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# Alternate Isotope-Coded Derivatization Assay: An Isotope Dilution Method Applied to the Quantification of Zearalenone in Maize Flour**

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The chromatographic quantification of analytes in complex matrices such as food or feed can be problematic as a result of the possible presence of interfering agents. The application of liquid chromatography coupled to mass spectrometric detection (LC–MS) is particularly helpful in these cases given the high selectivity of mass detectors. However, the response of a given amount of analyte at atmospheric pressure interfaces is quite variable, as ionization and evaporation depend on the different eluent mixtures used and small instrumental variations. They are also affected by the co-elution of interfering agents, which may result in ion-suppressive effects.[1] Moreover, serious recovery problems may arise from the extraction procedures performed to concentrate the analytes. The best way to handle these problems, given the ability of MS to detect simultaneously different isotopes of the same molecule, is to use the isotope dilution approach, in which the same analyte containing with stable isotopes is used as an internal standard.[2] The evident drawback, however, is that stable, isotope-labeled analogues of most analytes are either not available, not completely labeled, or are very costly.

Herein, we present an innovative approach for the application of isotope dilution methods to the quantification of analytes for which stable isotope-labeled analogues are not available. We were inspired by the isotope-coded affinity tagging (ICAT) method, which is widely used for the detection of differential protein expression in living systems:[3] isotope-labeled molecules can be obtained by using isotope-labeled derivatizing reagents starting either from standards or samples. The purpose of this method is to determine a suitable derivative of the analyte that is obtained by derivatization with reagents that are available in two pure isotopic forms, "light" and "heavy" (for example, protonated or deuterated). In the approach proposed herein, two standard analyte solutions containing the same amount of standard are used in two derivatization reactions, one with the "light" derivatizing reagent and the other with the "heavy"

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reagent. The derivatized products are added to the matrix in two portions, with the "light" derivatized standard added to one half and the "heavy" derivatized standard to the other half. The derivatization procedure is then repeated on both matrices, thereby derivatizing the part containing the "heavy" derivatized standard with the "light" derivatizing reagent and the part containing the "light" derivatized standard with the "heavy" derivatizing reagent. Both mixtures are then extracted by conventional methods and analyzed by LC–MS, monitoring the "light"- and the "heavy"-derivatized analytes with the former as standard for the latter in one case, and vice versa in the other (Figure 1).

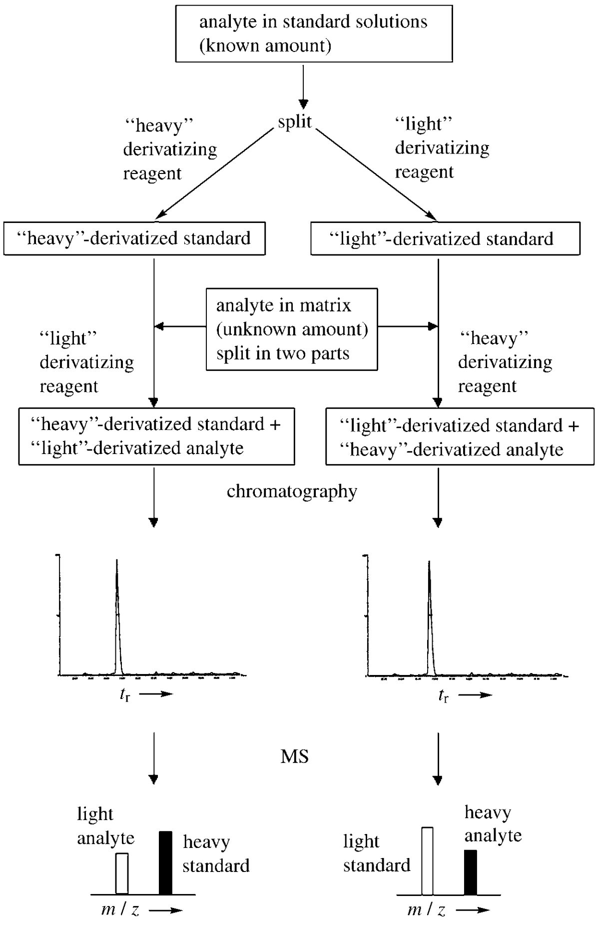


***Figure 1.*** Procedure for the application of the AIDA method.

In this way, two independent quantifications can be carried out on the same sample. We therefore termed this method the alternate isotope-coded derivatization assay (AIDA). The addition of the derivatized internal standard before the extraction procedure permits corrections for the recovery. In the LC–MS analysis, both derivatives, which are isotopomers are expected to have almost the same retention times. They will therefore experience the same instrumental and matrix conditions, thus allowing accurate results. This procedure may allow the adoption of a fast and simplified sample preparation technique that avoids loss of analytes, as any matrix interference during LC analysis will be corrected by the use of the isotopic internal standard. Indeed, the most important self-correction of the analytical data obtained in this way comes from the use of a double determination ("light" standard and "heavy" analyte and vice versa), which allows the determination of the relative response factor (the ratio of the response factor of the analyte to that of the standard) directly on the sample analyzed, without the need to calculate it from a calibration curve or by checking the linearity range.

