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Determination of daminozide residues in apples using gas chromatography with nitrogen–phosphorus detection

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Abstract

A method was developed for the determination of daminozide in apples using gas chromatography (GC) with nitrogen–phosphorus detection (NPD). Daminozide is hydrolysed to 1,1-dimethylhydrazine (UDMH) by alkaline digestion. The UDMH generated is distilled from the apple matrix, derivatized with 2-nitrobenzaldehyde and analysed by GC–NPD. The limit of detection (LOD) and the limit of quantitation (LOQ) for daminozide are 1.9 μg/kg and 6.2 μg/kg, respectively, in a 10-g sample. Average recoveries from apples spiked at the 12 and 60 μg/kg level are between 88% and 108% with R.S.D.s $\leq$6.9%. Additives used by others, such as calcium chloride, titanium chloride or zinc, have no positive effect on the extraction of daminozide from samples with incurred residues. The repeatability for these samples, containing 0.018, 0.13 and 1.8 mg/kg daminozide, is 4.0%, 4.4% and 2.5%, respectively.

Keywords: Derivatization, GC; Apples; Daminozide; 1,1-Dimethylhydrazine; Residue analysis

1. Introduction

Daminozide (N-dimethylaminosuccinamic acid, Alar) is a plant growth regulator used to improve the harvest quality of several fruits and vegetables. It is absorbed by the leaves and translocated throughout the plant. Daminozide is applied on apples to restrict vegetative growth, to increase flower development, to reduce premature fruit fall, to control fruit size, to synchronize maturity and to improve colour development and storage properties [1].

Daminozide (Fig. 1, 1) has been identified as a possible carcinogen [2], while its metabolite UDMH (Fig. 1, 2) has been identified not only as a toxin [3] but also as a potential carcinogen [4,5]. Although studies on daminozide degradation have shown hydrolysis of daminozide to UDMH in apples and apple products that had been subsequently boiled [6–8], no UDMH residues have been observed in (stored) fresh apples [7,8].

Several methods of analysis are available for the determination of daminozide residues in apples. Most methods are based on alkaline hydrolysis of daminozide to UDMH [6–13], followed by complexation of the UDMH generated with trisodium pentacyanoamine ferroate

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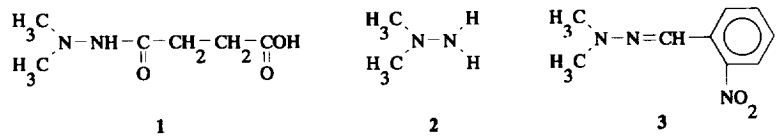

1 2 3

Fig. 1. Structures of daminozide (1), 1,1-dimethylhydrazine (2) and 2-nitrobenzaldehyde dimethylhydrazone (3).

[7,9], or derivatization with either pentafluorobenzoyl chloride [6,12,16], salicyl aldehyde [10,11,13] or 2-nitrobenzaldehyde [7,8,14]. The complex was determined by spectrophotometry [7,9], while analysis of the derivatives was performed by GC using electron-capture detection (ECD) [6–8,14], NPD [10,16] or mass spectrometric (MS) [11–13] detection. Direct conversion of daminozide to its methyl ester prior to GC–MS determination has also been applied [15].

Daminozide was determined in our laboratory by GC–NPD analysis of the pentafluorobenzoyl derivative of UDMH [16]. This method was sensitive enough to quantitate daminozide in apples below the Dutch maximum residue limit of 5 mg/kg [17]. However, since our laboratory became involved in the control of apples intended for export, residues of daminozide had to be quantitated at a level of 0.02 mg/kg. Although the sensitivity of our method was satisfactorily improved by using ECD instead of NPD detection, a clean-up was required due to matrix or reagent interferences. As recoveries turned out to be poor after clean-up, the method presented in this paper was developed. Sample pretreatment consists of alkaline hydrolysis of daminozide to UDMH, distillation of UDMH from the apple matrix and subsequent derivatization with 2-nitrobenzaldehyde to form 2-nitrobenzaldehyde dimethylhydrazone (Fig. 1, 3). Generation of a single, easily identifiable daminozide peak is achieved through analysis of the UDMH derivative using GC–NPD instead of GC–ECD [7,8,14]. Various validation parameters are reported, and the influence of the agents calcium chloride, titanium chloride and zinc on the extraction of daminozide was investigated.

