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Determination of maleic hydrazide residues in garlic bulbs by HPLC

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ABSTRACT

In recent years, the release of information about the preventative and curative properties of garlic on different diseases and their benefits to human health has led to an increase in the consumption of garlic. To meet the requirements of international markets and reach competitiveness and profitability, farmers seek to extend the offer period of fresh garlic by increasing post-harvest life. As a result, the use of maleic hydrazide (1,2-dihydropyridazine-3,6-dione) [MH], a plant growth regulator, has been widespread in various garlic growing regions of the world. The present work was undertaken to develop and validate a new analytical procedure based on MH extraction from garlic previously frozen by liquid nitrogen and submitted to low temperature clean-up. The applicability of the method by analysis of garlic samples from a commercial plantation was also demonstrated. The influence of certain factors on the performance of the analytical methodology were studied and optimized. The approach is an efficient extraction, clean-up and determination alternative for MH residue-quantification due to its specificity and sensitivity. The use of liquid nitrogen during the sample preparation prevents the degradation of the analyte due to oxidation reactions, a major limiting factor. Moreover, the method provides good linearity (r^2: 0.999), good intermediate precision (coefficient of variation (CV): 8.39%), and extracts were not affected by the matrix effect. Under optimized conditions, the limit of detection (LOD) (0.33 mg kg^{-1}) was well below the maximum residue level (MRL) set internationally for garlic (15 mg kg^{-1}), with excellent rates of recovery (over 95%), good repeatability and acceptable accuracy (CV averaged 5.74%), since garlic is a complex matrix. The analytical performance of the methodology presented was compared with other techniques already reported, with highly satisfactory results, lower LOD and higher recoveries rates. In addition, the extraction process is simple, not expensive, easily executable and requires lower volumes of organic solvent. The proposed methodology removes the need of extensive typical laboratory extraction procedures, reducing the amount of time needed for pesticide analysis and increasing sample throughput. Adopting this method gives food safety laboratories the potential to increase cost savings by a suitable technique in routine testing to determine MH residues in garlic.

1. Introduction

In recent years, the release of information about the preventive and curative properties of garlic on different diseases and their benefits to human health has led to an increased consumption of garlic [1]. To meet the requirements of international markets and reach competitiveness and profitability, farmers seek to extend the offer period of fresh garlic by increasing post-harvest life. As a result, the use of maleic hydrazide (1,2-dihydropyridazine-3,6-dione) [MH] (Fig. 1), a plant growth regulator, has been widespread in various garlic growing regions of the world, such as North America [2] and South America [3,4].

The biological effects of MH on plant growth and flowering were first described by Schoene and Hoffman [5]. MH acts as an antagonist of pyrimidine bases [6] by suppressing meristematic activity in the shoot apex thus reducing the associated deterioration in onion bulbs [7]. Following further absorption and translocation, the MH that becomes fixed within the plant is not completely metabolized but converted into β-D-glucoside as its predominant soluble metabolite [8].

It has been demonstrated that a pre-harvest foliar spray of MH induces a striking inhibition of sprouting and greatly reduces storage losses in potatoes [9,10], onions [11] and carrots [12]. Attempts to establish optimal application conditions using different cultivars of garlic have been conducted in experimental trials [13], with no

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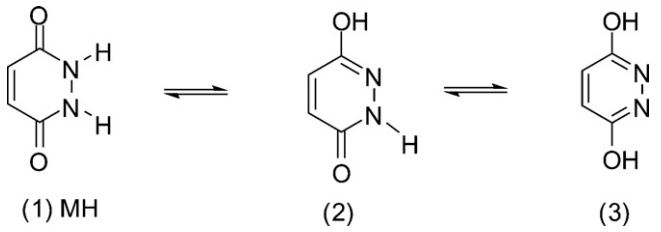

Fig. 1. Maleic hydrazide tautomeric structures (**1** – 1,2-dihydropyridazine-3,6-dione; **2** – 6-hydroxypyridazin-3(2*H*)-one; **3** – pyridazine-3,6-diol).

apparent abnormalities or losses of quality, even at high concentrations. In addition it has been shown that MH is highly efficient at the recommended doses, regardless of the application timing in the pre-harvest period [4].

As shown by Smith et al. [14] the time needed for MH uptake in tomatoes is 1–6 days, depending on the environmental relative humidity. Once MH is applied to the leaves, it is absorbed and reaches the phloem in less than 24 h [15]. Unfortunately macroscopical changes do not allow the differentiation of the presence of MH from growth inhibition, which might persist after the chemical itself disappears [14,16].

