Short communication

Determination of daminozide in apples and apple leaves by liquid chromatography–mass spectrometry

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Abstract

A straightforward and efficient method was developed for the determination of intact daminozide in apples and apple leaves. After extraction with methanol and a clean-up step using a graphitized carbon cartridge, the extract was analysed by ion-trap liquid chromatography–tandem mass spectrometry (LC–MS–MS) using atmospheric pressure chemical ionisation in the positive ion mode. Recoveries for apple were 98–102% with a R.S.D.≤11% ($n=6$) and for leaves were 112–116% with a R.S.D.≤18% ($n=6$). The limits of detection were 0.008 and 0.02 mg/kg for apples and leaves, respectively. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Daminozide (*N*-dimethylaminosuccinamic acid) is a plant growth regulator that can be used on several crops, including apples, potatoes, ground nuts and ornamentals. It is water-soluble and readily translocated to all parts of the plant. The application of daminozide in apple cultivation increases flower development, reduces premature fruit fall, and improves size, colour development and storage features [1].

Residues of daminozide in apples can be detected up to one year after application [2]. The parent compound is the only residue in (stored) fresh apples and the main residue in apple products like apple sauce and juice. In the latter, following heating during food processing, the degradation product, 1,1-dimethylhydrazine (UMDH), a toxin and potential carcinogen, can also be formed at a level of 2 to 8%

of the amount of daminozide present in the sample [3,4].

In the Netherlands, the application of daminozide has been restricted to ornamentals since 1996 and the maximum residue limit for daminozide in apples has been set to 0.02 mg/kg. Besides residue control on apples, the application of daminozide can also be demonstrated by analysing the leaves.

Daminozide cannot be directly analysed by gas chromatography (GC) due to its high polarity and thermolabile nature. Although determination of intact daminozide after esterification has been described [5], the most frequently applied methods involve an alkaline hydrolysis of daminozide to UMDH [3–10]. The degradation product is then derivatized with pentafluorobenzyl bromide [3], salicylaldehyde [6] or 2-nitrobenzaldehyde [7–10]. After a clean-up step, the extract is analysed by GC with either electron-capture (ECD), nitrogen–phosphorus (NPD) or mass spectrometric (MS) detection. Liquid chromatography (LC) allows the direct determination of

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daminozide, but the lack of a chromophoric group renders UV detection troublesome. The use of LC–MS would solve this problem, but, so far, only one application (apple juice), using a particle beam interface, has been described [11].

This paper describes a method for the determination of (intact) daminozide in apples and leaves using LC coupled to an ion-trap MS–MS with atmospheric pressure chemical ionisation (APCI). Compared to existing methodologies, the method is straightforward and involves a minimum of sample preparation.

2. Experimental

2.1. Chemicals

Daminozide (99%) was purchased from Promochem (Wesel, Germany). Methanol and water (both of HPLC grade) were obtained from Malinckrodt-Baker (Deventer, Netherlands). Daminozide standard solutions were prepared in methanol and stored in the dark at 4°C.

2.2. Instrumentation

The LC–MS system consisted of a LC pump and autosampler (model 2690 Alliance, Waters, Ettenleur, Netherlands) and a LC-Q bench top mass spectrometer (Thermoquest, Veenendaal, Netherlands). Liquid chromatography was performed on a 100×3 mm I.D., 5 μm aminopropyl column (Hypersil APS-2) with a Chromsep guard column (10×2 mm I.D.), both obtained from Chrompack, Middelburg, Netherlands. For optimisation of mass spectrometric detection, 50 μl of standard solutions were introduced using methanol–water (50:50, v/v) containing 10 mM of ammonium acetate as the eluent. In the case of apple and apple leave extracts, gradient elution was performed starting with 100% of solvent A (methanol) for 2 min, then to 100% solvent B [methanol–water (25:75, v/v) containing 10 mM ammonium acetate] in 1 min. The isocratic mode was used then for 7 min, and then the eluent was switched back to 100% solvent A in 1 min. The column was conditioned with solvent A for 9 min

before injection of the next sample. The total run time was 20 min. The flow-rate was 0.5 ml/min.