2. Experimental

2.1. Chemicals

Daminozide (purity 99%) was purchased from Promochem (Wesel, Germany). UDMH (purity 98%) and 2-nitrobenzaldehyde were obtained from Aldrich (Steinheim, Germany). Methanol (analyzed HPLC reagent) was obtained from J.T. Baker (Deventer, Netherlands). 2,2,4-Trimethylpentane (nanograde) was obtained from Mallinckrodt (Paris, KY, USA). Demineralized water was used throughout the study. L-(+)-Ascorbic acid, sodium hydroxide (NaOH) and hydrochloric acid (HCl, 37%) were purchased from Merck (Darmstadt, Germany).

A daminozide stock standard solution (0.30 mg/ml) and a daminozide working solution (6.0 μg/ml) were prepared in water. A stock standard solution of UDMH (1.2 mg/ml) was prepared in 1 *M* HCl. Two UDMH working solutions (12 μg/ml and 0.12 μg/ml) were prepared in 0.05 *M* HCl. Calibration solutions were prepared by derivatization of 20 μl 12 μg/ml and 500 μl 0.12 μg/ml UDMH working solutions as described under Derivatization.

Nitrobenzaldehyde reagent (20 mg/ml) was prepared in methanol, and an ascorbic acid solution (60 mg/ml) was prepared in water; both solutions were prepared daily.

Caution: UDMH is a suspected carcinogen and 2-nitrobenzaldehyde is poisonous. All work should be carried out in a fume cupboard. Furthermore, appropriate safety measures must be taken to protect eyes and skin from contact with caustic contents during distillation and cleaning of the glassware.

2.2. Instrumentation

Distillation apparatus

The distillation apparatus and set-up were as described [11], except for the stirring hot plate and the 1000-ml distillation flask. A 500-ml electric heating mantle (Heraeus, Heidelberg, Germany) was used instead of a stirring hot plate, while a 500-ml round-bottomed flask with a 24/29 ground glass joint was used as distillation reservoir.

Gas chromatograph

A Fisons HRGC Mega 2 (Milan, Italy) gas chromatograph, equipped with an AS-800 autosampler and two thermionic NPD-80 detectors, was used. Two fused-silica capillary columns, a DB-1701 (30 m × 0.25 mm I.D.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) and a CP-Sil 5 CB (25 m × 0.25 mm I.D.; 0.4 μm film thickness; Chrompack, Middelburg, Netherlands), were connected by a twin-hole ferrule to the injection port. The temperature programme of the oven was as follows: 95°C isothermal for 2 min, to 200°C at 30°C/min, followed after 2 min by 20°C/min to 260°C, and then 15 min isothermal at 260°C. When the program was finished, the oven was cooled down to 95°C, taking no stabilization time into account prior to the next injection. Helium was used as carrier gas with a head pressure of 140 kPa. The injection port temperature was 220°C and the injection volume was 8 μl. Splitless injection was applied with the valves closed for 20 s. The detector gases hydrogen and air had a flow-rate of 35 and 490 ml/min, respectively, while helium was used as make-up gas at 40 ml/min. The temperature of the detector base was 280°C.

2.3. Sample preparation

Hydrolysis and distillation

The apple samples were chopped up in a food cutter and 10 g homogenate was weighed into a 500-ml round-bottomed flask. Successively, 40 ml 50% aqueous NaOH, 10 g NaOH pellets and boiling stones were added to the homogenate. The flask was immediately coupled to the distillation equipment of which a 25-ml measuring cylinder, used for collection of the distillate, had already been filled with 10 ml ascorbic acid solution. The sample was slowly heated to the boiling point in about 30 min, followed by refluxing for 30 min. Next, the temperature of the system was increased slightly to distil the UDMH formed. Distillation lasted for about 15 min until the receiving cylinder contained 20 ml of liquid.

