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LC/ESI/MS Method for the Quantitative Detection of Guazatine Residues in Cereals

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Guazatine is a fungicide used in agriculture to control a wide range of seed-borne diseases of cereals and other vegetable foods. In this work, a LC-ESI-MS method was developed for the quantitative detection of guazatine residues in maize and hard wheat. Quantitative data were determined for the residues of the main diamines, triamines, and tetramines that cover more than 87% of the total contents of the mixture. The mean recoveries from the fortified cereals at 0.050 mg/kg ranged from 81 to 86%, with the coefficients of variation (CVs) ranging from 0.9 to 5.5% ($n = 5$). At 0.025 mg/kg, the recoveries ranged from 78 to 87%, with the CVs ranging from 0.8 to 6.3% ($n = 5$). The limits of quantification have been estimated to be 0.010, 0.004, 0.002, 0.002, 0.005, and 0.002 mg/kg, respectively, for GN, GG, GNG, GGN, GGG, and GGGG in maize and hard wheat (*S*/*N* ratio >10).

KEYWORDS: Residues; cereals; guazatine; fungicide; LC/ESI/MS

INTRODUCTION

In the last 50 years, a large group of organic compounds have been introduced in agriculture for the control or prevention of crop diseases. Even if applied in accordance with Good Agricultural Practices (GAP), these substances can leave residues, which can be dangerous for human health. To guarantee consumer protection, maximum residue limits (MRLs) have been established by both the European Union and the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (FAO).

Some of the most extensively applied agrochemicals in cereals are fungicides for treatment of diseases, such as seedling blight (*fusarium* spp.), glume blotch (*septoria*), common blunt (*tilletia* spp.), common root rot (*helminthosporium*), and smut (*ustilago*). The residues of these pesticides can persist to the harvest stage, so they may contaminate the cereal foods. Guazatine is a fungicide widely used in agriculture to control many seed-borne diseases of cereals and other vegetable foods. It is a nonsystemic contact fungicide that inhibits lipid biosynthesis, causing damage to fungal cellular membranes (*1*).

Guazatine acetate, the salt that is used in practice, is a mixture of reaction products from polyamines, comprising mainly octamethylenediamine, iminodi(octamethylene)diamine, octamethylenebis(imino-octamethylene) diamine, and carbamonitrile. A coding system, defined by the Codex Alimentarius Commission, is used for the compounds that make up guazatine. In this system "N" represents any amino group. Thus NN stands for H_2N-$(CH_2)_8$-NH_2, NNN stands for H_2N-$(CH_2)_8$-NH-$(CH_2)_8$-NH_2, and so on. "G" stands for any amino group (NH or NH_2) of the above which is guanidated. For example, GG stands for H_2N-C(NH)NH-$(CH_2)_8$-NH-C(NH)-NH_2 (*2*).

In literature, the separation and identification of the main components of this mixture have been obtained using GC-MS and FAB-MS analysis (*3*−*5*). In a more recent LC-MS method, each compound has been separated and identified with fragmentation studies using the spectra recorded with various fragmentor energies (*6*).

Although the identification of the main components of guazatine mixture is well-known, no papers describe methods for the residue analysis of the main compounds in cereals. The few published works have only quantified the GNG residue, although it represents a low percentage of total guazatine. These works described a GC method after derivatization with hexafluoroacetylacetone (*7*, *8*) and a LC method for the fluorometric determination of GNG residues in various crops (*9*). The GNG determination has been described mainly for water analysis using HPLC with fluorescence detector after solid-phase extraction and postcolumn derivatization (*10*, *11*). More recently, some authors have proposed a LC-MS method for GNG determination in tap water using hydrophilic interaction chromatography (*12*). The quantitative analysis of guazatine is also described in some unpublished studies that have quantified the GNG residues after the hydrolysis to bis(8-amino-octyl)amine (NNN) using GC analysis either directly or after derivatization (*2*). Better results were achieved by GC after derivatization using the marker GG for quantification. This method incorporates a correction factor because GG represents only 30% of the total guazatine (*2*).