Although studies on this subject have been carried out for more than five decades, the qualitative nature of MH residues in animals is not adequately understood and may be subject to further testing to better characterize effects related to endocrine disruption and its consequences for human health [17]. In addition, there is still great uncertainty about its chronic toxicity to non-target organisms [17–19]. Despite the lack of a clear understanding regarding its toxicity, maximum residue limits in garlic have been set at 15 mg kg $^{-1}$ by the Codex Alimentarius and the United States Environmental Protection Agency (US-EPA).

Different analytical techniques have been used in the analysis of MH residues in many vegetal matrices such as potato [10,20–23], tobacco [24–26], and mixed vegetal matrices [27–29]. However, the modified colorimetric method, which is nonspecific, and susceptible to interference [30,31] remains the official method [32]. Continual improvement in instrumentation and analytical techniques has provided more sensitive detection. On the other hand, the amount of solvents should be minimized even more to reduce waste and prevent pollution, and the throughput increasing samples is also required in routine analysis. Only one method has been reported for the determination of MH from garlic samples [2]. This method is based on the ion exchange liquid chromatography, employing a UV detector (IE/HPLC/UV). However, this method is laborious, expensive, and uses a large amount of solvent with a low rate of recovery.

The present work was undertaken to develop and validate a new analytical procedure based on MH extraction from garlic previously frozen by liquid nitrogen and submitted to a clean-up at low temperature. The applicability of the developed method by the analysis of garlic samples from commercial plantation treated with MH was also demonstrated. The approach presented in this work has taken particular account to the susceptibility of MH to be oxidized in homogenized plant tissue, a major limiting factor for this type of analysis. The analytical performance of the proposed methodology was evaluated in terms of accuracy, precision, and quantification and detection limits, linearity, and sensitivity.

2. Experimental

2.1. Equipment

A Shimadzu LC 20AT liquid chromatograph (Kyoto, Japan), equipped with a UV/vis Shimadzu detector (Kyoto, Japan), Shimadzu CTO 10ASVP column oven (Kyoto Japan), Shimadzu SIL 10AF (Kyoto, Japan) automatic injector were used. The system was operated by the Shimadzu Lab Solutions integration system software. A Certomat MV vortex mixer from B. Braun Biotech International (Melsungen, Germany) was used. Injections into the GC were made by using a 5 μL Hamilton syringe (Reno, USA). The GC–MS analyses were performed on a Shimadzu QP5050A gas chromatograph equipped with a mass selective detector (Kyoto, Japan).

2.2. Chromatographic conditions

For determination of MH, the HPLC chromatographic conditions were: 4.5 μm, 100 Å C18 column (150 mm × 4.6 mm; Shimadzu, Kyoto, Japan) with a mobile phase of deionized water: methanol, 97:3 (v:v) at 1 mL min^{-1}, column temperature: 25 °C, and injection volume: 20 μL. UV detection was at 303 nm [10,23,26,33,34]. Under these conditions the MH was quantified in 12.5 min.

For analysis by GC, the chromatographic conditions involved a DB-5 (J & W Scientific) fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The temperature program was: 80 °C, held for 5 min; increasing 4 °C min^{-1} to 140 °C, and kept at this temperature for 1 min, rating 15 °C min^{-1} to a final temperature of 285 °C and held for 25 min. Injector temperature was 290 °C. Helium (purity 99.999%) was the carrier gas at a flow rate of 1.6 mL min^{-1}. A volume of 1 μL was injected and the split ratio was 5. Mass detector conditions were as follows: temperature source 290 °C; electron impact (EI) mode at 70 eV; scan rate 1 scan/s; mass acquisition range 40–280 u. The identification of the components was performed by comparison of its mass spectrum with those on record in the Wiley library database (Wiley 330000) and by comparison with the data for a standard sample of MH.

2.3. Reagents and solutions

The standard 1,2-dihydropyridazine-3,6-dione (99%, w/w) was obtained from Fluka Analytical (Buchs, Switzerland). Methanol was HPLC grade from J.T. Baker (Philipsburg, USA). Before the analysis the mobile phase was filtered through a 0.45 μm nylon MF-Millipore membrane. Distillated water was filtered through 0.45 μm mixed cellulose esters hydrophilic MF-Millipore membrane. Water stock solutions, with concentrations of 100.0 mg L^{-1} of MH, were prepared daily and kept at 5 °C. Starting from the stock solution, working solutions at different concentrations were prepared immediately before use. The *N,O*-bis(trimethylsilyl)trifluoro acetamide used in the derivatization procedure was purchased from Merck (Darmstadt, Germany).