The LC-Q was used with APCI. Typical interface parameters were as follows: vaporizer temperature, 450°C; heated capillary temperature, 200°C; sheath gas, 40%; auxiliary gas, 5%; corona, 5 μA; capillary voltage, 38 V and tubelens offset, 10 V. The ion-trap instrument was used in positive ion MS–MS mode with precursor ion m/z 161 and product ion m/z 143. The ion injection waveform was on. The isolation width was 1.5 u and the relative collision energy 8%. The system was used with standard resolution settings, i.e. peak width of 0.7 u (at 50% peak height) for the low mass ions up to m/z 1200.

2.3. Sample preparation

Apples or leaves were chopped and homogenised in a food cutter. Extraction of 25 g of the homogenate was performed with methanol (50 ml for apple, 100 ml for leaves) in a centrifuge tube for 60 s with an Ultra Turrax. After centrifugation (5 min at 2000 rpm, 500 g), 2 ml of the extract were percolated through an unconditioned graphitized non-porous carbon solid-phase extraction cartridge (Supelclean Envicarb, 6 ml, 500 mg; Supelco, Leusden, Netherlands) and collected directly into an autosampler vial. A 250-μl volume of the extract was subjected to LC–MS–MS analysis.

3. Results and discussion

3.1. LC–MS–MS analysis of daminozide

During optimisation of mass spectrometric detection, electrospray (ESP) as well as APCI, both in positive and negative ion mode, were evaluated. A better signal-to-noise ratio was obtained using the APCI interface. The response in the positive and negative modes was similar for standards. However, in the case of spiked extracts, the signal-to-noise ratio decreased remarkably when using the negative ion mode. Matrix constituents prohibited ionisation of daminozide. Therefore, the positive ion mode was used in all further experiments.

Daminozide gave two main ions corresponding to $[M+H]^+$ and $[M+H-H_2O]^+$, i.e. m/z 161 and 143,

respectively (Fig. 1a). For optimum selectivity, selected reaction monitoring was performed with $[M+H]^+$ as the precursor ion. The product ion spectrum obtained, which is shown in Fig. 1b, consisted of an ion at m/z 143 (100%), corresponding to the $[M+H-H_2O]^+$ ion, and a second ion at m/z 61 (6%), corresponding to $[CH_3COOH+H]^+$.

When optimizing the LC eluent, it was found that daminozide was more strongly retained on the aminopropyl column when the amount of methanol in the mobile phase was increased. This phenomenon offered the possibility of introducing relatively large sample volumes, i.e. daminozide will be focused at the head of the analytical column and eluted when switching from methanol to 10 mM ammonium acetate in methanol–water (25:75, v/v). In so doing, the injection volume could be increased from 50 to at least 500 μl of a standard solution (dissolved in methanol–water; 70:30, v/v) without any band-broadening effect. The increased injection volume eliminated the need for a time-consuming evaporative concentration step while still achieving the desired detection limit. The possibility of large volume injection was the main reason for the selection of methanol as the extraction solvent rather then water, although with the latter solvent, cleaner extracts were obtained, especially for the leaf samples.

In order to remove co-extractants, like chlorophyll, from the methanol extract, a very straightforward, but effective clean-up step using an Envicarb cartridge was implemented. The clean-up reduced the background and also increased the response for daminozide (see matrix effects below).

The introduction of 500 μl of apple extract (containing water and methanol) spiked with daminozide into the LC–MS system led to a shift (decrease) in the retention time of daminozide compared to standard solutions, because the water content was too high, resulting in an early breakthrough of daminozide, i.e., a shorter retention time. This effect was not observed when restricting the injection volume to 250 μl and, therefore, this volume was used for the rest of the study.

3.2. Matrix effects

It is known that the matrix can affect the response obtained in LC–MS [12]. To evaluate this for the current application, the response of daminozide in blank apple and leaf extracts was compared to that of the same amount of daminozide in eluent. As can be seen from Fig. 2a and c, strong suppression of the response was observed in the case of the apple extract. In the case of the leaf extract, the effect was even more pronounced. With the system used, a supplementary voltage could be applied to the endcap electrodes of the ion trap to eliminate unwanted ions from the trap, e.g. matrix ions, while retaining the ions of interest. The application of this, so-called, ion injection waveform restored the response of daminozide in apple extract to approx. 80% of that in standard solution. (Fig. 2a–b). As expected, the ion injection waveform did not influence the response of daminozide in the standard solution (see Fig. 2c–d).

