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Evaluation of Alternate Isotope-Coded Derivatization Assay (AIDA) in the LC–MS/MS analysis of aldehydes in exhaled breath condensate☆

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ABSTRACT

We present the application of a novel isotope dilution method, named Alternate Isotope-Coded Derivatization Assay (AIDA), to the quantitative analysis of hydrazone derivatives of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in exhaled breath condensate (EBC) samples using liquid chromatography–tandem mass spectrometry. AIDA is based on the alternate derivatization of the analyte(s) with reagents that are available in two pure isotopic forms, respectively "light" and "heavy", by using light-derivatized standards for the quantification of the heavy-derivatized analytes, and *vice versa*. To this purpose, 2,4-dinitro-3,5,6-trideuterophenylhydrazine (d_3-DNPH) has been synthesized and used as "heavy" reagent in combination with commercial "light" DNPH. Using the AIDA method, any unknown concentration of the analyte in the matrix can be calculated without the need of a calibration curve. An external calibration method has been also investigated for comparative purpose. The stability of DNPH and d_3-DNPH derivatives was verified by excluding any exchange of hydrazones with each other. In the range of concentrations of biological interest, *e.g.*, 2–40 nM for MDA and 0.5–10 nM for 4-HNE, the derivatization reactions of MDA and 4-HNE with DNPH and d_3-DNPH showed overlapping kinetics and comparable yields. The MS response of both DNPH and d_3-DNPH hydrazones was similar. The precision of AIDA, calculated as %RSD, was within 3.2–8% for MDA and 4.5–11% for 4-HNE. Accuracy was tested by analyzing a spiked EBC pool sample and acceptable results (accuracy within 98–108% for MDA and 93–114% for 4-HNE) were obtained by AIDA after subtraction of the blank, which was not negligible. The results of quantitative analysis of MDA and 4-HNE in EBC samples obtained by AIDA assay with four analyses per sample were in good agreement with those obtained by external calibration method on the same samples.

1. Introduction

Biologically relevant aldehydes, namely malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), can be determined in exhaled breath condensate (EBC) samples by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after derivatization with 2,4-dinitrophenylhydrazine (DNPH) [1]. LC–MS(/MS) has been demonstrated to be a powerful technique for the quantitative determination of biomarkers in complex biological fluids, like blood and urine, without requiring extensive sample manipulation. Nevertheless, it is well known that precision and accuracy of LC–MS(/MS) quantitative bioanalysis could be affected by matrix effects, especially at very low concentration levels of analytes [2]. A matrix effect is considered to be an (unexpected) suppression or enhancement of the analyte response due to coeluting matrix constituents. Several approaches have been proposed to compensate for such effect, *i.e.*, an efficient sample clean-up, the introduction of additives into the mobile phase, the use of either ballistic gradients, high retention RP-HPLC, or 2D LC (LC–LC), the application of isotopically labelled internal standards (when available at the adequate isotopic purity) rather than analogue internal standards (ISs), the application of the standard addition method [3,4]. Even though EBC is a relatively clean matrix, being constituted mainly of water, the concentrations of biomarkers candidate for LC–MS bioanalysis are in the low nM range. Therefore, the use of stable isotope-labelled

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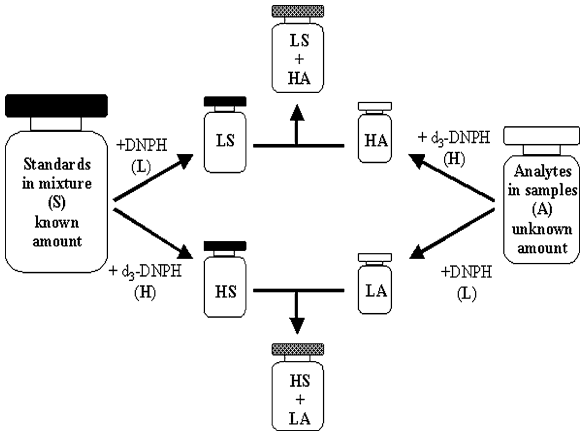

Fig. 1. Procedure for the application of the AIDA method. The method is based on an alternate derivatization of the analyte(s) with reagents that are available in two pure isotopic forms, "light" (L, DNPH) and "heavy" (H, d_3-DNPH), by using light-derivatized standards for the quantification of the heavy-derivatized analytes, and *vice versa* [10]. Legend: LS, light standard; HS, heavy standard; HA, heavy analyte; LA, light analyte.

analogues would be desirable to account for matrix effects that are known to affect precision and accuracy of LC–MS quantitative bioanalysis [4]. Unfortunately, these standards are often not commercially available (*e.g.*, for MDA) or extremely expensive (*e.g.*, for 4-HNE). The synthesis of 2,4-dinitro-3,5,6-trideuterohydrazine for derivatizing the carbonyl compounds into the corresponding deuterated hydrazones and subsequent use as internal standards in the LC–MS analysis of aldehydes and ketones in air samples has been proposed by Zurek and Karst [5], as an alternative to the use of labelled carbonyls as starting materials [6]. This second approach is less expensive and easily extendable to any carbonyl compound for which labelled carbonyl analogues are not commercially available.