2.4. MH extraction with clean-up by precipitation at low temperature

To establish the best conditions for extraction of MH, garlic samples were chopped, frozen with liquid nitrogen and powdered. A portion of 2 g of each defrosted sample was spiked with 0.1 mL of standard solution of MH (100 mg L^{-1}) and left standing for 3 h at room temperature. After this period the samples were submitted to the process of extraction with 15 mL of methanol [2,10,20–22,27] at room temperature (22–25 °C). The mixtures obtained in each assay were shaken for 1 min in a vortex mixer at high speed and then left in a freezer at −20 °C for 3 h. The sample was then filtered through a quantitative filter paper and the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator at 30 °C. The residue obtained was further dissolved in 10.0 mL of deionized water:methanol, 97:3 (v:v). The final solution was filtered through a 0.45 μm Millipore® membrane filters before injection into the chromatograph.

2.5. Analytical plots

Quantification of garlic extracts was carried out by the external standard method using analytical plots with eleven concentration levels over the range of 0.10–35 mg L^{-1} in deionized water:methanol, 97:3 (v:v).

2.6. Preparation of the spiked samples

In the optimization and validation processes of the method, MH-free garlic samples were obtained from garlic grown in open fields in Mendoza, Argentina, to which the pesticide had not been applied. Those samples were spiked with different volumes of a 100 mg L^{-1} MH solution. The spiked garlic samples were left at rest for 3 h before use. The spiking level was chosen to be lower than the Codex Alimentarius, European Union, United States Environmental Protection Agency (US-EPA), Brazilian ANVISA and Argentinean SENASA maximum residue level (MRL) for this pesticide in garlic [17,35–38].

2.7. Method optimization

To evaluate the extraction efficiency the following working parameters were investigated: volume of extraction solvent (60, 40 and 15 mL), number of filtrations (double filtration and single filtration, type of filter material (filter paper and glass wool), shaking time and mode (1 min of vortex; 30 min of orbital stirring table (OST); 15 min OST; 15 min OST and 15 min of ultrasound; 30 min of ultrasound; 15 min of ultrasound, and without shaking), weight of garlic (2, 3, 4 and 5 g) as well as freezing time (3, 4, 5, 6, 8, 12, 24, 96, 120 h). In each test area values were recorded and the percentage increase of the response was calculated. The optimization was carried out by univariated analysis.

2.8. Method validation

Validation was developed following the International Conference of Harmonization Tripartite Guideline and other publications [39–42]. Analytical parameters of the extraction technique with clean-up by precipitation at low temperature were evaluated, such as precision (repeatability and intermediary precision), accuracy, linearity, selectivity, limit of detection (LOD) and limit of quantification (LOQ). The accuracy of the method was evaluated with three repetitions by recovery tests at three concentration levels and by comparison with the official extraction method [30–32,43,44].

2.9. Application of the method with clean-up by precipitation at low temperature

Argentinean garlic samples were collected in the Valle de Uco region, in Mendoza State. The validated method was applied for the determination of MH in samples obtained in March 2010 (2009 harvest). All samples were placed in garlic-appropriate boxes for transport to the laboratory.

2.10. Evaluation of matrix effect

Three calibration curves were built to evaluate the effect of the matrix:

I. Analytical curve prepared from dissolution of MH in water at different concentrations (0.1–35 mg L^{-1}).
II. Analytical curve prepared by spiking methanol/water (97/3, v/v) garlic (untreated) extracts with MH at different concentrations (0.1–35 mg L^{-1}).
III. Analytical curve prepared from garlic samples (MH-free) spiked with working solutions containing MH to obtain the same final concentration of analytical curves 1 and 2 described previously.

2.11. Confirmation

In order to confirm the results obtained by HPLC, a derivatized 100 mg L^{-1} HM standard, a derivatized extract of untreated garlic and a derivatized (as a trimethylsilyl derivative) spiked garlic extract were injected in the gas chromatograph with mass spectrometry detector [24]. For injecting the garlic samples, after the solvent was completely removed under reduced pressure in rotary evaporator, as has been described, aliquots of the extracts or standard solution were weighed into a conical glass-house (suitable for this process) and then dissolved in 60 μL of pyridine and 100 μL of BSTFA (*N,O*-bis(trimethylsilyl)-trifluoracetamide) containing 1% chlorotrimethylsilane. The reaction mixture was heated to 70 °C for 30 min. From the solution obtained, 1 μL was injected into the GC–MS equipment.

2.12. Extract stability

After the application of the method, stock solutions and garlic extract were kept in vials in the dark at 5 °C, to evaluate the stability of the extract. The same garlic extracts were analyzed over a period of 51 days. The chromatographic peak area was the parameter used to assess the impact of different storage periods at low temperatures on the stability of the garlic extract.