Derivatization

The contents of the receiving cylinder were poured into a 35-ml glass tube and 1 ml 3 *M* NaOH was added. The contents of the tube were mixed so that the yellow colour of the liquid disappeared. Thereupon, 5 ml nitrobenzaldehyde reagent was added to the glass tube and the entire contents were mixed by turning the tube three times. The tube was placed in a water-bath for 2 h at 30°C. After incubation, 2 ml trimethylpentane was added to the "warm" tube and the contents were vortex-mixed for 10 s. When the layers had separated, about 0.5 ml of the upper layer was transferred to a 0.8-ml injection vial. Two calibration solutions, mentioned in Chemicals, were prepared with every series of samples.

2.4. GC determination

The GC system was stabilized by injecting a sample extract prior to the injection of the calibration solutions and the samples. Quantitation was performed by comparing the peak heights of the UDMH derivative in the sample to that of a calibration solution. The amount of daminozide in the sample was calculated as the average result of both columns.

2.5. Validation

Linearity of the GC system

Calibration curves were prepared by injecting six derivatized working solutions of UDMH over a concentration range of 5.7 to 285 pg/μl. The

peak heights were plotted against the concentrations in the UDMH working solutions for both GC columns.

Calculation of detection limit and quantitation limit

The LOD and the LOQ were determined by calculating the amount of daminozide required to produce a response that is three and ten times, respectively, the average of the baseline noise from eight unfortified apple samples. The response was measured from a calibration solution containing 120 ng/ml UDMH which corresponds to 64 μg/kg daminozide.

Determination of repeatability

The repeatability of the method was determined in two ways, i.e. by recovery studies and by multiple analysis of samples with incurred daminozide residues. Recovery studies were performed at fortification levels of 12 and 60 μg/kg by adding 20 μl or 100 μl daminozide working solution, respectively, to six 10-g portions of apple sample. At both levels, six spiked and two unspiked portions were assayed at the same day as described under Sample preparation and GC determination.

Repetitive analysis of three apple samples containing 0.018, 0.13 and 1.8 mg/kg daminozide was performed by determination of eight 10-g sample portions according to Sample preparation and GC determination.

Determination of reproducibility

The reproducibility of the method was determined by recovery studies at fortification levels of 12 and 60 μg/kg. Six 10-g portions of an apple sample were weighed. Two portions were spiked with 20 μl daminozide working solution (12 μg/kg), two portions were spiked with 100 μl daminozide working solution (60 μg/kg) and two portions were not fortified. The six portions were assayed as described under Sample preparation and GC determination. This procedure was performed eight times on various days over a one-month period, and the reproducibility of the method was calculated from the averages of the eight duplicate determinations.

3. Results and discussion

Prior to the development of this method, the derivatization of UDMH with salicyl aldehyde [10,11] was tested. Although recently described modifications of the derivatization reaction [13] were applied, determination of the UDMH-salicyl aldehyde derivative by GC–NPD was not sensitive enough to quantitate 0.02 mg/kg daminozide in apple without concentrating the extract.

3.1. Optimization of sample preparation

The derivatization time of the reaction between UDMH and 2-nitrobenzaldehyde was investigated to find out if the derivatization reaction was completed within the 2 h reported [7,8,14]. A solution containing 60 ng UDMH was derivatized in a water-bath at 30°C for different reaction times, i.e. 15 s, 5, 10, 15, 30, 45, 60, 90 and 120 min. The derivatization was completed in about 90 min.

Addition of agents such as calcium chloride, titanium chloride and zinc during extraction of the daminozide from samples is reported in several papers [8,9,11,13]. The influence of these additives was investigated by analysing in duplicate an apple sample with incurred daminozide residue. Prior to hydrolysis and distillation, calcium chloride, titanium chloride or zinc, or combinations of these three agents, were added to a sample containing 0.13 mg/kg daminozide. No positive effect of these agents was observed on the extraction of daminozide from the sample (cf. Table 1).