The theoretical background is derived directly from the following simple calculations: for the analysis of molecule **A** in the presence of an internal standard **B**, quantitative data are given by Equation (1):

$$q_A = (R_{fA}/R_{fB}) \times (A_A/A^S_B) \times q^S_B \quad (1)$$

($q$ : quantity; $R_f$ : response factor; $A$ : area; the superscript S indicates the use as internal standard)

The relative response factor $R_r = (R_{fA}/R_{fB})$ usually must be calculated previously from a calibration curve. According to our AIDA method, **A** and **B** are isotopomers, and **B** is used as a standard for **A** in one case, and **A** as a standard for **B** in the other. In the latter case, Equation (1) can be written as Equation (2):

$$q_B = (R_{fB}/R_{fA}) \times (A_B/A^S_A) \times q^S_A \quad (2)$$

Dividing the two equations gives Equation (3):

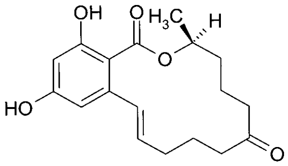
$$q_A/q_{B=}(R_{fA}/R_{fB})^2 \times (A_A/A^S_B) \times (A^S_A/A_B) \times (q^S_B/q^S_A) \quad (3)$$

Clearly, $q_A/q_B = 1$, as this value should always be the same regardless of whether the analyte has been treated with the "heavy" or the "light" derivatizing reagent. Moreover, also $q^S_B/q^S_A = 1$ if the "light" and "heavy" derivatized standards are used in the same amounts. Accordingly, Equation (4) can be derived from Equation (3), thus allowing the calculation of $R_r$.

$$(R_{fA}/R_{fB})^2 = (A^S_B/A_A) \times (A_B/A^S_A) \quad (4)$$

It is therefore possible to calculate the relative response factor directly from the area ratios **A/B** obtained from the two different determinations, in which **A** and **B** are alternatively used as analyte and internal standard. The quantitative data obtained can then be corrected according to the relative response factors ($R_{fA}/R_{fB} = R_r$ and $R_{fB}/R_{fA} = 1/R_r$). It is very important to ensure that the reaction gives the same conversion both for the matrix and the standard solution to avoid inaccurate results. Moreover, for the same reasons, both "light" and "heavy" derivatizing agents should also give the same conversion. Both these conditions appear to be easily achievable.

To test the applicability of the method, we addressed the quantification of the mycotoxin Zearalenone (ZEN) in food and feed. ZEN is a fungal toxin that is mainly produced by several *Fusarium* species, such as *F. culmorum, F. graminearum*, and *F. sporotrichioides*, which are known to colonize maize, barley, wheat, oats, and sorghum in both warm and temperate climates.[4] ZEN consists of a resorcinol moiety fused to a 14-membered macrocyclic lactone ring that includes a *trans* double bond, a ketone, and a methyl substituent on a stereogenic center. The structure is flexible enough to allow ZEN to adopt a conformation that is able to bind to the mammalian estrogen receptor; ZEN can therefore act as an endocrine disrupter.[5] The analysis of ZEN is commonly performed by applying a clean-up step with immunoaffinity chromatography (IAC) after extraction, followed by HPLC with fluorescence detection that takes advantage of the native fluorescence of this mycotoxin.[6] As an alternative to fluorescence detection, several LC–MS methods have been developed with various interfaces.[7] In all cases, however, quantification was hampered by the choice of an appropriate standard.



ZEN

To apply the AIDA method to the ZEN molecule, we chose as derivatizing reagent a compound that can easily react with the hydroxy functional groups, and which is commonly available in two isotopic forms: acetic anhydride and its deuterated [$D_6$] analogue. A standard solution of ZEN was derivatized either with [$H_6$]- or [$D_6$]acetic anhydride,[8] and LC–MS analysis[9] revealed only the presence of the [$H_6$]- or [$D_6$]bisacetylated compound, respectively, with no chromatographic peaks attributable to nonderivatized or monoacetylated toxin (Figure 2). NaCl was added to the eluent to enhance detection of the sodiated compounds according to a method recently published by our research group[10]: infusion experiments of ZEN and bisacetylated ZEN performed by adding NaCl to the solvent confirmed that, under these conditions, only the sodiated molecular ion is present; LC–MS experiments performed with and without NaCl added to the eluent showed an average 20-fold increase of the chromatographic peak intensity for the former relative to the latter case (data not shown).