Nevertheless, with the waveform on, the response was still affected by the matrix. Different calibration curves were obtained for daminozide dissolved in eluent and in matrix (Fig. 3). The linearity of the system for daminozide, either in eluent or in matrix was good over two orders of magnitude ($R>0.998$ for eluent and apple, $R=0.982$ for leaves). Quantification, however, should be performed using standards prepared in the applicable matrix.

3.3. Analytical performance

Homogenised apple and leaf samples were spiked with daminozide at two levels in six-fold. After sample preparation, as described in Section 2, LC–MS–MS analysis was performed and the recoveries and repeatabilities were calculated. The results are given in Table 1. Quantitation was performed without the use of a deuterated internal standard because of the unavailability of such a compound. The recovery and repeatability were excellent for apple samples and were acceptable for leaf samples, especially taking into consideration that quantitation is done via external standard calibration. The limit of detection (LOD)[1] was calculated to be 0.008 mg/kg

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[1]LOD was determined at a S/N ratio of 3. Average noise (peak-to-peak) was determined from the analysis of blanks and standards. The instrumental detection limit, determined from injections of standard solutions in methanol–water, however, was around 100 pg of daminozide.
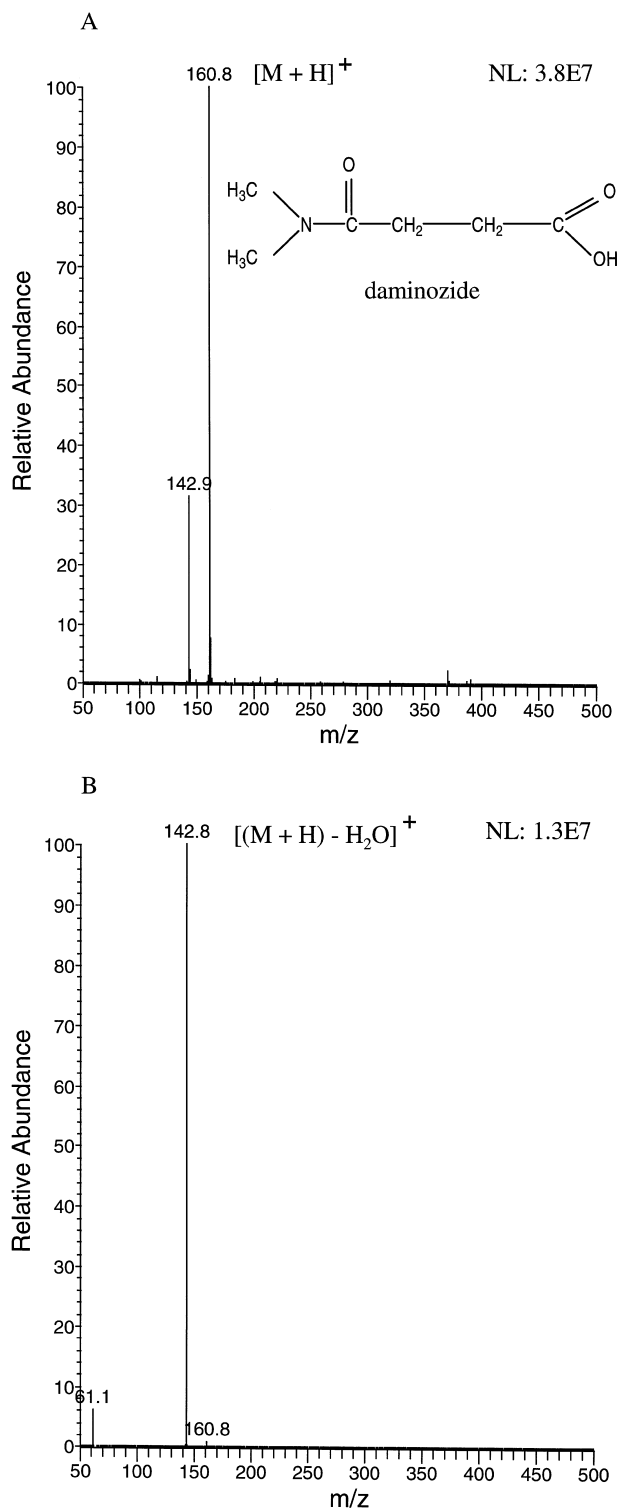
A