In this study, we have investigated another approach, that is the possibility of applying a novel isotope dilution method, named Alternate Isotope-Coded Derivatization Assay (AIDA), originally proposed for the quantification of zearalenone in maize flour after derivatization with acetic anhydride [7], to the quantitative analysis of MDA and 4-HNE in EBC. The method is based on the alternate derivatization of the analyte(s) with reagents that are available in two pure isotopic forms, "light" and "heavy" (L and H, respectively). The analyte (A) in matrix sample is split in two fractions, which are derivatized with either a non-labelled or a stable isotope-labelled reagent (LA or HA). To the non-labelled sample fraction (LA), a known amount of analyte in standard solution (S) derivatized with the stable isotope-labelled reagent (HS) is added, and *vice versa* (LS added to HA, Fig. 1). The unknown concentration of the analyte in the matrix can be calculated from the ratios of non-labelled analyte and labelled standard in one fraction and of labelled analyte and non-labelled standard in the other fraction: the combination of the two measurements allows to calculate the response factor and thus the true analyte content without the need of a calibration curve. To this purpose, 2,4-dinitro-3,5,6-trideuterophenylhydrazine (d_3-DNPH) has been synthesized and used as H-reagent in combination with commercial DNPH, used as L-reagent. The results of quantitative analysis of MDA and 4-HNE in EBC samples obtained by AIDA assay have been compared with those obtained by external calibration method on the same samples.

2. Materials and methods

2.1. Synthesis of 2,4-dinitro-3,5,6-trideuterophenylhydrazine

The d_3-DNPH was synthesized essentially as described by Zurek and Karst [5], whereas compound purification was carried out by flash chromatography on a glass column packed with a 15-cm bed of silica gel by using a ethyl acetate:hexane 1:9 mixture as eluent. The compound purity was verified by TLC (silica gel plates, eluent mixture ethyl acetate:hexane 1:2, R_f = 0.5, single spot both by UV and ninhydrine detection). Its identity was verified by electrospray ionization MS (ESI–MS) (positive-ion mode, calculated for MH^+ m/z 202.0, found m/z 202.1). No bis-, mono- or undeuterated compound was detectable by ESI–MS analysis.

2.2. Chemicals and standards

2,4-Dinitrophenylhydrazine (DNPH, purity 97%), malondialdehyde (MDA) tetrabutylammonium salt (98%), acetic acid (>99%) and formic acid (>98%) were obtained by Sigma–Aldrich (Milan, Italy). 4-Hydroxy-2-nonenal (4-HNE, 98%) dissolved in ethanol (87.6 mM) was purchased from Cayman Chemicals (Michigan, USA). This solution is stable for 6 months at −80 °C. A standard stock solution of MDA (about 10 mM) was prepared in acetonitrile and stored at −20 °C for up to 1 month. A standard mixture containing MDA (40 μM) and 4-HNE (10 μM) in CH_3CN (final volume, 1 mL) was prepared weekly and stored in dark vial at −20 °C. Daily, this solution was diluted 50-fold with water (final volume, 1 mL) in order to obtain a working solution containing MDA and 4-HNE at concentrations of 800 and 200 nM, respectively.

DNPH or d_3-DNPH (25 mg) was dissolved in 10 mL of acetonitrile and acidified with 0.2 mL of formic acid to obtain a 12 mM stock solution, which was stable for one working week when stored at +4 °C in dark vials. Daily, a DNPH or d_3-DNPH working solution (1.2 mM, final volume 5 mL) was prepared by 10-fold dilution of the stock solution with acetonitrile/formic acid (98/2, v/v). HPLC-grade methanol and acetonitrile were purchased from Carlo Erba (Milan, Italy). LiChrosolv HPLC water was obtained from Merck (Darmstadt, Germany) and was used in the preparation of mobile phases and for standards.

2.3. EBC sample collection and preparation

EBC samples were collected using a commercially available TURBO DECCS breath condenser (Medivac, Parma, Italy), which is a transportable device equipped with temperature controls. Ten volunteers were asked to breathe tidally through the mouthpiece without nose clip for 15 min at the collecting temperature of −5 °C, in order to obtain an EBC volume of at least 0.5 mL. Aliquots (100 μL) of each sample were stored at −20 °C until analysis.

For validation of the AIDA assay, a pooled EBC sample (10 mL) was obtained by mixing EBC samples from 20 healthy non-smoking subjects and was used for the validation of the AIDA assay (see Section 2.5).