The derivatization reaction was also repeated in food matrices by spiking several toxin-free maize flour samples with ZEN. The samples were blended and the toxins extracted in a 1:4 water/acetonitrile mixture, filtered, evaporated to dryness, and derivatized with acetic anhydride.[11] Again, only the bisacetylated compounds were observed by LC–MS both with [$H_6$]- and [$D_6$]acetic anhydride (Figure 2). To ensure that the AIDA method gives no artifacts, some important controls were performed. The most important was the stability of the acetyl groups during the derivatization reaction. As after the addition of the [$D_6$]bisacetylated standard to the food matrix, a second acetylation with [$H_6$]acetic anhydride is carried out (or vice versa), it is essential that the acetyl groups do not undergo exchange with each other. This control can be done rapidly in the case of ZEN: if an exchange does occur, it would be possible to detect bisacetylated molecules that bear one [$H_3$]acetyl group and one [$D_3$]acetyl group. As shown in Figure 3, no species of this type were detected after the derivatization reaction. Full-scan MS data of the [$D_6$]bisacetylated ZEN also confirmed that the protons on the acetyl groups do not exchange with the solvent, as the molecule shows only minimal traces of a [$D_5H$]compound that was already present in the starting [$D_6$]acetic anhydride preparation and no sign of other [$D_{6-x}H_x$]derivatives.

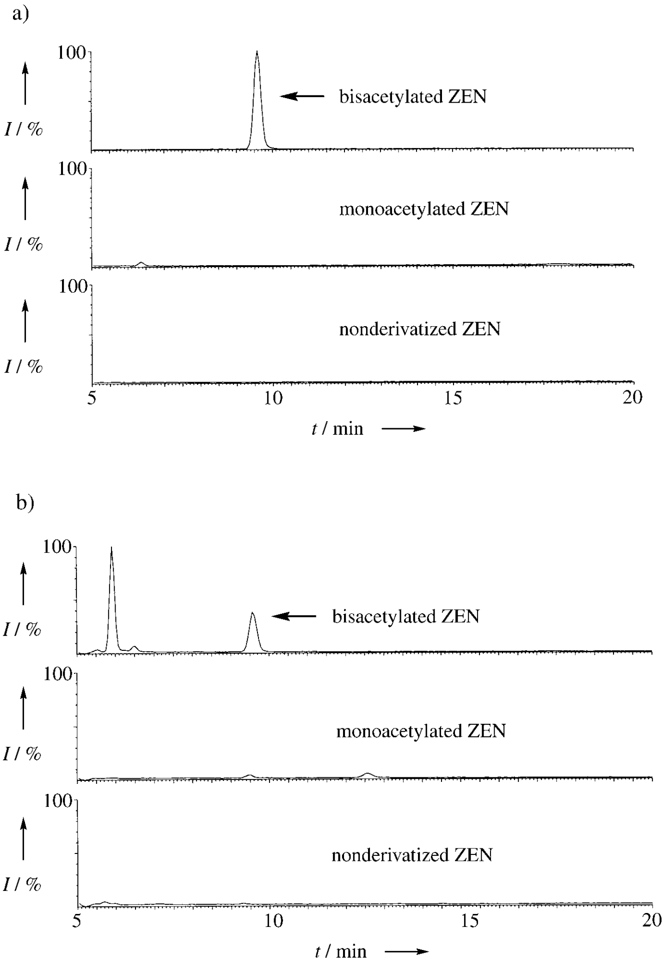


***Figure 2.*** LC–MS chromatograms (single-ion mode acquisition of sodiated molecular ions) of the acetylation reaction in a) standard solution and b) with spiked flour. Bisacetylated, monoacetylated, and nonderivatized ZEN were monitored, thus confirming that in both cases only the bisacetylated compound was present.