3. Results and discussion

3.1. Chromatographic analysis

The optimized chromatographic conditions for MH analysis provided a good separation of the principal components of the sample. The retention time was 4.64 min. Clean-up of the extracts was performed as described by Lewis [20] with some modifications. In the development of this technique, different cartridges were tested, without significant improvement in terms of co-extractive garlic samples. Instead, a low temperature clean-up method provided a clean extract. With respect to the wavelength, a number of researchers have investigated the detection of MH at different wavelengths such as: 254 nm (UV); 303 nm (Fl); 313 nm (UV); 330 nm (DAD); 430 nm (UV); 460 nm (UV): 455 nm (UV) and 490 nm (UV) [2,10,20,21,23,27,30–34]. In this study, the detection of MH was carried out at 303 nm, resulting from a spectral scan of a standard MH that indicated an absorption maximum at this wavelength. Cessna [2] reported to their operational conditions that MH retention time was dependent on the pH of the mobile phase. In this study, UV spectra were obtained for different pH values. No significant change in the UV spectrum between pH 2 and 4 was observed. However, for pH 5, it was possible to see certain modification in the spectrum, probably because the pK_a of maleic hydrazide is 5.62 at 20 °C [17]. In conclusion, the pH should be adjusted between 2 and 4. The effect of column temperature on HPLC analysis of MH was studied from 18 to 35 °C, in increments of 2 °C. Area and peak width variations observed during the study of MH analytical standard show no statistical significance between 24 °C and 30 °C, while at lower temperatures, increases in the area and peak width adversely affected the chromatogram resolution. Higher temperatures may affect other components of garlic extract. Therefore, the analyses were carried out at 25 °C (Fig. 2).

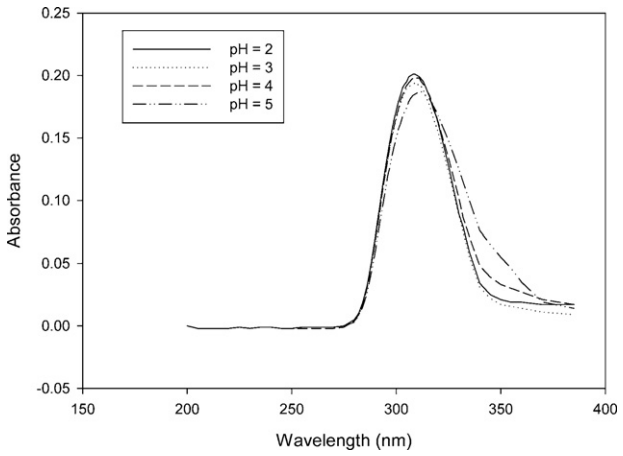

Fig. 2. Maleic hydrazide UV spectrum at different pH values.

3.2. Use of liquid nitrogen for sample preparation

In order to maximize the effectiveness of the extraction of MH from the garlic matrix, enhancement of the contact among the tissue surface and the extraction solvent is crucial. For this purpose, liquid nitrogen was chosen to optimize contact of the cell contents with the extracting solvent [45]. Furthermore, liquid nitrogen avoids MH oxidation reactions that could affect the residue assessment [14,30,46].

3.3. Optimization of the extraction technique

The study and optimization of the mentioned variables were evaluated using univariated analysis. The chromatographic peak area was the parameter used to evaluate the influence of those variables on the relative recovery of the technique.

The volumes of extractor solvent evaluated were 60, 40 and 15 mL. Lewis et al. [20], Cessna [2], Newsome [27], Nagami [21], Kubilius and Bushway [22] used large solvent volumes, sometimes above 60 mL, which makes analysis more expensive, increases the analytical time, and generates more waste pollutants. No significant differences were observed in the percentage of extraction, with three repetitions. Acceptable extraction rates, with reduced solvent volume have been already observed in the determination of pesticides by HPLC [47–49].

To assess the influence of the number of filter stages in the extraction of the analytes, 15 mL of solvent were used. The assessed levels were double filtration [2], and single filtration. The double filtration consisted of vacuum filtration in a Büchner funnel followed by filtration through glass wool. The single filtration was through a glass wool. There were no significant differences between the treatments at the 95% level of confidence ($\alpha = 0.05$) by the Student t test, in the chromatographic responses of the MH. However, with single filtering, the standard deviation was lower and the extraction process, simpler and faster.

To evaluate the influence of the filter material, glass wool and filter paper were tested by means of a single filtration with 15 mL of methanol. Filtration through glass wool resulted in recoveries of 125% (CV = 8%) and with filter paper 97% (CV = 2%) recoveries were obtained. By using the filter paper material, the method proved to be more reproducible and also exclude operational problems related to glass wool as its inadequate compression.