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Table 1. Data on Regression Equations of the Six Compounds Extracted from Maize and Hard Wheat[a]

		maize		hard wheat	
compound	spiked conc (mg/kg)	equation	correlation coefficient (R^2)	equation	correlation coefficient (R^2)
GN	0.010–0.25	$y = 692320x + 26369$	0.997	$y = 695410x + 23584$	0.996
GG	0.004–0.30	$y = 7170212x + 17615$	0.999	$y = 7256322x + 12478$	0.995
GNG	0.002–0.20	$y = 11614218x + 21810$	0.998	$y = 11638751x - 25672$	0.999
GGN	0.002–0.30	$y = 12273043x - 9092$	0.998	$y = 12387746x + 8654$	0.998
GGG	0.005–0.25	$y = 5202216x + 25783$	0.997	$y = 5214632x - 7458$	0.997
GGGG	0.002–0.25	$y = 10076630x - 23505$	0.998	$y = 10106807x - 16843$	0.997

[a] The data were subjected to linear regression analysis of peak area (y) of the compound against the spiked concentration (x). For the equations, six plots (each point represents the mean of triplicate determinations) with different concentrations for each compound were used.

On this basis, it is possible to assert that the quantification of the single components of the mixture represents an open problem, probably because of the difficulty in choosing a method for residue analysis of this complex mixture or because of the lack of commercial standards. The aim of this study was to develop a sensitive and specific method for the quantitative detection of guazatine residues in maize and hard wheat at the guideline established by the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (residue of GG in cereal crops, 0.05 mg/kg) (*13*). Quantitative analysis was carried out for GN, GG, GNG, GGN, GGG, and GGGG (more than 87% of the total contents of the mixture) using an external standard method with the six compounds obtained, characterized, and purified as reported in an our work published previously (*6*).

MATERIALS AND METHODS

Chemicals and Reagents. All of the reagents and solvents (Chromasolv HPLC grade) were from Sigma-Aldrich Srl (Milan, Italy). Milli-Q quality water (Millipore, Milford, MA) was used.

The compounds: GN, GG, GNG, GGN, GGG, and GGGG, used as standards for the quantitative analysis, were obtained from guazatine acetate Pestanal standard (Riedel-de Haën, Sigma-Aldrich Srl, Milan, Italy) as reported in a previous work (*6*).

Apparatus and Chromatographic Conditions. The chromatography−mass spectrometry (LC-MS) system consisted of an Agilent 1100 series liquid chromatograph system (Agilent Technologies, Palo Alto, CA) including a vacuum solvent degassing unit, a binary high-pressure gradient pump, and an 1100 MSD model VL benchtop mass spectrometer with a API-ES interface.

The Agilent 1100 series MSD single-quadrupole instrument was equipped with the orthogonal spray API-ES. Nitrogen (purity 99.995%) was used as the nebulizer gas and the drying gas (350 °C). The nebulizer gas, the drying gas, the capillary voltage, and the vaporizer temperature were set at 40 psi, 9 L/min, 3000 V, and 350 °C, respectively. The LC-ESI-MS determination was performed by operating the MSD in positive ion mode. Mass spectra were acquired over the scan range m/z 100−1500 using a step size of 0.1 u. Quantitative analysis was carried out using the signal of base peak ions of various compounds.

The chromatographic separation was performed on an Alltima C_{18} column (250 × 10 mm; 5 μm) (Alltech Italia Srl., Sedriano, Milan, Italy). The sample was injected (20 μL) after filtration.

The separation was performed by using a linear elution gradient for 30 min with a mobile phase of 0.2% (v/v) formic acid in water and acetonitrile (from 90:10 to 30:70 v/v in 30 min) at a flow rate of 3.5 mL/min. After the chromatographic separation, an aliquot of the eluent (400 μL/min) was directed to the MSD for spectral analysis.