2.4. AIDA protocol for the quantification of aldehydes in EBC

For AIDA, 5 mL of a standard solution containing MDA (10 nM) and 4-HNE (2.5 nM) was prepared daily in water. Then, 2.5 mL of this solution were derivatized with an equal volume of 1.2 mM DNPH (light standard, LS) and the remaining 2.5 mL with d_3-DNPH (heavy standard, HS). Both reactions were incubated for 2 h at room temperature.

The sample aliquot reserved for the AIDA assay was split into two fractions (50 μL each), one of them were derivatized at room temperature with an equal volume of DNPH (yielding the light analyte, LA) and the other with d_3-DNPH (yielding the heavy analyte, HA). After 2 h, a volume of 100 μL of HS was added to the LA fraction, and *vice versa,* a volume of 100 μL of LS was added to the HA fraction. Two independent determinations (one with HS and one with LS) were run in duplicate for every sample. The quantification of each sample required four analyses. The scheme of the AIDA

sample preparation is shown in Fig. 1. The AIDA protocol for EBC samples was also applied to water used in the preparation standard solutions to estimate the aldehyde concentrations of the solvent blank.

2.5. Validation of the AIDA assay: kinetics of the derivatization reactions, stability of L- and H-hydrazones, accuracy and precision

To verify the absence of any exchange between DNPH and d_3-DNPH derivatives, 100 μL of d_3-DNPH (12 mM) were added to 100 μL of a mixture of MDA and 4-HNE (10 nM and 2.5 nM) previously derivatized with DNPH (1.2 mM), and *vice versa,* 100 μL of DNPH (12 mM) were added to 100 μL of the same mixture of MDA and 4-HNE previously derivatized with d_3-DNPH (1.2 mM).

The kinetics of the derivatization reactions of both L- and H-hydrazones was established by injecting every 11 min for 6 h the freshly prepared LS and HS solutions (see Section 2.4). The long-term stability of both L- and H-hydrazones was tested by repetitive injection of the LS and HS solutions for 72 h.

Accuracy and precision of the AIDA assay were determined by using the pooled EBC sample, whose aldehyde concentration (MDA: 10.62 nM, 4-HNE: 2.24 nM) was determined by applying the standard addition method, *i.e.*, by adding mixtures of aldehydes in order to obtain concentration increases of 2, 4, 10, 20 and 40 nM for MDA and 0.5, 1, 2.5, 5 and 10 nM for 4-HNE.

Accuracy and precision of the AIDA assay were assessed at 6 concentration levels to demonstrate the absence of any concentration-dependent trend for both parameters. To this purpose, the pooled EBC sample was spiked with the 6 calibrating solutions used for external calibration and derivatized according to the AIDA protocol described for samples, with some modifications concerning the number of both independent derivatizations (n = 1–3) and sample analyses (n = 2, 3). Precision of the AIDA assay was calculated as %RSD of six (four or two) independent reacted samples, half of them derivatized with the L- and half with the H-reagent, each injected in triplicate (or in duplicate) for each of the six concentration levels (6×3, n = 18; 4×2, n = 8; or 2×2, n = 4 analyses per sample). The accuracy of the method was assessed by comparing the known aldehyde concentrations of the pooled EBC sample spiked with the 6 calibrating solutions with those determined by applying AIDA assay on the same samples.

The pooled EBC sample was used to prepare quality control samples, both unspiked (low QC level) and fortified with MDA and 4-HNE concentrations of 10 and 2.5 nM, respectively (high QC level). Aliquots (100 μL) of QC samples were prepared and stored at −20 °C for further analyses and were used to establish the inter-day precision of the AIDA assay.

2.6. Quantification of EBC aldehydes by external calibration method

For external calibration, a set of 6 calibrating standard solutions was prepared by dilution of the working solution containing MDA (800 nM) and 4-HNE (200 nM) with water to produce the following aldehyde concentrations: 0, 2, 4, 10, 20, and 40 nM for MDA, and 0, 0.5, 1, 2.5, 5, and 10 nM for 4-HNE. These standards (100 μL) were derivatized with an equal volume of either DNPH or d_3-DNPH (1.2 mM) at room temperature. After 2 h, both sets of derivatized standards were injected into the LC–MS/MS system in order to obtain external calibrations for L- or H-hydrazones, respectively, whose slope values were used to compare the MS response of DNPH and d_3-DNPH hydrazones over a wide range of concentrations.

Similarly, an aliquot of each EBC sample (100 μL) was derivatized with an equal volume of DNPH (1.2 mM) and analyzed in triplicate using the external calibration method for L-hydrazones and results were compared with those obtained by the AIDA assay. Three calibration sets were run, at the beginning, in the middle (after 5 samples), and at the end of the sample series. A quality control sample was run in the middle of each of the sample series.