Different shaking modes to produce greater contact of garlic solid particles with the liquid phase methanol were investigated. This stage can significantly affect the mass transfer process of the MH in the organic phase. The kind of turbulent fluid movement that penetrates near the surface of the particle can increase the local rate of mass transfer process of the target analyte to the liquid phase. The shaking mode was evaluated with 15 mL of methanol, and a single filtration with filter paper. Treatment without shaking showed recoveries of 70% and treatments that involved the use of ultrasound increased the chromatogram baseline. The results showed that either vortex treatment; 15 min orbital stirring table; or 30 min orbital stirring table could be used as shaking mode. Because of the analytical frequency, vortex stirring for 1 min was chosen.

The weight of sample was evaluated using 15 mL of methanol, and single filtration with filter paper after a 1 min stirring with a vortex equipment. There were no significant differences between the different weights of garlic tested, according to the Tukey test. However, treatment with 5 g of sample had a coefficient of variation 12 times higher than treatment with 2 g of sample. Therefore, 2 g of sample was chosen for the following studies.

3.4. Low temperature clean-up

The low temperature clean-up is based on the precipitation of co-extractives of garlic. With cooling, larger impurities and particles end up at the bottom of the glass container and methanol extract can be clearly seen. The minimum time that was necessary for samples to be kept in freezer to achieve good results was studied. Conversely, it was also evaluated the response if necessary to store samples for 5 days. The freezing time was evaluated at 3, 4, 5, 8, 12, 24, 96, 120 h of freezing. There were no significant differences among the treatments; therefore it is possible to maintain a minimum of 3 h of freezing. The clean-up effect was not observed for a cooling time less than 3 h, and it was not feasible to inject the extract into the chromatograph.

3.5. Validation of the optimized method

3.5.1. Selectivity

The selectivity of a method indicates its ability to accurately measure the analyte response in the presence of all potentially interfering sample components [50]. In this study the selectivity of the method was verified by comparison of the chromatograms obtained from fortified garlic samples with those of samples free of MH. The samples did not present peaks at the retention times of the MH, moreover, the chromatograms of the extracts presented satisfactory chromatographic resolution (Fig. 3).

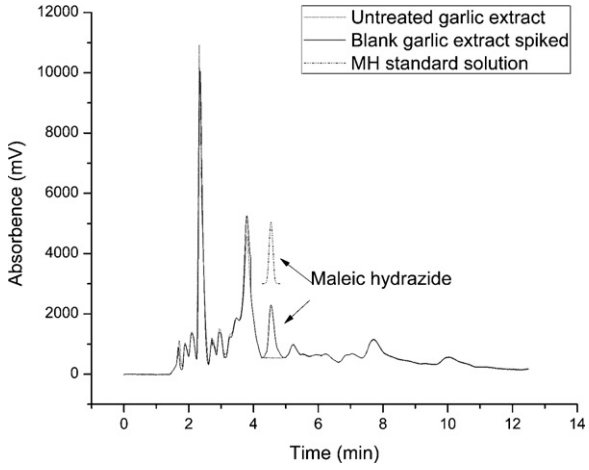

Fig. 3. Chromatogram of an untreated garlic extract (···) in dot line, compared with a blank garlic extract spiked with maleic hydrazide 1 mg kg^{-1}, (—) in solid line. On top of that, chromatographic peak of maleic hydrazide standard solution.

Table 1
Quantitative features for maleic hydrazide.

Parameter	Unit	Maleic hydrazide response
Linear range	mg kg^{-1}	1–175
LOD	mg kg^{-1}	0.33
LOQ	mg kg^{-1}	1.0
Linearity	r^2	0.999

LOD: limit of detection; LOQ: limit of quantification.

3.5.2. Detection and quantification limits

The detection and the quantification limits (LOD and LOQ) of the proposed technique were determined under the guidelines given by the ICH [39]. They were calculated based on the Standard Deviation of the Response and the Slope, with the Standard Deviation of the Response based on the standard deviation of the target. Garlic samples fortified with the analyte at decreasing concentrations were subjected to extraction procedure and quantification. Concentrations used were between 0.1 and 2 mg L^{-1}. It was considered that concentration LOD and LOQ producing 3.3 and 10 times the ratio of standard deviation from the response and the slope of the calibration plot. The resulting LOD for MH was 0.33 mg kg^{-1} and the LOQ for MH was 1 mg kg^{-1} (Table 1).

