2 (s, 9H, NMe$_3$), 2.1 (s, 3H, COC$\underline{H}_3$). Extended splitting of some ^{1}H-signals is well-known for choline-type structures.[26]

*4-(2-(Trimethylammonio)ethoxy)benzenaminium Chloride Bromide (***1***, 4-APC).* A procedure adapted from Marlow et al. with major modifications was used.[25] Salt **C** (4.0 g, 12.6 mmol) was heated at reflux with hydrochloric acid (5 N, 40 mL) for 30 min. After cooling, the solvent was evaporated from the solution leaving a solid residue, which was recrystallized from hot EtOH/water. Filtering, washing, and drying afforded shiny beige crystals with excellent purity (1.9 g). Elemental analysis revealed that this particular crystal lattice consisted of the desired dication with 0.43 chloride counterion and 1.57 bromide counterion. Thus, for the yield calculation and throughout our work, a mass of 337.0 was used for the final product. Yield 45% (5.64 mmol, 1.9 g). The product is stable for at least several weeks (as judged from ^{1}H NMR) in aerated D_2O solution and for months as a solid at room temperature in the dark. λ_{max} (H_2O) 277 nm. ^{1}H NMR (D_2O, 200 MHz) δ 7.4 (d, 2H, C$\underline{H}$CNH$_3$), 7.1 (d, 2H, C$\underline{H}$COCH$_2$), 4.5 (m, 2H, OC$\underline{H}_2$), 3.8 (m, 2H, C$\underline{H}_2$NMe$_3$), 3.2 (s, 9H, NMe$_3$). ^{13}C NMR (D_2O, 50 MHz) δ 158.8, 125.9, 124.9, 117.4, 66.5, 63.6, 55.5. Extended splitting of some ^{1}H-signals is well-known for choline-type structures.[26] HR-MS ($C_{11}H_{19}N_2O^+$): calcd 195.1492, found 195.1497. Elem. Anal. ($C_{11}H_{20}Br_{1.57}Cl_{0.43}N_2O$): calcd (%) C 39.21, H 5.98, Br 37.23, Cl 4.52, N 8.31, O 4.75; found (%, $n = 2$) C 39.22, H 6.27, Br 37.25, Cl 4.43, N 8.38, O 4.82. LC−MS purity: >98% (MS, BPI).

Preparation of Malondialdehyde (MDA) Standard and Aldehyde Solution. TMP was used to prepare a malondialdehyde stock solution. A volume of 17 μL was dissolved in HCl 0.1 M (10 mL), and this solution was incubated at 40 °C for 60 min to hydrolyze TMP to MDA (final concentration ~10 mM). The concentration of MDA was determined by measuring its absorbance at 245 nm ($\epsilon = 31\,800$ mol L^{-1} cm^{-1}) according to Esterbauer et al.[27] Before analysis, MDA stock solution was diluted with H_2O to working solution. For each aldehyde, a stock solution of 10 mM in MeOH was prepared and stored in the freezer at −20 °C. The stock solution was diluted with H_2O to the final working mixed aldehydes solution.

Optimization of Reaction Conditions. To determine the derivatization speed and stability, the formed derivatives were measured at 1, 10, 30, 60, 120, and 600 min. Alternatively, derivatizations were left for 35 hours and measurements taken every hour. Repeatability of the derivatization was determined by performing the derivatization in triplicate and analyzing the formed derivatives. Detection limits and linearity of the selected aldehydes were determined by a calibration series ranging from 0 to 500 nM aldehydes. The calibration series were measured in buffer and in urine.

Sample Pretreatment and Derivatization. For the optimization reactions, 200 μL of 2 mg/mL biotin hydrazide, amine PEO$_2$ biotin, phenethylamine, pyridoxamine, aniline, or 4-APC in 50 mM ammonium acetate buffer pH 5.7, 50 μL of $NaBH_3CN$ (4 mg/mL in MeOH), 50 μL of 2 μM benzoylcholine chloride in H_2O as internal standard (IS) and 250 μL of mixed aldehyde standard consisting of *trans*-2-pentenal, pentanal, hexanal, cyclohexylcarboxaldehyde, heptanal, octanal, nonanal, decanal, and MDA with a final concentration of 500 nM were mixed and vortexed for 60 s. The urine samples were centrifuged at 13 600 rpm for 15 min at 10 °C before the derivatization reaction. The derivatization was carried out at 10 °C in the cooled autosampler of HP1100. After 3 h, the first sample was injected in the LC−MS/MS for analysis.

Pooled Urine Samples. Pooled urine sample of five healthy volunteers were collected and stored at −20 °C. The 250 μL of urine was mixed with 200 μL of 4-APC (2 mg/mL) in buffer pH 5.7, 50 μL of $NaBH_3CN$ (4 mg/mL) in MeOH, and 50 μL of aldehyde spike consisting of pentanal, hexanal, octanal, and decanal (final concentration 10 nM). The derivatization was performed as mentioned above.

HPLC. All HPLC separations were performed on an Agilent 1100 HPLC system (Agilent Technologies, Amstelveen, The Netherlands) controlled by Chemstation Rev B.01.09. A Waters XTerra MS reversed-phase column (C18 100 × 2.1 mm, 3 μM) at 45 °C and with a flow rate of 150 μL/min was used for the separation of the derivatized aldehydes standards and urine samples. Samples were injected (10 μL) from a thermostatic autosampler kept at 10 °C.

The gradient elution was programmed as follows: After injection, 100% mobile phase A (95% H_2O + 5% MeOH + 0.1% formic acid) was maintained for 5 min and then solvent B (5% H_2O + 95% MeOH + 0.1% formic acid) was increased from 0 to 90% in 10 min with a 5-min hold at 90% B. After this, the column was reconditioned for 10 min at 100% mobile phase A. The effluent from the LC column was directed to the mass spectrometer.