Standard Solutions. Each single standard was obtained using a method published previously (*6*) and stored in a desiccator until used. The standard solutions were prepared by dissolving each compound with methanol in a volumetric flask and diluting to make the working solutions.

Sample Extraction. The samples (maize and hard wheat) were obtained from an organic farm. In the first step, a representative portion of the samples (200 g) was mixed well with a food chopper. Then, a 10 g portion was placed in a centrifuge tube and 25 mL of 0.5 N NaOH/methanol was added. The extraction was carried out using an IKA Labortechnik homogenizer model T25 basic (IKA WERKE GmbH & Co.: Staufen, Germany) for 5 min at 13 500 rpm. The supernatant was transferred after centrifugation, and another aliquot of extraction mixture was added to the residue and homogenized as described previously. The separated fractions were collected in a centrifuge tube, and after the addition of 50 mL of water the extraction was carried out by two portions (25 mL) of CH_2Cl_2. (Caution, avoid human exposure; dichloromethane may be a carcinogen.) The organic fractions were evaporated to dryness under vacuum by rotary evaporation (temperature of the bath, 20 °C), and the residue was redissolved in 500 μL of 0.2% (v/v) formic acid in water and acetonitrile (50:50 v/v). The sample was filtered with 0.45 μm Minisart SRP 4 (Sartorius: Goettingen, Germany) and used for the LC/ESI/MS analysis.

Quantification and Recovery. The quantitative analysis of GN, GG, GNG, GGN, GGG, and GGGG was based on calibration curves obtained analyzing spiked samples at different concentrations in the range reported in Table 1. For the equations, six plots (each point represents the mean of triplicate determinations) with different concentrations were used. Extraction recoveries were determined by spiking untreated powdered samples (10 g) with standard solutions to have two different final concentrations (0.025 and 0.050 mg/kg for each investigated compound). After spiking, the samples were mixed for 2 min. After the solvent was evaporated, the samples were extracted as described previously. Control samples were prepared in the same way as the fortified samples, except that methanol, without analytes, was used to spike the cereals.

Recovery values were calculated as the ratio of the peak area obtained from the extraction of the fortified samples to the corresponding peak area determined by a single-point calibration standard.

RESULTS AND DISCUSSION

In an our work published previously, we reported a LC−MS method for the separation and identification of 20 components of the guazatine mixture (*6*). The standards of GN, GG, GNG, GGN, GGG ,and GGGG (more than 87% of the total contents of the mixture) were also obtained, characterized, and purified. The same LC/ESI/MS analysis was applied in the present study to develop a multiresidue method, which allowed the quantification of these compounds in hard wheat and maize at the limits