3.5.3. Sensitivity and linearity

The linear response method is the ability to show that the results are directly proportional to the concentration of analyte in the sample, within a specified range. In a chromatographic analysis, this response refers to the peak area for the compound. The linear response method was determined by injection and analysis of extracts obtained from spiked samples at different concentrations of the active ingredient undergoing optimal extraction technique (1–175 mg kg^{-1}). After the chromatographic analysis of these extracts was built a standard curve, and obtained the equations of straight lines and correlation coefficients. The detector proved a satisfactory linearity with a coefficient of estimation (r^2) of 0.999. Tests were made to concentrations of 250 mg kg^{-1}, and linearity was also seen in that range. However, the linear range was determined from 1 to 175 mg kg^{-1}. A large r^2 does not necessarily imply that the regression model will provide accurate predictions of future observations (Table 2). Fitting a calibration function by ordinary least squares method requires several assumptions related to the residuals: normality, homoscedasticity and independency [51,52]. A statistical study demonstrated that the residues of the calibration curve follow a normal distribution. Autocorrelation between the residuals was not observed, and the variances of the standard deviations were not different. Therefore, the assumptions of independence and homoscedasticity were satisfied.

Matrix effects have been reported in the analysis of pesticides, often in gas chromatography [53–55] and high performance liquid chromatography with a mass detector [56]. This effect could be important in vegetables samples since these are complex matrices and systematic errors can affect the result. However, Hajslová [54] reported a greater impact on the matrix effect when using mass detector, which pursuant to the high selectivity and specificity of the detector, in the presence of substances co-eluting matrix could generate errors in the detection of analytes. Matrix interferences can be detected by comparing the slope of the standard calibration curve with the slope of the curve prepared by spiking the extract of untreated garlic. The similarity between the slopes of the curves described above, discard any suppressor or enhancer effect of the analytical signal due to the matrix (Table 2). The matrix effect should be investigated by conventional bio-analytical methods such as HPLC with fluorescence detector and HPLC with UV detector [57] (Fig. 4).

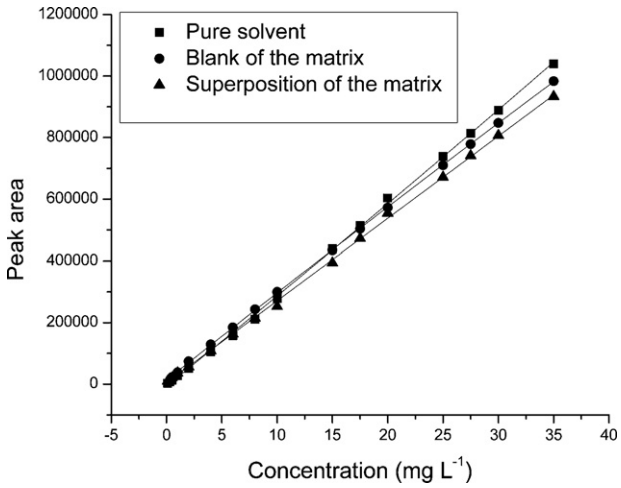

Fig. 4. Calibration curves of maleic hydrazide in pure solvent (dissolution of the MH in water at different concentrations), blank of the matrix (by spiking methanol/water (97/3, v/v) garlic (untreated) extracts with MH at different concentrations), and superposition of the matrix (by garlic samples (MH free) spiked with working solutions containing MH.

3.5.4. Precision

The precision of the method was evaluated in seven replications spiked at 2 mg kg^{-1}. Intermediate precision of the method was verified by the recovery percentages of the analytes and their variation coefficients obtained by the preparation, injection and analysis of extracts of garlic samples subjected to extraction technique optimized on three different days (days 1, 7 and 30). It can be stated that the extraction technique provides recovery percentages statistically equal (at 95% probability by the *t* test) and that the variation coefficients are satisfactory (Table 3).

3.5.5. Accuracy

In recovery experiments, the pesticides were added to samples of garlic at 3 concentrations levels: 1 mg kg^{-1}, 2 mg kg^{-1}, and 10 mg kg^{-1}. The results for MH extraction of garlic are statistically equivalent to each other, at 95% probability by the *t* test, showing accuracy of the method. Moreover, the coefficients of variation were lower than 10% (Table 4).

The analytical performance of HPLC–UV for MH determination in garlic samples was compared with other analytical methodologies previously reported for MH. It can be observed that the analytical performance for the proposed method is comparable

Table 2
Correlation coefficient and equation of the straight line for the analytical curves.

Analytical curve	Equation	Correlation coefficient
Pure solvent	$y = 29964x - 9593.2$	0.999
Blank of the matrix	$y = 27727x + 17{,}032$	0.999
Superposition of the matrix	$y = 26689x + 4171.3$	0.999

Table 3
Recovery percentages and coefficients of variation (CV), obtained from the application of the method in garlic samples spiked with maleic hydrazide (2 mg kg^{-1}) on different days (*n*: number of replicates).