Mass Spectrometer. A Micromass (Wythenshawe, Manchester, U.K.) Q-TOF2 mass spectrometer equipped with a Micromass Z-spray ESI source was used for detection. MassLynx software (version 3.5) running under Windows NT was used for control of

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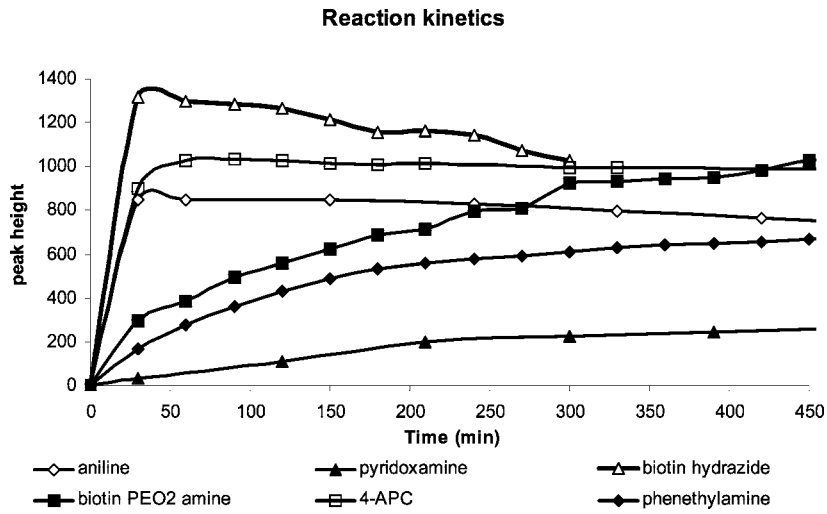

Figure 1. Time curves with derivatization speed and stability of nonanal derivatized with biotin hydrazide, aniline, pyridoxamine, biotin PEO2 amine, 4-APC, and phenethylamine.

the system and data acquisition. The time-of-flight analyzer was operated at a 20-kHz frequency with a spectrum integration time of 1 s in "full spectrum" MS in the positive-ion mode in the range m/z 150−450 ("interscan" time, 0.1 s). The ESI source conditions for the HPLC analysis were as follows: source temperature 100 °C, desolvation temperature 250 °C, and capillary voltage 2.5 kV. The sampling cone voltage was 20 V. For the MS/MS experiments, the collision energy was set on 20 V. Nitrogen (99.999% purity; Praxair, Oevel, Belgium) was used with flow rates of 20 L/h for nebulization, 50 L/h for cone gas, and 350 L/h for desolvation. Argon (99.9995% purity; Praxair) was used in the collision cell.

RESULTS AND DISCUSSION

Initial Screen for Candidate Derivatization Agents. At the onset of our project, we screened several existing derivatization agents for selective aldehyde derivatization (Figure 1). Because other biomarkers will be determined in the same reaction mixture in the near future, we selected positive-ion ESI-MS as our method of choice. Therefore, since benchmark DNPH derivatives are typically analyzed in negative-ion APCI-MS, another hydrazide had to be chosen. A possible agent is biotin hydrazide, which is typically used for biotinylation of carbohydrates and carbonyl compounds in combination with positive-ion ESI-MS.[28,29] Biotin hydrazide rapidly reacts with aldehydes and ketones at pH 4−6 and attaches the biotin group through stable hydrazone bonds. However, our experiments show that these hydrazones in an aqueous environment are only stable in the presence of biotin hydrazide or at alkaline pH (data not shown). Furthermore, the LC separation of the aldehyde derivatives at basic pH results in doublet peaks for a single aldehyde adduct. The identical m/z for the two peaks indicate that stereoisomers are formed (cis/trans). Actually, Uchiyma et al.[23] reported a similar effect with the derivatization of aldehydes with DNPH. Reduction of the hydrazone bond with sodium cyanoborohydride ($NaBH_3CN$) is required to avoid stereoisomer formation and to obtain biotin derivatives also stable in acidic environment. The biotin derivatives can be detected with high sensitivity in positive-ion ESI-MS, the derivatization reaction is fast, and the reaction conditions are mild. Unfortunately, ketones are also derivatized under these conditions, whereas biotin derivatives of larger aliphatic aldehydes such as nonanal and decanal show solubility problems, as can be concluded from Figure 1 where the biotin hydrazone derivative of nonanal is declining over time. Smaller aliphatic aldehydes were also measured and showed same derivatization rates as nonanal but stable derivatives. These findings illustrate that derivatization with neither DNPH nor biotin hydrazide was suitable for our purposes. This prompted us to develop a tailor-made derivatization reagent that would meet the criteria listed below.

Selection Criteria for a Tailor-Made Derivatization Agent. The development of our new derivatization agent for aldehydes was based on a number of important criteria: The derivatization agent should contain a group reactive to aldehydes, but it should not react with other carbonyl-containing compounds such as ketones and carboxylic acids. The reaction time for derivatization should be in the same order as the benchmark DNPH derivatization method, but the reaction should take place under less harsh conditions, i.e., mild pH and temperature. The fragmentation pattern should give additional conformation to identify possible derivatized aldehydes. It should involve a convenient one-pot reaction, preferably not requiring additional extraction procedures. Furthermore, the formed derivatives should be water-soluble and be stable for a significant amount of time to allow large sample sets to be derivatized simultaneously and analyzed subsequently. Finally, with the aim of maximizing MS sensitivity in positive-ion ESI-MS analysis, the compound has to be equipped with a permanent positive charge.