2.7. LC–MS/MS analysis

LC–MS/MS analysis was carried out as previously described [1], with some modifications. The LC–MS/MS system consisted of an Agilent HP 1100 Series binary pump (Palo Alto, CA, USA), equipped with a thermostated auto-sampler and a vacuum degasser, coupled with a PE-Sciex API 365 triple-quadrupole mass spectrometer (Sciex, Concord, Canada) equipped with a TurboIonSpray™ interface (TIS). A Power Macintosh G3 computer was used for instrument control, data acquisition and data processing. Chromatography was performed on a Supelcosil™ LC-18-DB column (75 mm × 3.0 mm i.d., 3 μm; Supelco, Bellefonte, PA, USA) using variable proportions of 20 mM aqueous acetic acid and methanol/acetonitrile (95/5, v/v) at a flow-rate of 0.5 mL/min. The injection volume was 20 μL. The first (0–2.6 min) and the last (8–11 min) parts of the chromatographic run were diverted to waste using a 10-port valve (Valco Systems, Houston, Texas, USA). MDA and 4-HNE hydrazones were ionized in positive-ion (PI) and in negative-ion (NI) mode, respectively. ESI conditions: for MDA (PI), ionspray voltage 5500 V, orifice voltage 30 V; for 4-HNE (NI), ionspray voltage −4000 V, orifice voltage −20 V. The TIS temperature was set at 350 °C. The detection was obtained in selected-reaction monitoring (SRM) mode by following the reactions m/z 235 → 159 and m/z 238 → 162 (collision energy, 21 eV) for DNPH–MDA and d_3-DNPH–MDA, respectively, and m/z 335 → 167 and m/z 338 → 170 (20 eV) for DNPH–4-HNE and d_3-DNPH–4-HNE, respectively. For comparative purposes, some experiments were also performed on a Quattro micro triple-quadrupole mass spectrometer (Waters, Milford, MA, USA). Source parameters: capillary voltage 3.2 KV; sample cone voltage 28 V; source temperature 120 °C; desolvation gas temperature 350 °C. For MS/MS experiments, argon (99.995%) was used as collision gas with a pressure of 3.3×10^{-3} mbar in the collision cell; the collision energy was 20 eV.

3. Calculation

According to the formulas originally proposed for the quantification of zearalenone in maize flour after derivatization with acetic anhydride [7], quantification of the L-derivatized analyte (LA) in the presence of the H-derivatized internal standard (HS), is given by Eq. (1):

$$q_{LA} = \left(\frac{Rf_L}{Rf_H}\right) \times \left(\frac{A_{LA}}{A_{HS}}\right) \times q_{HS} \quad (1)$$

where q: quantity, Rf: response factor, A: peak area. L and H are "light" and "heavy" hydrazone derivatives, that is in this case "light" analyte (LA) and "heavy" standard, respectively.

Similarly, when the role of H- and L-derivatized molecule has been reversed according to the AIDA protocol, the quantity of the H-derivatized analyte (HA) in the presence of L-derivatized internal standard (LS), is given by Eq. (2):

$$q_{HA} = \left(\frac{Rf_H}{Rf_L}\right) \times \left(\frac{A_{HA}}{A_{LS}}\right) \times q_{LS} \quad (2)$$

where L and H are standard and analyte, respectively.

Dividing the two equations:

$$\left(\frac{q_{LA}}{q_{HA}}\right) = \left(\frac{Rf_L}{Rf_H}\right)^2 \times \left(\frac{A_{LA}}{A_{HS}}\right) \times \left(\frac{A_{LS}}{A_{HA}}\right) \times \left(\frac{q_{HS}}{q_{LS}}\right)$$

and after assuming that q_{LA}/q_{HA} = 1 and q_{HS}/q_{LS} = 1, since the analyte amount in the sample or the standard is the same, irrespective of whether it was derivatized using a "light" or a "heavy" DNPH, a third

equation is obtained which allows the calculation of the response factor Rf:

$$\left(\frac{\mathrm{Rf_L}}{\mathrm{Rf_H}}\right)^2 = \left(\frac{A_{\mathrm{HS}}}{A_{\mathrm{LA}}}\right) \times \left(\frac{A_{\mathrm{HA}}}{A_{\mathrm{LS}}}\right) \quad (3)$$

Since the concentration of MDA and 4-HNE in the water used to prepare both L and H standards was non negligible, the equations originally used for the AIDA calculations have been adapted, by subtracting the area of the blank derivatized with d_3-DNPH from the area of that of heavy standards used in the calculation of light analytes, and *vice versa*.