B

Fig. 1. (A) Precursor spectrum of daminozide and (B) product spectrum of daminozide using m/z 161 as the precursor.
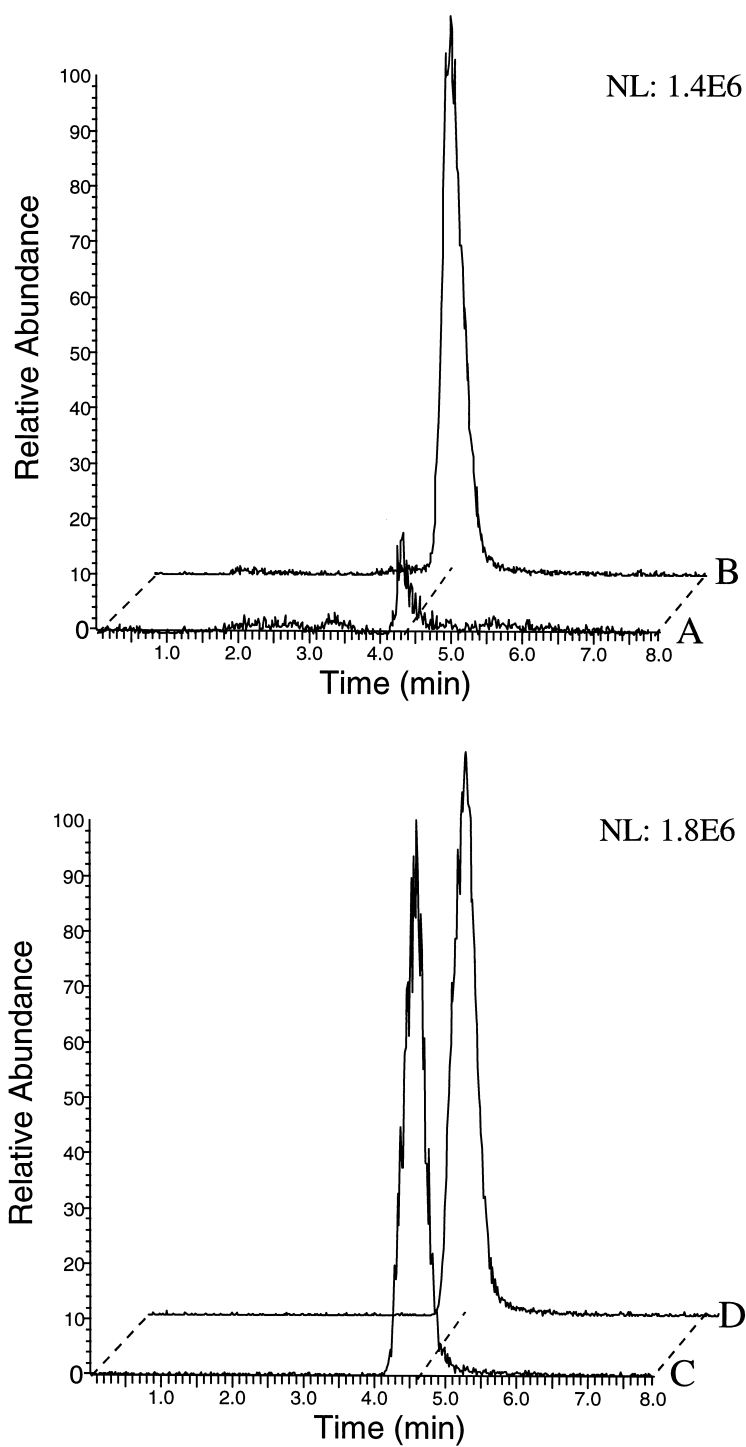
Fig. 2. Effect of ion injection waveform on the response of product ion m/z 143 of daminozide. (A) Daminozide in apple extract, waveform off, (B) daminozide in apple extract, waveform on, (C) daminozide in methanol–water (70:30, v/v), waveform off and (D) daminozide in methanol–water (70:30, v/v), waveform on.
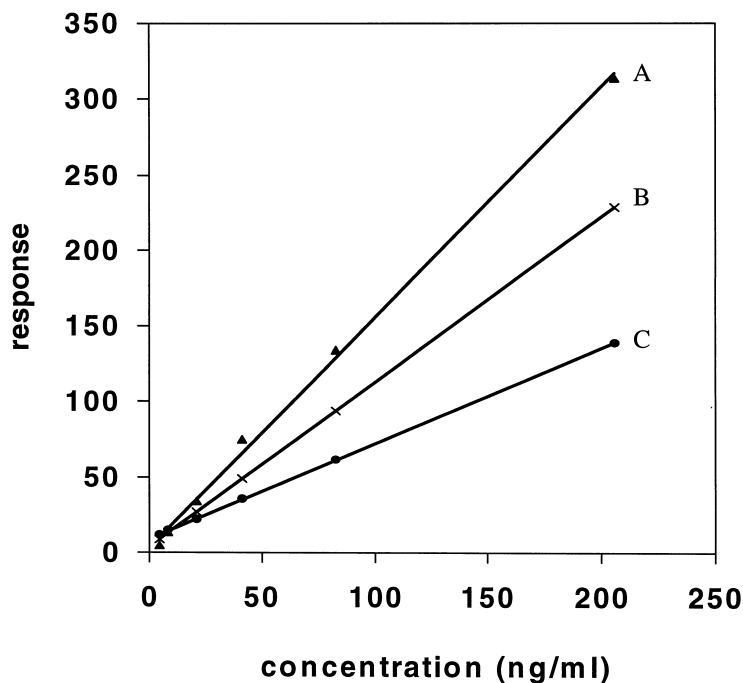
Fig. 3. Calibration curves of daminozide ($n=6$) in (A) methanol–water (70:30, v/v), (B) apple extract and (C) leaf extract.