Selection of the Nucleophilic Group. We decided to explore reversible imine formation from an aldehyde and a nucleophilic NH_2 group followed by an in situ irreversible reduction by $NaBH_3CN$ to yield stable secondary amines. This is attractive for selectivity reasons as it is known that many ketones tend to react more slowly than aldehydes in this sequence.[30] A key obstacle is

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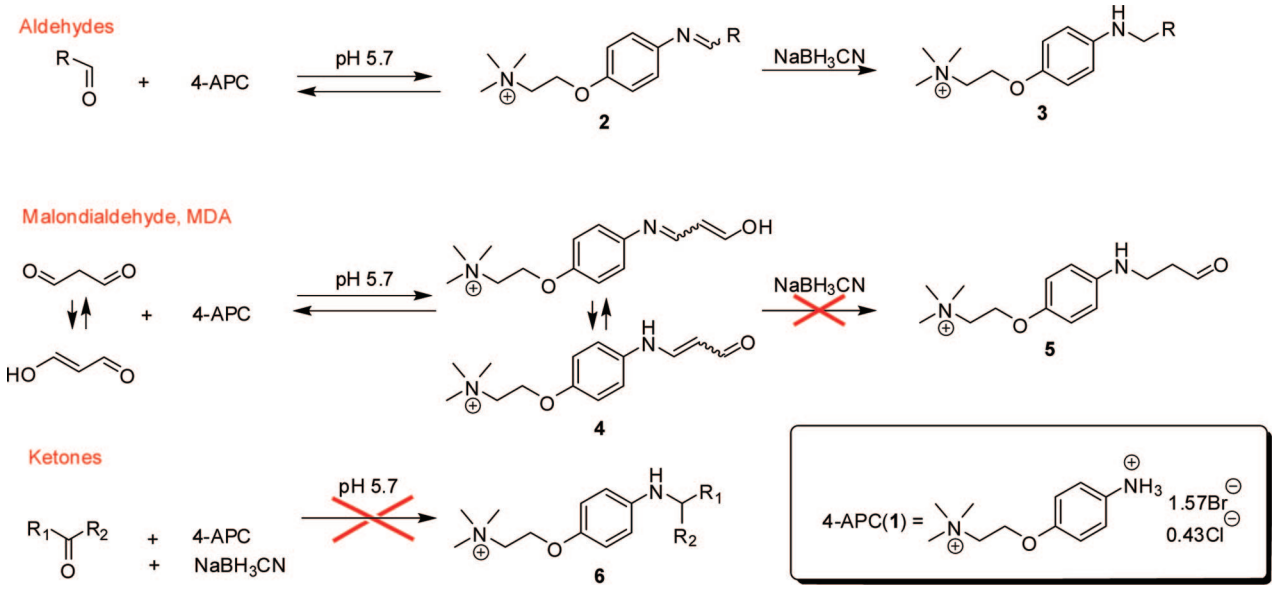

Figure 2. Pathways for imine formation and reduction using aldehydes/ketones/malondialdehyde and 4-APC.

that efficient reduction by $NaBH_3CN$ only takes place at protonated imines, i.e., at pH <7.[30] We considered various nucleophilic NH_2 groups for derivatization at such subneutral pH values. The protonation requirement did not bode well for use of an alkylamine ($pK_a \approx 10-11$) in our reagent, because such a group will be fully protonated at subneutral pH and thereby lose its nucleophilic character. To confirm these expectations, we selected three different primary alkylamines, amine PEO_2 biotin, phenethylamine, and pyridoxamine, as derivatization agents and evaluated the reaction time. As shown in the Figure 1, the reaction times of all the primary alkylamines were longer than 8 h for a complete derivatization of nonanal.

Other potential NH_2 groups of interest are primary aromatic amines (the class of anilines) because these have lower pK_a values. Two recent papers by Dirksen et al. describe the elegant use of aniline as a very effective catalyst for the condensation of aldehydes with oximes or hydrazides.[31,32] Key to the acceleration is the in situ formation of protonated, highly reactive aniline imines.[32] While trying to use this aniline methodology in catalyzing our early-stage derivatizations with biotin hydrazide as mentioned above, we were surprised to see that in situ treatment with $NaBH_3CN$ gave no reduced biotin hydrazones at all. Rather, only clean and rapid formation of the reduced imine resulting from the aldehyde and the catalyst aniline was observed. This is fully in line with the observations by Dirksen et al., who noticed the exact same behavior when aniline was applied as a catalyst in the reaction of glyoxylyls and hydrazides.[31] This unexpected outcome is a direct result of the pK_a of aniline ($pK_a = 4.6$), which is low enough to retain nucleophilic character yet high enough to warrant fast reduction of the resulting imine by $NaBH_3CN$, all at pH 4.5 or, in our case, at pH 5.7.[31] This dual reactivity clearly renders aniline a superior candidate for our purposes. Indeed, when nonanal was mixed with just aniline and $NaBH_3CN$, a very rapid formation of the stable reduced aniline adduct was observed (Figure 1). It was therefore decided to take advantage of this unique pH profile of aniline by incorporating it into the new derivatization reagent.

Additional Structural Features. To meet our criteria, aniline needed to be subjected to other structural demands, e.g., inclusion of a permanent positive charge. For this, we turned to *p*-MeO-aniline (anisidine, $pK_a = 5.3$), which was also reported by Dirksen et al.[32] Replacing the MeO group by a substituted alkoxy group will have little effect on the pK_a and thus NH_2 reactivity but provides a point for convenient synthetic elaboration. The unit providing the permanent charge is a quaternary tetraalkylammonium group, which will also increase the water solubility of adducts. The ammonium group was connected to the oxyaniline-head through an ethyl spacer to minimize any potential inductive effects from the positive charge on the nucleophility of the NH_2. In the end, this led to 4-APC (**1**) shown in Scheme 1 as our novel derivatization agent. Although 4-APC has previously been studied in research on (acetyl)choline[25,33] and multilamellar vesicles,[34] to our knowledge it has never been reported as a derivatization agent nor has any aniline in a reductive-amination based protocol for aldehyde biomarker research.