$$q_{\mathrm{LA}} = \left(\frac{\mathrm{Rf_L}}{\mathrm{Rf_H}}\right) \times \left[\frac{A_{\mathrm{L}}}{A_{\mathrm{HS}} - A_{\mathrm{blkHS}}}\right] \times q_{\mathrm{HS}} \quad (1a)$$

$$q_{\mathrm{HA}} = \left(\frac{\mathrm{Rf_H}}{\mathrm{Rf_L}}\right) \times \left[\frac{A_{\mathrm{H}}}{A_{\mathrm{LS}} - A_{\mathrm{blkLS}}}\right] \times q_{\mathrm{LS}} \quad (2a)$$

$$\left(\frac{\mathrm{Rf_H}}{\mathrm{Rf_L}}\right)^2 = \times \left[\frac{A_{\mathrm{HS}} - A_{\mathrm{blkHS}}}{A_{\mathrm{L}}}\right] \times \left[\frac{A_{\mathrm{H}}}{A_{\mathrm{LS}} - A_{\mathrm{blkLS}}}\right] \quad (3a)$$

where blk: blank.

4. Results and discussion

4.1. Improvements in the LC–MS/MS determination of aldehydes in EBC

The method initially proposed for the characterization of profile of aldehydes in EBC by LC–MS/MS [1] has subsequently been modified and its sensitivity improved by using a TurboIonSpray™ ion-source and a 3 mm i.d. column instead of APCI and a 4.6 mm i.d. column. To avoid some memory effects observed during the analysis of large batches of samples, the concentration of DNPH has been reduced 10-fold and a 10-port valve has been implemented to divert unreacted DNPH to waste. These memory effects were found not to be dependent on the ion-source geometry as they were also observed in a series of experiments performed using a Quattro micro system, featuring a Z-spray source design rather than the turbo-ionspray/curtain plate design of the API365. At the end of the analytical session, an orange deposit was observed on both the curtain plate of API365 and the sample cone of the Quattro Micro, probably due to the deposition of unreacted DNPH and perhaps other non-volatile compounds. Unreacted DNPH elutes prior to MDA and 4-HNE. Although it was not detected, it is known that even undetected compounds can still exert a significant matrix effect [4]. The use of a lower DNPH excess in the derivatization reaction in combination with the divert valve was effective in preserving the ion-source from contamination. The reduction of DNPH concentration (from 12 to 1.2 mM) did not impair the yield of the derivatization reaction (data not shown), as the derivatizing agent was still in large excess as compared to the aldehyde levels in EBC (nM range). The possibility to reduce the concentration of the derivatization agent should be re-evaluated in the case that more complex matrices containing aldehydes and other carbonyl compounds in the μM or mM concentration ranges, like urine or plasma, are analyzed.

Since EBC is a relatively clean matrix, therefore presumably less prone to matrix effects, internal standardization seemed not to be necessary. In fact, good and reliable results were obtained and reported in previous studies [1,8–12]. The levels of the aldehyde biomarkers of lipid peroxidation, such as MDA and 4-HNE, were found to be significantly increased in patients with several lung pathologies compared to healthy controls [8,9] and were positively associated with exposure biomarkers in workers exposed to oxidizing agents, such as cobalt and chromium(VI) [10,11], and in hairdressers potentially exposed to irritant agents [12]. When aldehydes were determined in more complex biological matrices, like induced sputum, we prepared matrix-matched standards for LC–MS/MS calibration [13]. The application of the standard addition method to every sample would have been desirable, but it was impossible, owing to the small sample volume available. It is recognized that internal standardization using isotope-labelled compounds represents the most effective approach to address matrix effects in quantitative bioanalysis [4]. The use of 2,4-dinitro-3,5,6-trideuterohydrazones as internal standards in the LC–MS analysis of aldehydes and ketones in air samples has been proposed by Zurek and Karst [5] as an alternative to the use of (expensive) labelled carbonyls as starting materials [6]. In this study, we have investigated the possibility of applying an AIDA assay to the analysis of aldehydes in EBC samples, using d_3-DNPH obtained by synthesis and commercial DNPH as H- and L-reagents, respectively.

4.2. Applicability of AIDA assay to aldehyde derivatization with DNPH

In order to apply the AIDA method, it is necessary that the derivatizing agent rapidly and irreversibly reacts with the analytes. This is the case for carbonyl derivatization with DNPH [14]. In addition, the reaction should give the same conversion both for the matrix and the standard solution. Considering that EBC samples mainly constitute of water, this second condition is also satisfied, as we previously reported [1].

The stability of DNPH and d_3-DNPH derivatives was verified by excluding any exchange of hydrazones with each other. To this purpose, d_3-DNPH (12 mM) was added to a mixture of MDA and 4-HNE (10 nM and 2.5 nM) previously derivatized with DNPH (1.2 mM), and *vice versa*. The results of this experiment are shown in Fig. 2. When samples after 2 h incubation were analyzed by LC–MS/MS, SRM chromatograms only revealed the traces of L-derivatives (or *vice versa*, the traces of the H-derivatives), demonstrating that the derivatization reaction was complete and DNPH and d_3-DNPH did not mutually exchange. As already reported [5], deuterium/hydrogen exchange reactions of trideuterated DNPH are highly unlikely due to the extremely low acidity of the aromatic hydrogen or deuterium atoms (pK_a value of 43).