Table 1
Influence of reagents on extraction of daminozide ($n = 2$)

Reagents added	Extraction (%)
None	100
$CaCl_2 \cdot 2H_2O$ (15 g)	105
$CaCl_2 \cdot 2H_2O$ (35 g)	80
$CaCl_2 \cdot 2H_2O$ (15 g) + 20% $TiCl_3$ (5 ml)	97
20% $TiCl_3$ (5 ml)	101
20% $TiCl_3$ (5 ml) + Zn (7 g)	88

3.2. Optimization of GC determination

Determination of the UDMH derivative is usually performed by GC–ECD [7,8,14]. Nevertheless, an additional clean-up on aluminum oxide was required, otherwise co-extractives interfered with the determination of the UDMH derivative. A clean-up step was not necessary in our method as NPD instead of ECD was employed to determine the UDMH derivative. The chromatograms obtained from both GC columns contained numerous reagent peaks except on the place where the derivative eluted (see Fig. 2).

An aliquot of the extracts could directly be injected onto the GC system as trimethylpentane was used as extraction solvent. Unlike some methods reported previously [6,8,11–13], there was no need to evaporate the extracts prior to GC determination. Furthermore, the trimethylpentane extracts of the derivatized samples retained their concentration for 14 days if stored at 4°C.

3.3. Linearity of the GC system

Linearity of the GC–NPD response was observed in the range investigated, corresponding to 3.0–152 μg/kg of daminozide in apple. The