An added bonus of this type of structure is the known and predictable fragmentation pattern of the loss of the quaternary amine, which is known from e.g. tandem MS on acetylcholine and choline.[35,36] We introduced strategic changes into an existing synthetic procedure[25] to give gram amounts of 4-APC without a need for chromatographic purification.

Derivatization Reactions of Aldehydes, Ketones, and MDA. The 4-APC derivatization reagent in combination with $NaBH_3CN$ (both in excess) was subjected to a diverse set of carbonyl compounds at pH 5.7. Figure 2 shows the reaction pathways involved. Striking differences in reaction outcome with

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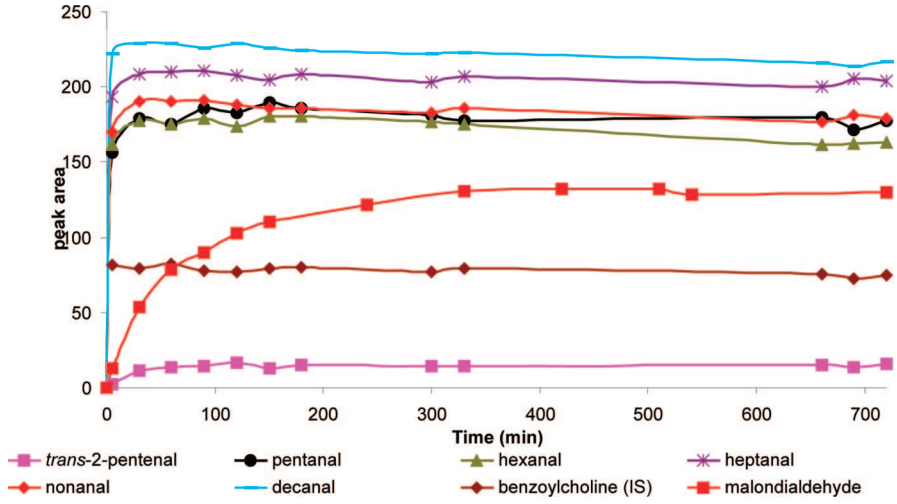

Figure 3. Reaction kinetics of derivatization of 4-APC and *trans*-2-pentenal, pentanal, hexanal, heptanal, nonanal, decanal, malondialdehyde, and benzoylcholine as IS.

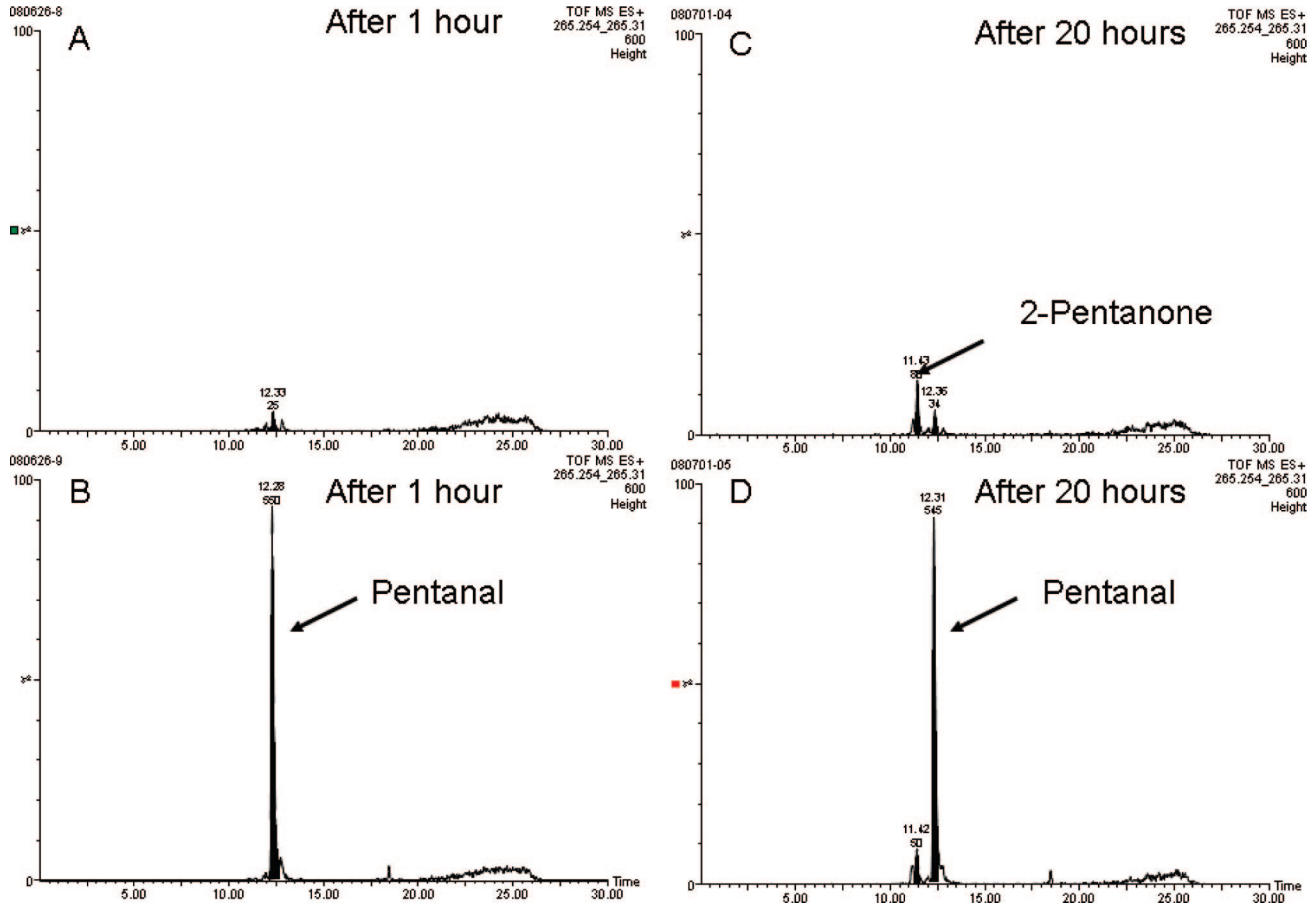

Figure 4. Adduct intensity with (A) 500 nM 2-pentanone after 1 h, (B) 500 nM pentanal after 1 h of derivatization, (C) 500 nM 2-pentanone after 20 h, and (D) 500 nM pentanal after 20 h of derivatization.