The derivatization reactions of MDA and 4-HNE with either of the two derivatizing reagents, DNPH and d_3-DNPH, exhibited a superimposable kinetics, the reactions being complete in 120 and 30 min for MDA and 4-HNE, respectively, and a comparable yield, as shown by the comparison of the areas of L- and H-hydrazones which differed for less than 5%. The results are shown in Fig. 3. Once derivatized, L- and H-hydrazones are stable for at least 72 h, the %RSD of the areas ranging between 6.4% and 7.4%, without any time-dependent trend. The MS response of both DNPH and d_3-DNPH hydrazones was similar in a wide range of concentrations, as shown by comparison of the slopes of the calibration lines of analytes derivatized with either DNPH or d_3-DNPH reagent, *i.e.*, 147.5 ± 1.1 *vs* 149.7 ± 1.7 for MDA (in the 0–200 nM range, $n = 63$, $r^2 > 0.993$), and 184.1 ± 1.9 *vs* 195.1 ± 1.8 for 4-HNE (in the 0–50 nM range, $n = 63$, $r^2 > 0.997$).

4.3. Precision and accuracy

Two factors contribute to precision of the AIDA assay: the repeatability of sample analysis and the reproducibility of the derivatization reaction. The AIDA method was initially proposed for the LC–MS determination of zearalenone in authentic maize flour samples after derivatization with acetic anhydride but without any sample clean-up and without calibration curve [7]. Since it was expected that the matrix could significantly interfere with the derivatization reaction and affect the analytical precision, a protocol with six independent determinations (three with deuterated and three with protonated standards) for every sample was

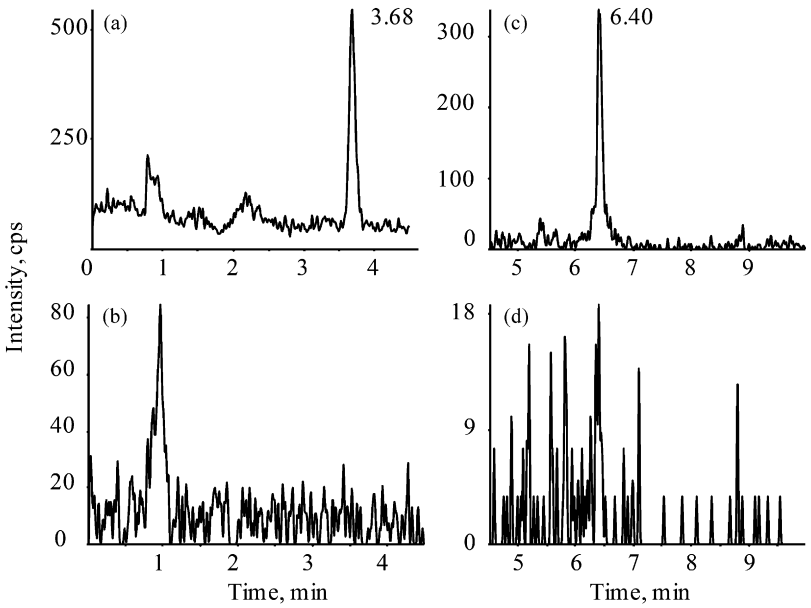

Fig. 2. Stability of derivatized hydrazones. SRM chromatograms of a mixture of MDA (10 nM) and 4-HNE (2.5 nM) derivatized with DNPH (1.2 mM) and added with d_3-DNPH (12 mM) after 2 h. SRM transitions: (a) m/z 235 → 159 for DNPH–MDA and (b) m/z 238 → 162 for d_3-DNPH–MDA; (c) m/z 335 → 167 for DNPH–4-HNE and (d) m/z 338 → 170 for d_3-DNPH–4-HNE.

proposed to obtain accurate results. The contribution of sample analysis repeatability on precision was assumed to be negligible and replicate injections were not performed [7].