for apples, which is below the European Union (EU) maximum residue limit of 0.02 mg/kg. In the case of leaves, the LOD was calculated to be 0.02 mg/kg. Although the sensitivity obtained for daminozide in leaves was slightly lower compared to apples (Fig. 3), the main reason for the higher LOD was that the leaves were extracted with twice the amount of methanol.

Fig. 4 shows chromatograms of a blank apple sample, two spiked apple samples and a spiked leaf sample. With the exception of a small peak at 11.7 min, no other peaks appeared in the chromatogram. When analysing leaf extracts, the retention time of

daminozide increased slightly and caused daminozide to co-elute with the small interference at 11.7 min. The reason for this increase in retention time lies in the fact that the apple leaf extracts contained more methanol and, therefore, daminozide was trapped better on the column upon injection and eluted later.

The system was used to analyse apple and leaf samples. No maintenance (exchange of heating capillary, etc.) was required during the analysis of over one hundred samples, indicating that the method is reliable and robust and is therefore applicable to routine analysis.

Table 1
Recovery and repeatability of analysis of apples and leaves

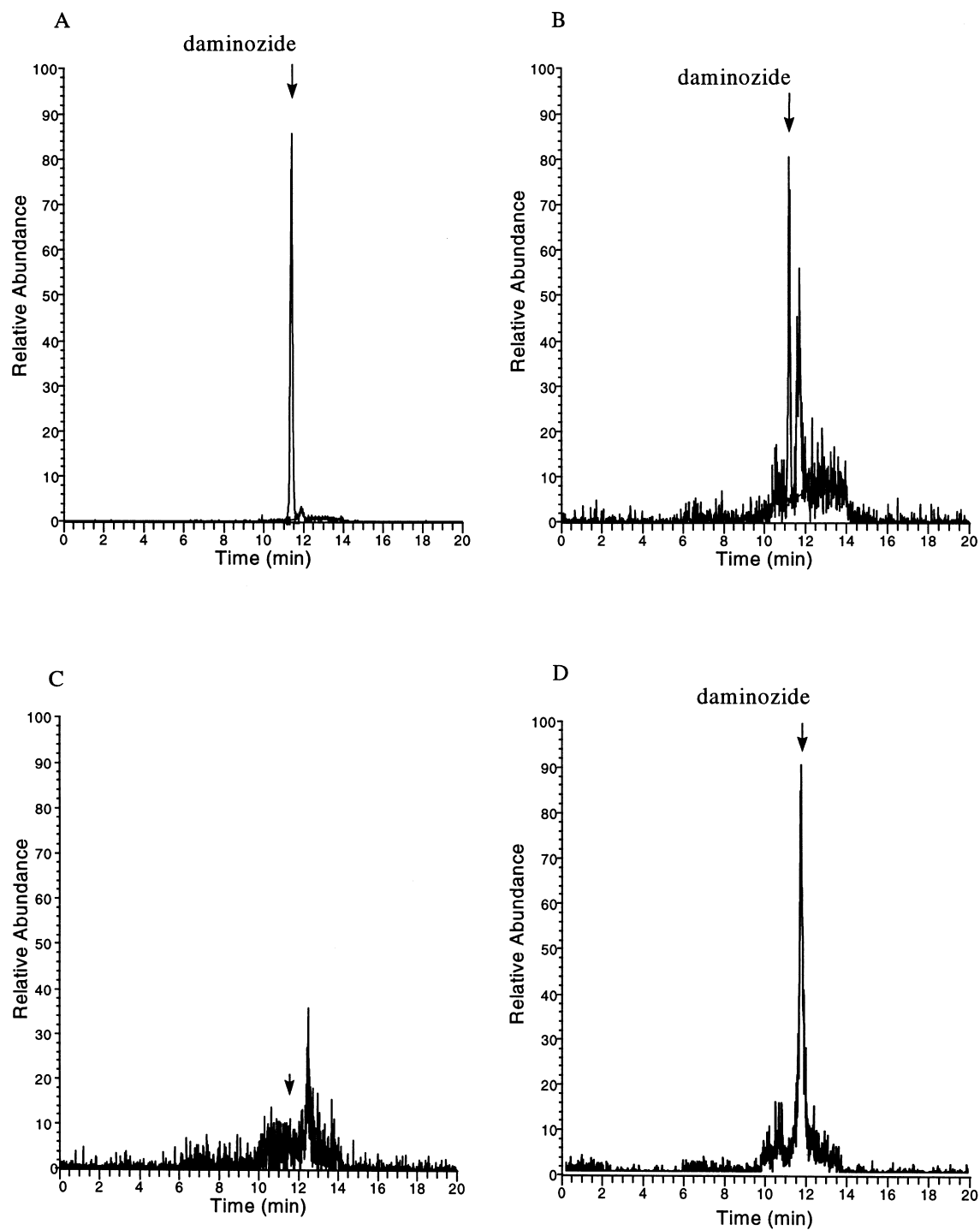| Sample	Level (mg/kg)	Recovery (%) ($n=6$)	R.S.D. (%) ($n=6$)
Apple	0.02	102	11
	0.5	98	7.1
Leaves	0.1	112	18
	1.0	116	15

Fig. 4. LC–MS–MS chromatograms of daminozide in apple and leaf samples. (A) Apple spiked at 0.5 mg/kg, (B) apple spiked at 0.02 mg/kg, (C) apple blank and (D) leaves spiked at 0.1 mg/kg.

4. Conclusion

The presented method enables the determination of (intact) daminozide in apples and apple leaves. The method is reliable and straightforward and much less time-consuming than the conventional method in which the hydrolysis product UMDH is measured. Because of the unavailability of a deuterated standard, quantitation was performed by external calibration. Although higher R.S.D.s can be expected when external calibration is used, good and reliable results were obtained when standards were prepared in matrix. The total analytical performance of the method described follows good and accepted criteria in pesticide analysis.

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