aldehydes, ketones, and MDA were observed (see Figure 3). As anticipated, aldehydes are rapidly and irreversibly reduced by $NaBH_3CN$ to amines **3** through intermediacy of imines **2**. Ketones, on the other hand, were only poorly derivatized and no amines **6** or the precursor imines were observed by MS at nanomolar range. Figure 4 shows that, at a concentration of 500 nM of 2-pentanone, no peak was detected after 1 h whereas pentanal gave a large peak within the same time. After 20 h, a small peak of 2-pentanone adduct was detected where pentanal gave the same amount as after 1 h. Based on a previous report, it is anticipated that this low reactivity of ketones is due to difficulties in the initial imine formation between the ketone and the aniline moiety in aqueous environments.[30] Intriguingly, MDA did react with 4-APC, although slower than for saturated aldehydes, but the resulting imine/enamine **4** was left untouched by $NaBH_3CN$. In general, enamines conjugated with a carbonyl are

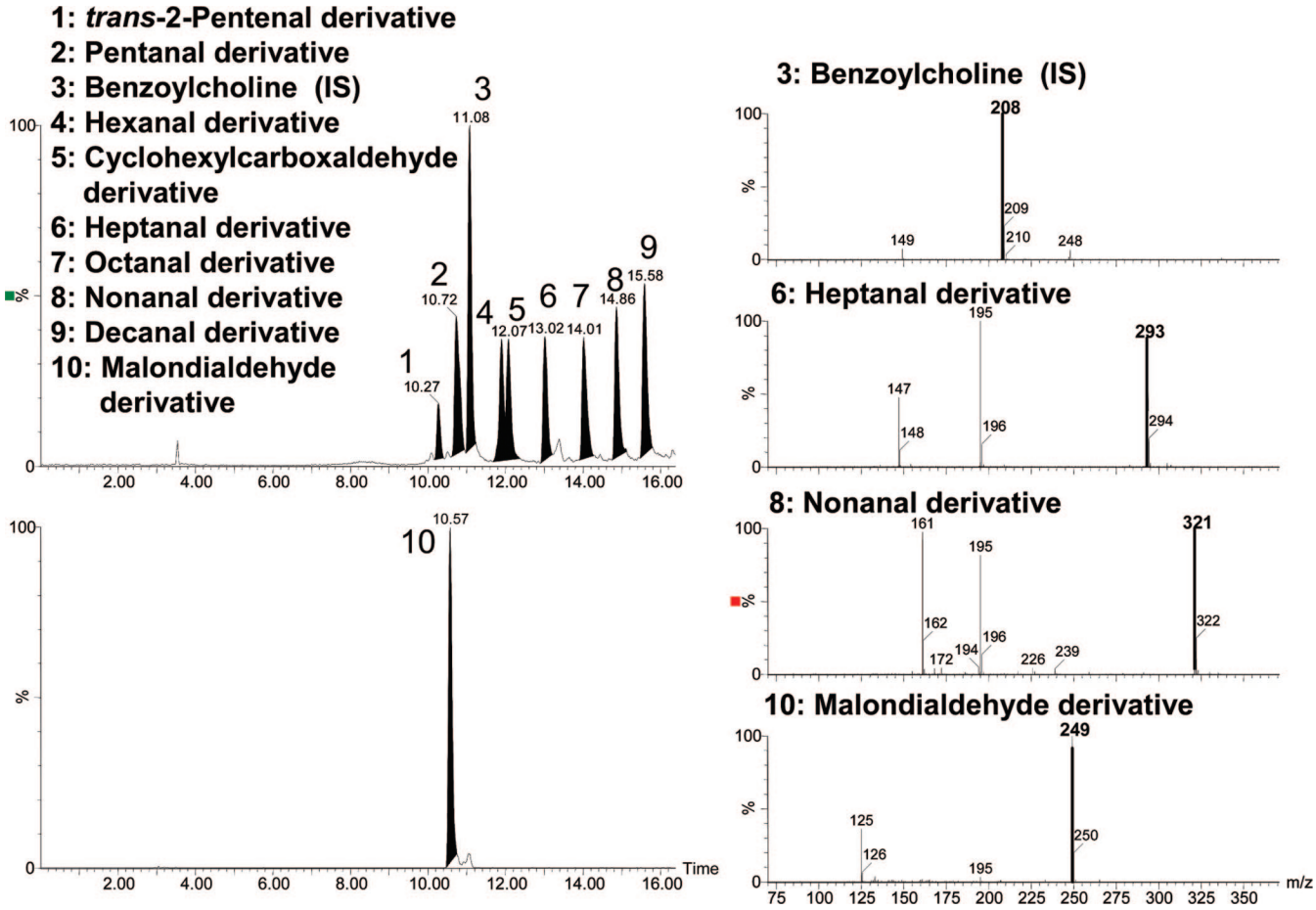

Figure 5. Typical LC−MS (combined) XIC chromatogram of nine aldehyde standards (50 nM) and benzoylcholine as IS (80 nM).