	Recovery (%)	CV (%)	*n*
Day 1	113	9.5	7
1 Week	108	7.4	7
1 Month	99	8.2	7

Table 4
Recovery percentages and coefficient of variation (CV), obtained from the extraction of maleic hydrazide in spiked garlic samples, by the method proposed. (*n*: number of replicates).

Concentration (mg kg^{-1})	Recovery (%)	CV (%)	*n*
1	113	4.9	3
2	104	7.5	3
10	95	4.8	3

Table 5
Recovery percentage and coefficients of variation (CV) obtained from extraction of maleic hydrazide in spiked garlic samples, (2 mg kg^{-1}) using the method proposed and AOAC official method.

	Recovery (%)	CV (%)
AOAC method	75.1	47.5
This work	99	8.2

with methodologies previously used for MH determination. It is noteworthy that in the AOAC official method [32] based on the technique developed by Wood [30], garlic was not included in the group of vegetables tested. However, the method of Wood [30] is described for different plant and animal tissues. The results obtained by the proposed methodology were compared with those from the AOAC official method [32] for MH extraction. The AOAC method consists of a reduction with zinc and hydrolysis in boiled alkali to hydrazine. The hydrazine is isolated by distillation and determined by photocolorimetry (Table 5). The official method presents an acceptable recovery, but a very high coefficient of variation, complex extraction procedure, poor linearity and lack of selectivity. Additionally, the analytical performance of the proposed methodology was compared with the methodology of Cessna [2] and Wood [30] (Table 6).

It should be emphasized that in previously reported techniques the analysis time is usually longer due to a more laborious extraction process involved. Furthermore, the technique proposed in this study employs conventional chromatographic equipment, usually found in most analytical laboratories. The extraction process is simple and cheap, and the fact that the use of organic solvent is minimized provides a low cost and environmentally responsible method. Therefore, we have presented a method for determining sample garlic MH which is accurate, not expensive, reproducible, easily executable and with good analytical frequency and recoveries, ideal for application in a routine laboratory.

3.6. Application to real samples

The method of MH extraction and quantification was used in test samples, which were applied at concentrations of 4 kg ha^{-1} of commercial product with 49% purity, following manufacturer's recommendations. The quantification was carried out by using a calibration curve on garlic matrix. The application was made taking into account the usual operation carried out by many farmers from garlic producing regions. MH residues were found in the samples analyzed. In order to verify the recoveries, extracts from garlic samples (MH-free) spiked were injected simultaneously with the samples from treated garlic (Table 7).

Table 7
Untreated and treated garlic samples analyzed for the presence of maleic hydrazide.

	Level found (mg kg^{-1})	Recovery (%)	CV (%)	*n*
Untreated samples	nd	–	–	3
Spiked (1 mg kg^{-1})	0.9	95	9.66	3
Treated garlic (4 kg ha^{-1})	8.59	–	9.96	3

nd: not detected.

3.7. Gas chromatography–mass spectrometry (GC–MS) analysis as a confirmation method

The confirmatory method chosen was GC–MS due to its good selectivity based on selecting the target molecule by the molecular mass. The bis(trimethylsilyl) derivative of MH and compounds present in sample extracts were analyzed by GC with mass-selective detection. Mass spectra of the silylated derivatives of MH standard and MH present in garlic were similar. Untreated garlic samples were analyzed too. By use of mass spectrometry software and library and by the analysis of the fragmentation pattern the presence of MH in the garlic investigated was confirmed. The mass spectrum presented a signal at $m/z = 256$ corresponding to the molecular weight and a base peak was observed at $m/z = 241$ (Figs. 5 and 6).

3.8. Extract stability

Assessment was made of the stability of the extracts stored in cold conditions at −5 °C. Garlic extracts fortified at concentrations of 2.5 and 7.5 mg L^{-1} were analyzed. In addition, standards solutions stored at concentrations of 1 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1} were similarly evaluated. On average, after 10 days, the decrease in chromatographic peak area in garlic extracts was 8% and 5% for extracts of 2.5 and 7.5 mg L^{-1} respectively. From day 11 up to day 51 of storage, the peak area was reduced by 32% in the extract of 2.5 mg L^{-1} and 22% for the concentration of 7.5 mg L^{-1}. In the case of analytical standards solutions, up to 10 days storage, the decrease was 4% for 1 mg L^{-1}, 5% for 5 mg L^{-1}, and 7% for 10 mg L^{-1}. At 51 days, the largest decrease was 26% on the first day of injection at concentration of 1 mg L^{-1}. In view of these results and considering that the variations in the peak areas are common in liquid chromatography, it can be assured that, up to 10 days of storage no significant changes in the peak area occur. For longer periods, the resolution and peak shape may be affected. Matrix interferences could affect the chromatograms, as well. As a result, the area values are increased, and quantification would not be reliable. It is possible that even at low temperature, chemical changes occur in the extracts tested.