Even though EBC is a relatively clean matrix and aldehyde determination does not require any sample clean-up (and therefore we expected that variability among independent derivatizations would be of the same order as the variability in sample analysis), the AIDA assay is novel and its precision needs to be established carefully during method validation. The experiment performed with the pooled EBC sample spiked at six concentration levels was useful: (i) to separately evaluate the relative contribution of both factors, *e.g.*, the repeatability by analyzing in triplicate each independently reacted sample (derivatized with both reagents) and the variability of derivatization by analyzing three independent reacted samples (derivatized with both reagents); (ii) to demonstrate the absence of any concentration-dependent effect on precision; and (iii) to evaluate the precision of the overall AIDA assay. The %RSD of triplicate sample analyses calculated for one independent reaction with either DNPH or d_3-DNPH was 4.3% or 4.0% for MDA, and 3.8% or 4.2% for 4-HNE. The reproducibility of the derivatization reaction, obtained by a single sample analysis of three independent reacted samples was 4.9% for MDA (for both L- and H-reagents), and 5.5% and 6.7% for 4-HNE when reacted with DNPH and d_3-DNPH, respectively. Besides to demonstrate the similar weight of the two factors, the sample analysis and the derivatization reaction, in contributing to the precision of the method, this experiment showed a comparable precision for both reagents. In the light of these results, we can consider the reactions with either DNPH or d_3-DNPH as two independent reactions of the same sample.

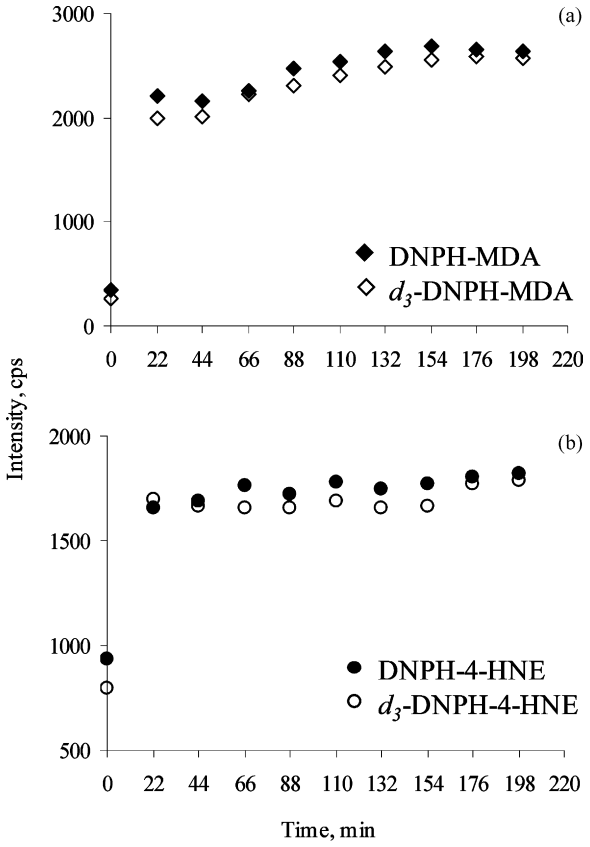

Fig. 3. Time course of the derivatization reactions of (a) MDA and (b) 4-HNE with DNPH and d_3-DNPH (1.2 mM for both). Aldehyde concentrations: MDA, 10 nM; 4-HNE, 2.5 nM.

The precision of the overall AIDA assay was established during method development by initially applying a highly conservative protocol, which required six independent determinations (three with deuterated and three with protonated standards) as initially proposed by Sforza et al. [7] and triplicate analyses for every sample. The %RSD of six independent reacted samples each injected in triplicate (6×3, $n = 18$) at six concentration levels was within 3.5–8% for MDA and 5–11% for 4-HNE. Since the high number of derivatizations and injections per sample could be considered as the main drawback of AIDA assay, particularly when a little sample volume is available (like in the case of EBC), we have verified that reducing the number of independent derivatizations to 4, or even to 2 and the number of injections to 2, did not result in a loss of analytical performances in terms of precision. In the case of 4 derivatizations × 2 analyses per sample (4×2, $n = 8$), the precision was within 3.7–7.2% for MDA and 4.5–10% for 4-HNE,

Table 1
Exhaled breath condensate analyses by AIDA and external calibration methods.

Sample	MDA (nM)			4-HNE (nM)		
	AIDA[a]	Ext. calibration	% Diff.[b]	AIDA[a]	Ext. calibration	% Diff.[b]
#1	7.25 ± 0.65	7.58 ± 0.19	4.5	3.06 ± 0.31	2.79 ± 0.68	9.7
#2	9.51 ± 0.49	8.98 ± 0.79	7.9	3.29 ± 0.33	2.99 ± 0.53	10.0
#3	11.00 ± 0.66	11.83 ± 1.04	7.0	4.28 ± 0.73	3.64 ± 1.13	17.6
#4	9.77 ± 0.72	9.22 ± 0.70	6.0	3.92 ± 0.83	3.44 ± 0.94	14.0
#5	10.49 ± 0.62	10.53 ± 0.70	9.5	3.33 ± 1.05	3.25 ± 0.53	2.5
#6	10.62 ± 0.98	11.04 ± 0.15	3.8	4.26 ± 0.37	3.62 ± 0.82	17.7
#7	12.22 ± 0.63	13.38 ± 0.52	8.7	3.19 ± 0.90	2.67 ± 0.70	19.5
#8	11.67 ± 0.76	12.57 ± 0.48	7.2	3.23 ± 1.09	3.29 ± 0.51	1.8
#9	11.61 ± 0.70	11.13 ± 0.50	4.3	3.82 ± 1.28	3.65 ± 0.63	4.7
#10	14.13 ± 0.86	14.18 ± 0.91	0.4	4.7 ± 0.99	4.17 ± 0.39	12.7