known to resist $NaBH_3CN$ reductions at mild pH,[30] and in our case, a set of papers by Tamura et al. on the reaction of MDA with 4-MeO-aniline (anisidine) can be invoked to rationalize our findings with MDA in greater detail.[37,38]

Tamura et al. showed that the equilibrium constant K_{eq} for acid-catalyzed hydrolysis of β-(anisidino)acrolein to MDA and anisidine is 1.31×10^{-6} (1% EtOH/H_2O, 25 °C), meaning that the reverse reaction (imine formation from anisidine and MDA) is thermodynamically highly favored.[37] They also showed that the pK_a value for protonated β-(anisidino)acrolein is 1.26.[38] Given the high structural similarity between anisidine and 4-APC, these numbers are directly applicable to our case. The estimated pK_a of an alkylimine such as **2** is 3.3,[32] meaning that at pH 5.7 sufficient amounts of protonated **2** are present to warrant a fast reduction by the excess $NaBH_3CN$ to adduct **3**.[30] In contrast, imine **4** is more stable and likely has a p$K_a \approx 1.3$. As a result, no appreciable reduction of **4** to **5** takes place at pH 5.7.[30] These combined data clearly indicate that reaching equilibrium in our reaction of MDA with 4-APC means that virtually all MDA will be in the stable imine form (**4**) and that this does not appreciably react further in a reduction, which is supported by the experiments shown in Figure 3.

Optimization of Reaction Conditions. The data in Figure 3 clearly demonstrate that the aldehydes pentanal, hexanal, heptanal, nonanal, decanal, and *trans*-2-pentenal were derivatized within 30 min to stable products which last for at least 10 h. Further stability studies have shown (see Supporting Information Figure S5) that the derivatives are stable for at least 35 h. The participation of *trans*-2-pentenal is noteworthy as the double bond does not interfere in the derivatization. As explained above, MDA reacts significantly slower than the other aldehydes: a plateau is reached after 300 min. A 250-fold molar excess of 4-APC and 125-fold molar excess of $NaBH_3CN$ is used in the derivatization for three reasons. Lower amounts give the same results but in the end biological samples with unknown amounts of aldehydes will be used. Furthermore, low concentrations of endogenous primary amines can be present in these biological samples that could also react with the aldehydes. Last, employing an excess of 4-APC will effectively block a potential second reductive amination with **3** as the nucleophile.

MDA, *trans*-2-pentenal, pentanal, hexanal, heptanal, octanal, nonanal, and decanal are potential lipid peroxidation biomarkers in biological samples. Therefore, cyclohexylcarboxaldehyde was added to biological samples prior to derivatization as a nonendogenous aldehyde for control. Benzoylcholine was used as internal standard to correct for any signal fluctuations during long sequence runs in the LC−MS. Because trace amounts of formaldehyde, acetaldehyde, propionaldehyde, and butyralde-

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Table 1. Linear Dynamic Range, Correlation Coefficients (r^2), Limit of Detection (LODs), and Characteristic Ions of LC–MS/MS of 4-APC Derivatives of Aldehydes

aldehydes	linear range (nM)	r^2	LOD (nM)[b]	M^+	$[M^+-59.07]$	t_R RSD (%)	area RSD (%)[a]
malondialdehyde	10–500	0.9998	3	249.2	190.1	0.71	5.23
trans-2-pentenal	100–500	0.9957	33	263.2	204.1	1.04	18.72
pentanal	10–500	0.9998	3	265.2	206.1	0.67	8.72
hexanal	10–500	0.9968	3	279.3	220.2	0.39	5.32
heptanal	10–500	0.9983	3	293.3	234.2	0.29	2.51
cylcohexylcarboxaldehyde	10–500	0.9972	3	291.3	232.2	0.40	2.12
octanal	10–500	0.9928	3	307.3	248.2	0.18	5.48
nonanal	10–500	0.9999	3	321.3	262.2	0.11	4.96
decanal	10–500	0.9994	3	335.4	276.3	0.08	4.68
benzoylcholine (IS)	80		1.5	208.1	149	0.70	4.43

[a] RSDs are average of the calibration series. [b] Defined as 3 times the signal-to-noise ratio.