Table 6
Determination of maleic hydrazide in garlic samples by using different analytical methodologies.

Methodology	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Mean recovery	RSD (%)	Extraction time (min)	References
IE/HPLC/UV	Not reported	1	75.8	6.1	80	[1]
UV	Not reported	1	90–100	10	120	[30]
RP/HPLC/UV	0.33	1	99	8.3	15	This work

IE/HPLC/UV: ionic exchange high performance liquid chromatography and ultraviolet detection; UV: spectrophotometric method; RP/HPLC/UV: reverse phase high performance liquid chromatography and ultraviolet detection.

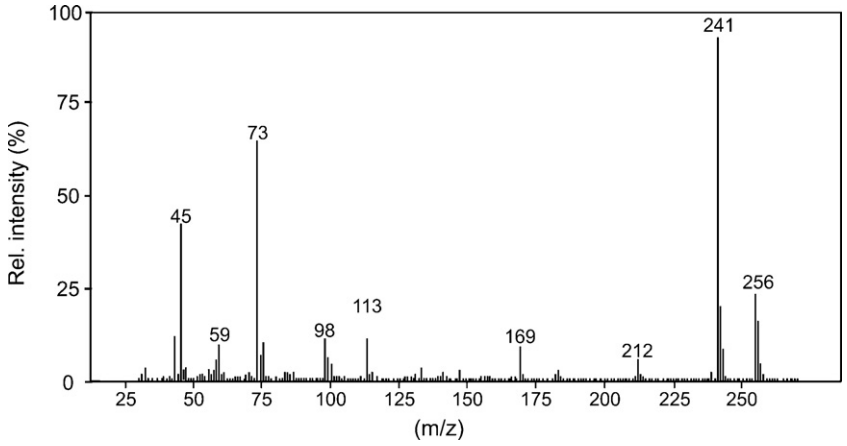

Fig. 5. Mass spectrum of maleic hydrazide after derivatization with BSTFA.

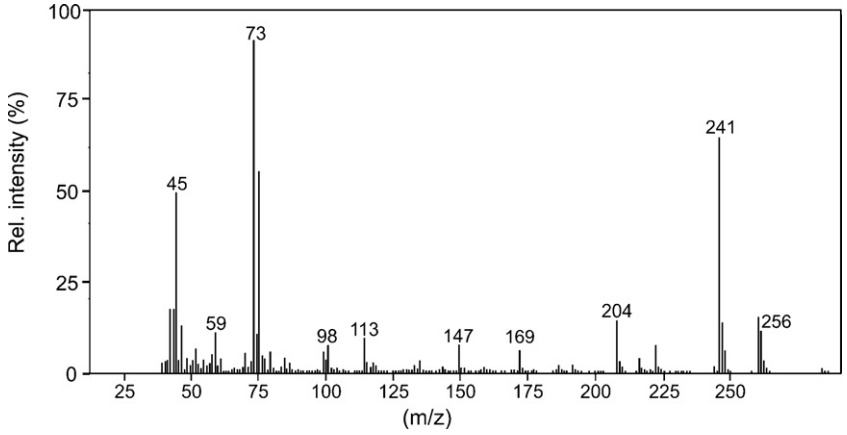

Fig. 6. Mass spectrum of bis(trimethylsilyl) derivatives of an extract from a garlic sample spiked with MH.

4. Conclusions

We have established that the proposed method for the determination of MH in fresh garlic based on cold extraction, clean-up and quantification by HPLC–UV is highly efficient due to its specificity and sensitivity. Using liquid nitrogen during the sample preparation ensures no degradation of the analyte due to oxidation reactions. Moreover, the developed method provides good linearity, good intermediate precision, and extracts were not affected by matrix effect. Under optimized conditions, the LOD was well below the MRL set internationally for garlic, with rates of recovery over 95%, good repeatability and acceptable accuracy. The analytical performance of the methodology presented was compared with other techniques already reported, with highly satisfactory results, as lower LOQ and higher recoveries rates. In addition, the extraction process is simple, not expensive, easily executable and requires lower volumes of organic solvent. Real garlic samples were analyzed and the presence of MH residues was reported. The proposed methodology removes the need of extensive typical laboratory extraction procedures, reducing the amount of time needed for pesticide analysis and increasing sample throughput. Adopting this method gives food safety laboratories the potential to increase cost savings by a suitable technique in routine testing to determine MH residues in garlic.

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