[a] Calculated after subtraction of the blank.
[b] % Diff, calculated as $[(Conc_{ExtCal} - Conc_{AIDA})/Conc_{ExtCal}] \times 100$.

whereas in the case of 2 derivatizations × 2 analyses per sample (2 × 2, $n = 4$) the precision was 3.2–6.5% for MDA and 4.8–8.6% for 4-HNE. In all cases, precision of the AIDA assay was comparable to that obtained with external calibration with measurement of calibrating standards prior, during, and at the end of the series, *i.e.*, 5.2% and 7.9% for MDA and 4-HNE, respectively [1]. Interestingly, the results of AIDA calculations differed by less than 1% by comparing the 6 × 3 or 4 × 2 or 2 × 2 sample preparation approaches. Since the precision of the AIDA assay for the quantification of MDA and 4-HNE in EBC is not affected by the number of independent derivatizations performed on the same sample, a simplified protocol with only two independent determinations (one with deuterated and one with protonated standards) and duplicate analyses could be proposed for aldehyde quantifications in authentic EBC samples. The resulting number of analyses per sample ($n = 4$) is comparable with that required by applying the external calibration method. The inter-day precision of the AIDA assay evaluated on QC samples and expressed as %RSD was <15% for both analytes.

Using the AIDA method, the unknown concentration of the analyte in the matrix can be determined without the need of a calibration curve [7]. However, during method development, it was found that none of the high-purity water lots from different suppliers tested was aldehyde-free. The aldehyde content of these high-purity water ranged between 1.3 and 2.5 nM for MDA and between 0.6 and 0.85 nM for 4-HNE. Therefore, the equations used for AIDA calculations have been modified to take into account the aldehyde content of the blank (see Section 3). The blank correction step is critical since the original AIDA calculations are actually based on a straight (calibration) line passing through two points, that is the origin and the point corresponding to the concentration of the standard. If the blank is not negligible, the area of the IS corresponding to the nominal concentration, *e.g.*, A_{HS} corresponding to q_{HS} in (1), is due to sum of the nominal concentration and the blank concentration of the standard. Since in the (original) AIDA calculations the straight line is forced through the origin, its slope would therefore increase, eventually leading to inaccurate and underestimated concentrations. As in both (1a) and (2a) the term corresponding to the IS area is in the denominator, both formulas used in the calculation of the unknown concentration of analyte would give the same underestimated result, without compensating the underestimation effect among each other. By correcting for the aldehyde concentrations in the blank, this effect is annihilated.

The accuracy of the method was tested by analyzing an EBC pool sample, whose aldehyde concentration (MDA: 10.62 nM, 4-HNE: 2.24 nM) had been previously determined by the standard addition method, spiked with the six calibrating standards used for external calibration. Good results (accuracy within 98–108% for MDA and 93–114% for 4-HNE) were obtained by AIDA after blank subtraction. Comparable results were obtained when QC samples were analyzed in the middle of a sample series and quantificated by external calibration.

Finally, a comparison of results obtained by both the AIDA method and external calibration applied to the same EBC samples are summarized in Table 1. Results obtained by two methods were in good agreement, with deviations <10% for MDA and <20% for 4-HNE, which is acceptable and in accordance to the recommendations by the Food and Drug Administration in the Guidelines for bioanalytical method validation [15].

5. Conclusion

This study demonstrates that the AIDA method, initially proposed for the derivatization of hydroxyl groups in zearalenone with acetic anhydride, is applicable to other analytes and reagents, *e.g.*, the derivatization of the aldehyde function with DNPH. Therefore, the AIDA approach appears to be potentially applicable to a wide range of compounds and derivatizing reactions. In the case of the determination of nM concentrations of MDA and 4-HNE in EBC samples, AIDA calculations have been adapted to take into account the aldehyde content of the blank, which is not negligible in the high-purity water used for standard preparation. Comparable results were obtained by AIDA and external calibration methods on the same samples, indicating that both methods are applicable to the quantification of low concentrations of MDA and 4-HNE in EBC. In terms of the number of analyses per sample, satisfactory precision was obtained by analyzing in duplicate two independently reacted sample, one derivatized with DNPH and the other with d_3-DNPH. The resulting number of analysis ($n = 4$) is comparable with that required with external calibration method. Finally, AIDA can compensate for matrix effects that are marginal in EBC samples, but not negligible in other more complex biological fluids, like plasma, urine, induced sputum, or bronchoalveolar lavage.

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