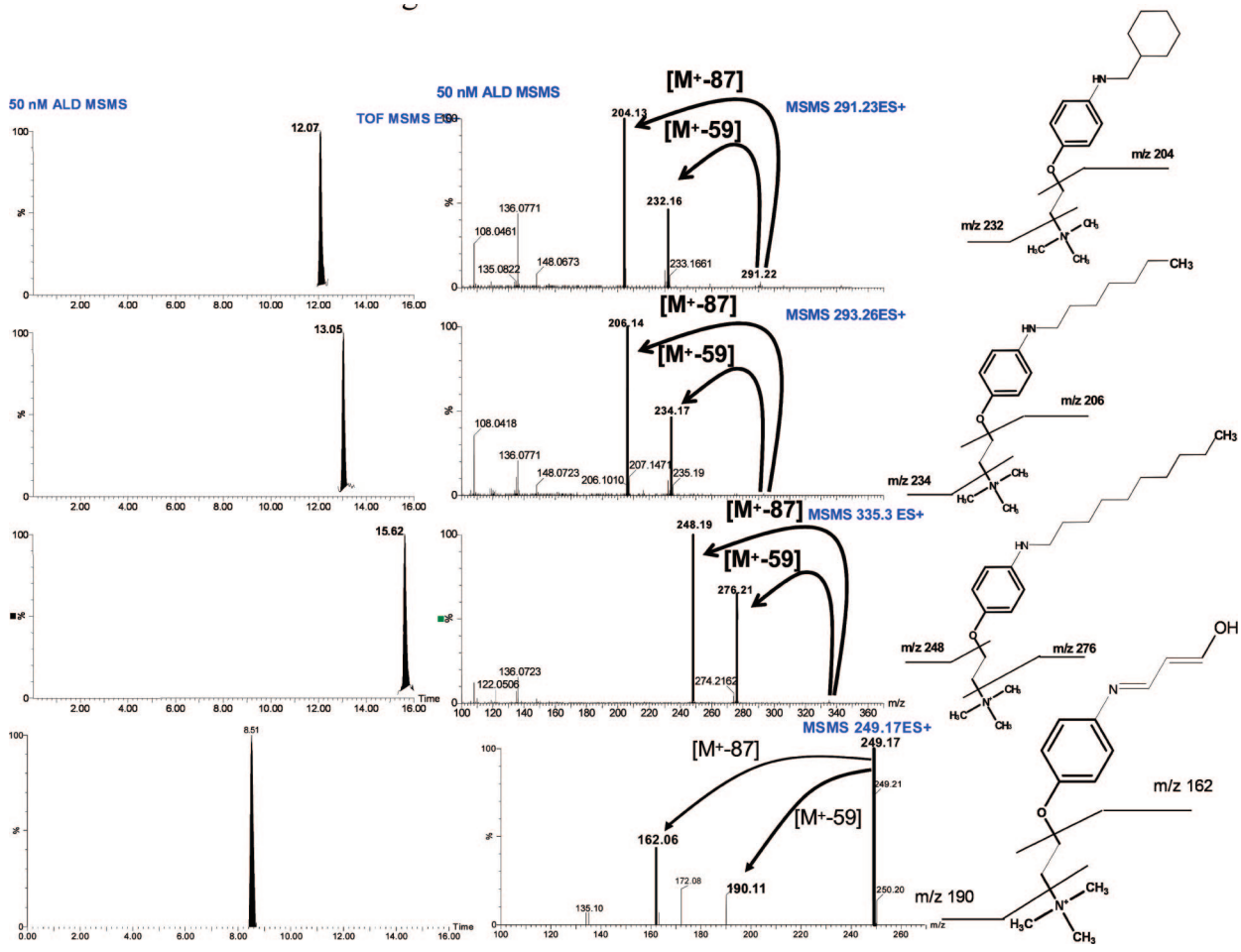

Figure 6. Tandem MS spectra of 50 nM cyclohexylcarboxaldehyde, heptanal, decanal, and malondialdehyde derivative standard.

hyde are present in methanol and formic acid used as reagents, these aldehydes were excluded in this study.

Determination of Aldehyde Derivatives. From the in-vial derivatization reaction mixture, a 10-μL injection is performed onto the C18 column of the LC system. Figure 5 shows a combined extracted ion chromatogram of the nine aldehyde derivatives of *trans*-2-pentenal, pentanal, hexanal, heptanal, cyclohexylcarboxaldehyde, octanal, nonanal, decanal, malondialdehyde and IS, benzoylcholine. All derivatized aldehydes examined showed an M^+ ion. The MS spectra of benzoylcholine, heptanal, nonanal, and malondialdehyde derivatives are also presented in Figure 5. The derivatives show ions with m/z 208, 293, 321, and 249, respectively. The intense ion with m/z 195 is due to the 4-APC derivatization reagent. Table 1 shows the linearity data of the nine derivatized aldehyde standards in buffer. All aldehydes show good linearity over almost 3 orders of magnitude. Limit of detection, defined as 3 times the signal-to-noise ratio, of these derivatives were determined at 3 nM, except for *trans*-2-pentenal, which was 33 nM. The RSD of the retention times were all within 1.05%, which indicates a robust separation system. The average RSD of