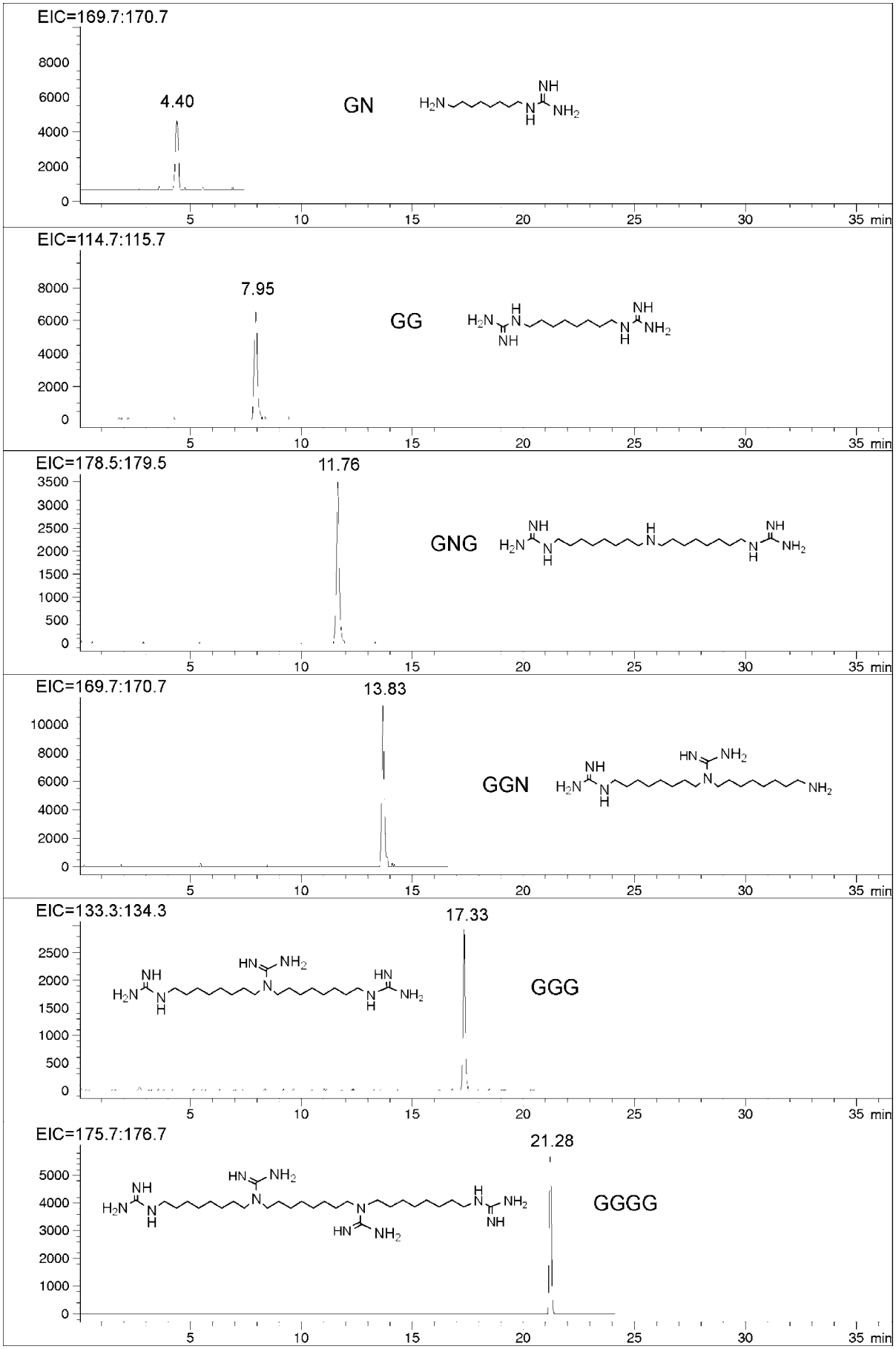

Figure 1. Chemical structures and chromatographic profiles obtained for GN, GG, GNG, GGN, GGG, and GGGG standards at the quantification limits.