The above method was then applied to the determination of ZEN in several spiked maize flours by LC–MS.[11] The limits of detection (S/N = 3:1) and quantification (S/N = 10:1) of the method were determined by spiking toxin-free flour samples at the appropriate level and were found to be 5 ppb (absolute amount injected: 100 pg) and 10 ppb (absolute amount injected: 200 pg), respectively. Notably, a calibration curve was not calculated before the analyses, and six independent determinations (three with the deuterated standard and three with the protonated standard) were run for every sample. The chromatographic peak areas were then measured for both isotopes by extracting the chromatograms given by the corresponding sodiated ions. The response-factor ratios were calculated from Equation (4) and the amounts

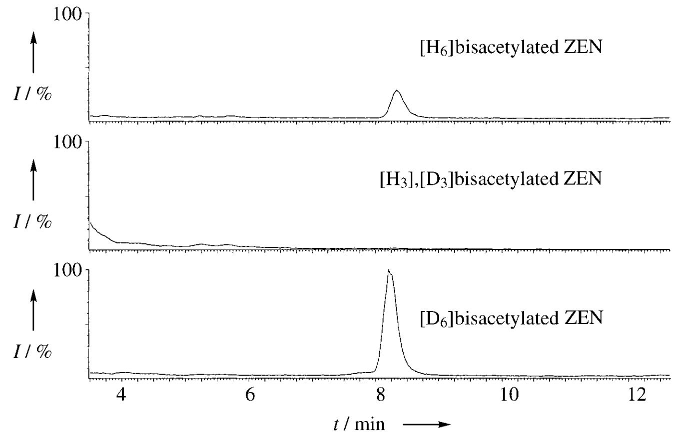


***Figure 3.*** LC–MS chromatograms (single-ion mode acquisition of sodiated molecular ions) of a flour preparation spiked with [$D_6$]bisacetylated ZEN and derivatized with [$H_6$]acetic anhydride. [$D_3$],[$H_3$]bisacetylated ZEN was not detected, thus indicating the absence of acetyl group exchange.

were then calculated from Equations (1) and (2). The true results were taken as the average of these six determinations. The data are reported in Table 1, together with the results for a FAPAS-certified reference material (FAPAS = food analysis performance assessment scheme). The results are quite outstanding, considering that no calibration curve or linearity tests were performed. The response ratio between protonated and deuterated compounds was calculated in every sample analyzed and are also reported in Table 1. These ratios always appear to be slightly, yet significantly, different from unity, which implies that the methodology proposed herein must be followed with the subsequent response-factor correction for the results to be accurate. These data constitute definitive proof of the validity of the AIDA concept. The chromatograms of the FAPAS-certified reference material corresponding to the two cases ([$H_6$]acetylated ZEN as standard with [$D_6$]acetylated ZEN as analyte and [$D_6$]acetylated ZEN as standard with [$H_6$]acetylated ZEN as analyte) are shown in Figure 4. Notably, in all these cases, no IAC purification was carried out before the LC–MS analyses, which is usually performed before ZEN analysis by LC-FLD (FLD = fluorescence detection), which demonstrates that the acetylation procedure used is also suitable for the detection of ZEN in natural samples by using raw extracts. This allows one to avoid the cumbersome and expensive IAC clean-up procedure that can also prevent a full recovery of the analyte.

***Table 1:*** Determination of ZEN in spiked samples and in a FAPAS-certified reference material.

| Quantity spiked [ppb] | Quantity detected [ppb] | Average response factor ratio [$H_6$]$Ac_2$-ZEN/[$D_6$]$Ac_2$-ZEN |
|---|---|---|
| 10 | 13 ± 3 | 1.20 ± 0.14 |
| 50 | 48 ± 7 | 1.20 ± 0.17 |
| 100 | 104 ± 5 | 1.26 ± 0.05 |
| 500 | 516 ± 58 | 1.34 ± 0.05 |
| 1000 | 1039 ± 72 | 1.41 ± 0.02 |
| certified flour (648 ± 140) | 709 ± 24 | 1.07 ± 0.04 |