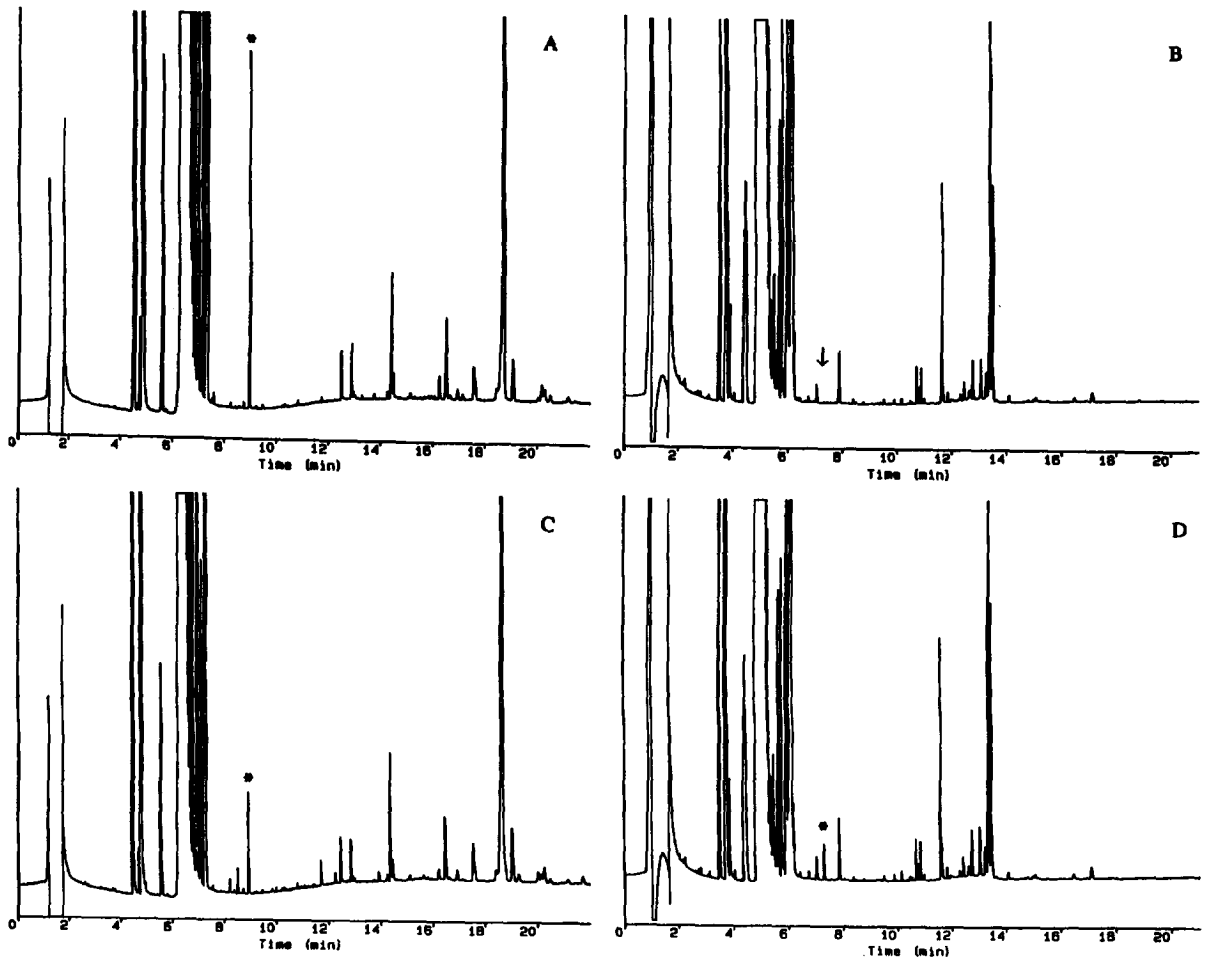

Fig. 2. Chromatograms of (A) calibration solution (64 μg/kg daminozide), (B) unfortified apple sample, (C) apple sample containing 18 μg/kg daminozide, (D) apple sample fortified with 12 μg/kg daminozide (same sample as in B). Conditions: (A, C) GC column, DB-1701; (B, D) GC column, CP-Sil 5 CB; all other conditions as described in the Experimental section.

response on the CP-Sil 5 CB column had a linear correlation coefficient (r) of 0.9993 ($n = 6$), while r was 0.9995 ($n = 6$) for the response on the DB-1701 column.

3.4. Detection limit and limit of quantitation

The LOD of the method varied from 1.5 to 1.9 μg/kg daminozide and the LOQ was 4.9–6.2 μg/kg daminozide, depending on the conditions of the GC system and based on a 10-g sample. The LOQ of the analysis method is comparable to the LOQs obtained by other methods employing derivatization of UDMH with 2-nitrobenzaldehyde [7,8,14]. However, these methods require an additional clean-up prior to GC–ECD determination.

3.5. Repeatability

The recovery studies of an apple sample fortified at 12 μg/kg showed that the average recovery was 108% ($n = 6$) with a precision of 6.3% R.S.D.. At a fortification level of 60 μg/kg, the average recovery was 88% ($n = 6$) with a R.S.D. of 13%. The precision of repetitive analysis of three apple samples containing 0.018, 0.13 and 1.8 mg/kg daminozide was 4.0% ($n = 7$), 4.4% ($n = 8$) and 2.5% ($n = 8$) R.S.D., respectively (a chromatogram of the first apple sample is shown in Fig. 2). All recovery and precision values presented here are within internationally accepted limits for performance of analytical methods [18,19]. Hence, the presented method of analysis is repeatable, in contrast to previously reported methods [8,12,13,15] that lack accuracy and/or precision to quantitate daminozide at a level of 0.01 mg/kg.

3.6. Reproducibility

Recovery data obtained from studies performed on various days showed an average recovery of 104% ($n = 8$) with a precision of 5.5% R.S.D. for a sample spiked at 12 μg/kg. The average recovery of an apple sample spiked at 60 μg/kg was 99% ($n = 8$) with a R.S.D. of 6.9%. Chromatograms of an unfortified and a fortified sample can be seen in Fig. 2. In conclusion, the method of analysis is reproducible since the data are within internationally accepted limits for analytical methods [18,19].

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