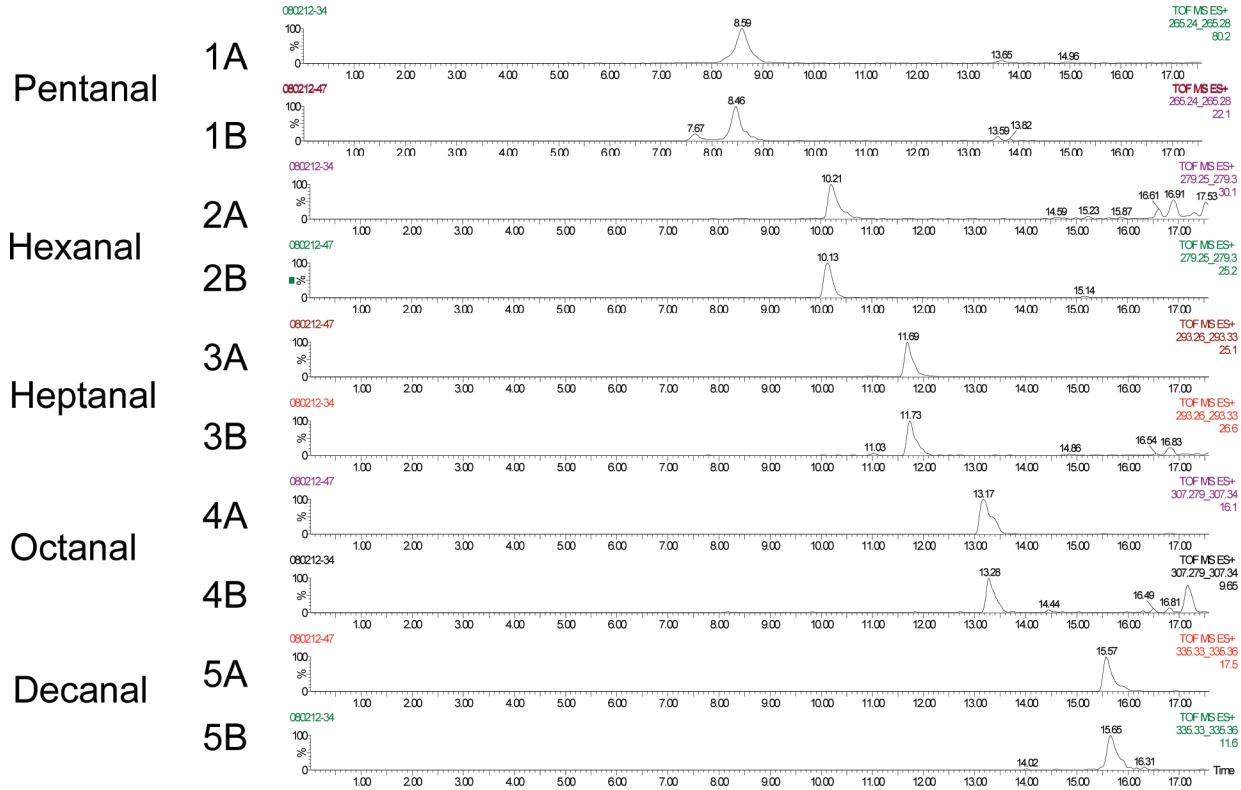

Figure 7. (A) Pooled urine spiked with 10 nM of aldehydes, (B) 10 nM aldehyde standard. 1, Pentanal; 2, hexanal; 3, heptanal; 4, octanal; and 5, decanal.

the peak areas ($n = 3$) were within 5.5% for all compounds except for pentanal and *trans*-2-pentenal, which were 8.72 and 18.72%, respectively. The larger variation can be explained by the lower concentration range. With these detection limits, aldehydes can be determined in biological samples.[12,39]

An important additional feature of tandem MS is the ability to provide analyte identification or to confirm its identity. Tandem MS experiments on aldehyde-4-APC derivatives show that typical neutral losses of 59 and 87 Da, consistent with losses of C_3H_9N and $C_5H_{13}N$, respectively, are observed for all derivatives. All aldehyde derivatives show the two abundant fragment ions at a collision voltage of 20 V. Figure 6 show the MS/MS spectra of the 50 nM derivatized cyclohexylcarboxaldehyde, heptanal, decanal, and malondialdehyde, providing the abundant fragment ions with m/z 204.1 and 232.2 for cyclohexylcarboxaldehyde (selected precursor ion with m/z 291.2), with m/z 206.1 and 234.2 for heptanal (m/z 293.2), with m/z 248.2 and 276.2 for decanal (m/z 335.3), and with m/z 162.1 and 190.1 for malondialdehyde (m/z 249.2). Although the losses of $[M^+ - 59]$ and $[M^+ - 87]$ are observed for all 4-APC-derivatized aldehydes, the abundance ratio of the fragment ions changes with increasing aliphatic chain length. Increasing collision energies could be used, but in this case, we have chosen for a fixed collision energy. Also, the $[M^+ - 59]$ gave excellent linearity of $R^2 > 0.99$ for all the tested derivatized aldehydes.

Detection of Aldehyde Derivatives in Biological Samples. To determine the aldehyde concentration in biological samples, standard addition was applied. Comparing the calibration curves in buffer and in urine, the derivatives show matrix-induced ionization suppression of the signal but the linearity ($R^2 > 0.99$) for all the tested aldehydes was not affected. Benzoylcholine concentration was kept constant in all samples; its response did not change in time and showed a signal loss of 10% by the ionization suppression in urine. Because the matrix effects were not constant for all the aldehydes and different from the IS, standard addition for each aldehydes was used to determine the concentration in urine. The extracted ion chromatograms in Figure 7 represent pentanal, hexanal, heptanal, octanal, and decanal with a concentration of 10 nM spiked in urine (A) and 10 nM in buffer (B). All selected aldehydes are detected in both urine and buffer sample. Pentanal and hexanal were detected at elevated concentration in the pooled urine samples, i.e., 61 ± 10 nM for pentanal and 26 ± 2 nM for hexanal. Konidari et al.[40] also reported pentanal as biomarker of oxidative stress in human urine. The peak heights for pentanal and hexanal are 80.2 and 30.1, respectively, in spiked urine, and 25.2 and 22.1 in the aldehyde standard.

Furthermore, malondialdehyde was detected in the derivatized urine and was conformed by the retention time of derivatized malondialdehyde standard (data shown in Supporting Information Figure S6).

Again, for identification, the specific losses of $[M^+ - 59]$ and $[M^+ - 87]$ in tandem MS can be used to confirm identity of the aldehyde derivatives in urine, where a pooled urine sample was spiked with 100 nM hexanal, cyclohexylcarboxaldehyde, heptanal, octanal, and decanal, and all MS/MS spectra (Supporting Information Figure S7) showed these typical losses.

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CONCLUSION

We successfully designed and prepared a novel derivatization reagent, 4-APC. The key advantage of 4-APC is that it allows highly sensitive and selective MS detection of aldehydes. The derivatization reaction is a fast and convenient one-pot reaction at a mild pH of 5.7. As a result, no additional extraction steps are necessary before analysis with an LC−MS/MS system for the quantification of aldehydes as biomarkers for lipid peroxidation in urine at low-nanomolar range. As a result of the chosen design of the derivatization agent, tandem MS can be used to confirm identity of the aldehydes based on the typical neutral losses of 59 and 87 Da from the aldehyde derivative and to screen for "unknown" aldehydes from lipid peroxidation in urine samples. Although some of the aldehydes show a 2−4 times response loss due to matrix effect, quantification was still possible with the use of standard addition. The quantification levels of this method are sufficient to determine aldehydes in urine. In future studies, the possibilities of isotopically labeled internal standards or fast and simple SPE-based sample pretreatment will be explored to correct for the ionization suppression effects. Future efforts will also be directed at structural refinement of 4-APC as well as at the use of this type of reagent in the quantification or screening of aldehydes in large sets of biological samples for lipid peroxidation-related biomarkers.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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