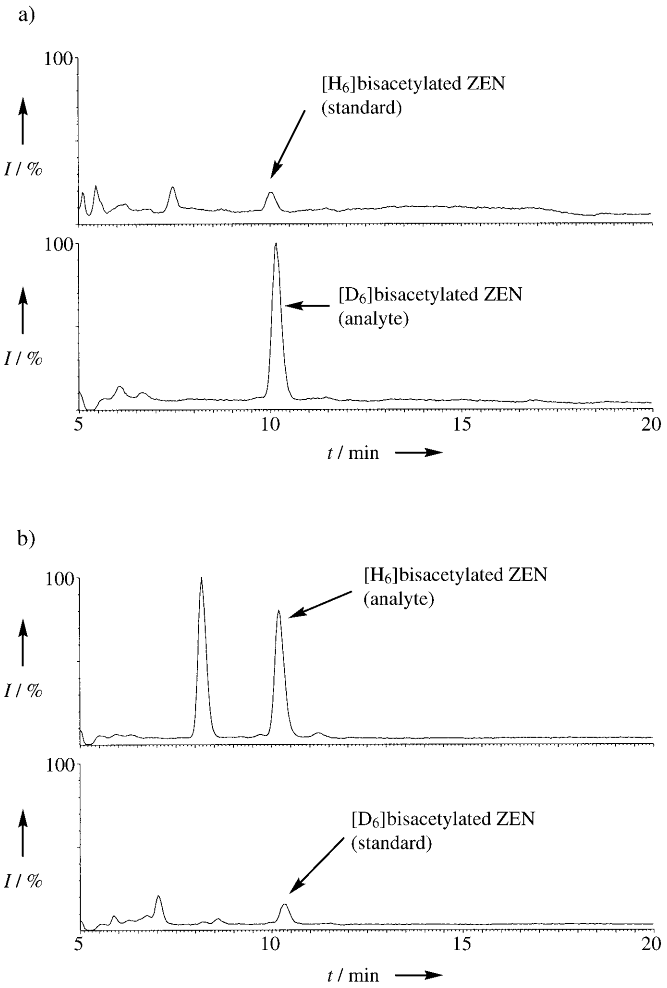


***Figure 4.*** LC–MS chromatograms (single-ion mode acquisition of sodiated molecular ions) of a) the FAPAS-certified flour with added [$H_6$]bisacetylated ZEN (standard, 100 ppb) and derivatized with [$D_6$]acetic anhydride and b) the same sample with added [$D_6$]bisacetylated ZEN (standard, 100 ppb) and derivatized with [$H_6$]acetic anhydride.

The data presented herein on the quantification of ZEN in flour samples demonstrate how the AIDA method may be applied for the accurate quantification of analytes in complex matrices, such as food or feed, by LC–MS. This method avoids errors due to instrumental and matrix effects, yields accurate results with minimal sample preparation, and exploits all the advantages of isotope dilution methods in a low-cost, easy-to-perform procedure, although two independent experiments have to be performed for each sample analyzed. This procedure is currently being extended to the detection of other mycotoxins in food samples.

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[8] Procedure for the preparation of derivatized standards: 100 µL of a 10-ppm standard solution of ZEN in dry acetonitrile was added to 35 µL triethylamine, 270 µL dry acetonitrile, and 95 µL either [$H_6$]- or [$D_6$]acetic anhydride. The mixture was left at room temperature for 60 min, then evaporated to dryness and redissolved in 1 mL water/acetonitrile (1:4) to be added to the food/feed samples or directly analyzed by LC–MS.

[9] LC–MS conditions: C18 column (Waters, 250 × 4.6, 5 µm) on a Waters Alliance 2695 separation module, isocratic elution with 0.2 mM aqueous NaCl/$CH_3OH$ (35:65); flow: 1 mL min$^{-1}$. Injected volume: 10 µL. A Micromass ZMD mass spectrometer equipped with an ESI interface was used as MS detector by splitting 90 % of the flow with a T-valve before the probe. Acquisition in positive ion mode; capillary voltage: 3.5 kV (pos); cone voltage: 30 V (pos); source temperature: 80 °C; desolvation temperature: 150 °C; cone gas ($N_2$): 100 L h$^{-1}$; desolvation gas ($N_2$): 400 L h$^{-1}$. Acquisition in single-ion monitoring: $m/z = 425.3$ for sodiated [$H_6$]bisacetylated ZEN and 431.3 for sodiated [$D_6$]bisacetylated ZEN; dwell time: 0.5 s; span: 0.2. Acquisition in full scan: range: 150–1000; scan time: 3 s; inter-scan delay: 0.1 s.

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[11] Sample preparation: 10 g flour was mixed with 39 mL water/acetonitrile (1:4) and 1 mL of a water/acetonitrile (1:4) solution containing 1 µg of [$H_6$]- or [$D_6$]bisacetylated ZEN, prepared as reported in Ref. [8]. The mixture was blended for 2 min in a high-speed blender. After filtration through paper filters, the filtrate (4 mL) was dried under a nitrogen stream. The residue was redissolved in 35 µL triethylamine, 370 µL dry acetonitrile, and 95 µL [$H_6$]acetic anhydride (in the case of [$D_6$]bisacetylated standard) or [$D_6$]acetic anhydride (in the case of [$H_6$]bisacetylated standard). For acidic food matrices, which may give incomplete derivatization, the amount of triethylamine was doubled. The mixture was left at room temperature for 60 min, then evaporated to dryness and redissolved in 500 µL water/acetonitrile (1:4) to be analyzed by LC–MS.