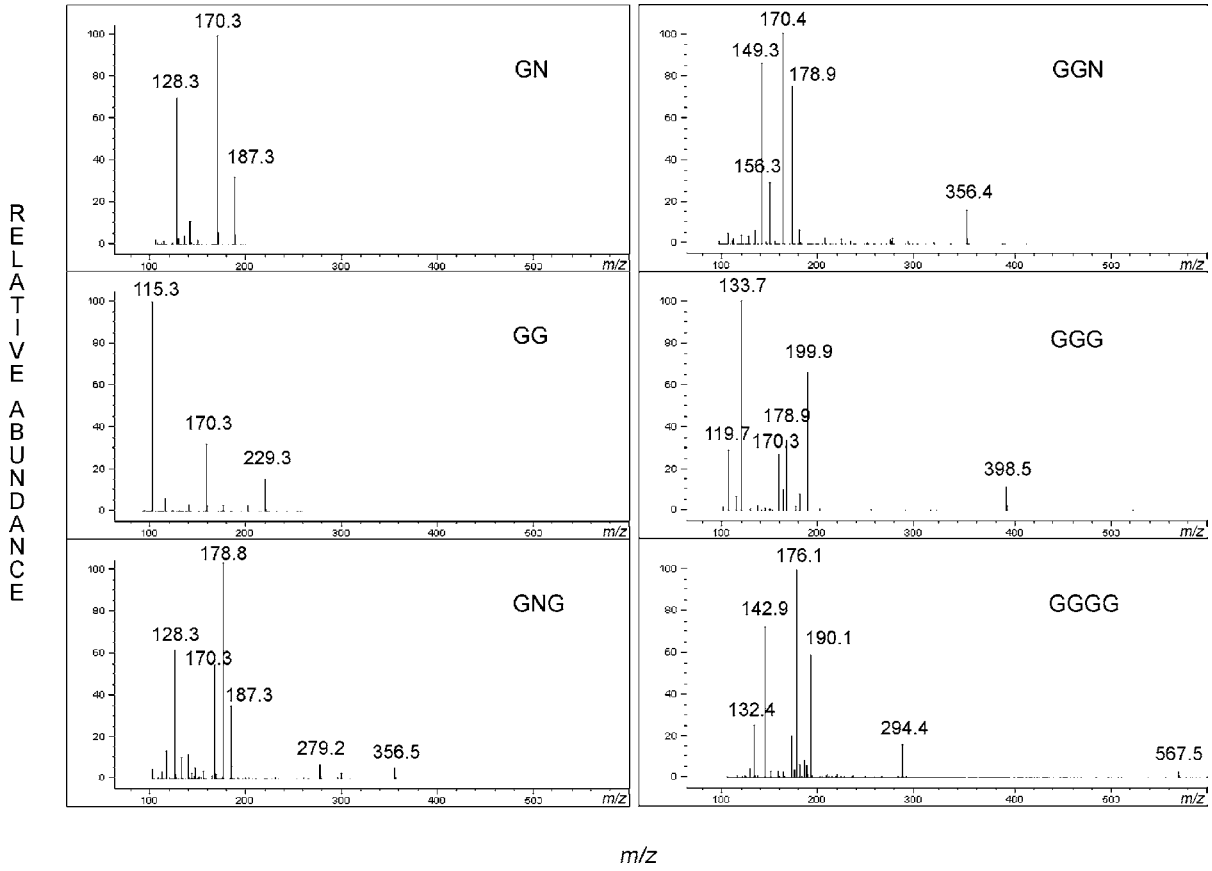

Figure 2. LC/ESI/MS spectra for GN, GG, GNG, GGN, GGG, and GGGG standards.

Table 2. Recoveries of the Various Compounds Analyzed from Maize and Hard Wheat

	spiked concentration		maize		hard wheat	
compound	(mg/kg)[a]	(mg/kg)[b]	recovery (%)[c]	CV (%)[d]	recovery (%)[c]	CV (%)[d]
GN	0.050		81.19	5.11	81.24	5.52
	0.025		78.40	5.95	79.50	6.32
		0.010	77.60	5.48	78.47	5.39
GG	0.050		82.93	3.95	84.58	3.74
	0.025		81.49	4.79	83.19	5.14
		0.060	83.42	4.09	82.97	4.55
GNG	0.050		85.12	0.93	86.32	1.25
	0.025		86.49	1.32	87.48	2.47
		0.010	83.29	1.24	85.39	1.84
GGN	0.050		85.04	1.34	85.88	1.72
	0.025		85.32	1.05	86.27	0.83
		0.020	84.54	1.22	84.87	1.22
GGG	0.050		81.21	3.94	83.69	4.04
	0.025		80.84	4.38	84.12	5.13
		0.060	82.39	4.27	83.92	5.44
GGGG	0.050		81.34	2.28	82.89	5.80
	0.025		82.15	5.97	81.65	5.39
		0.010	82.36	3.48	81.22	5.64

[a] The spiking concentrations were selected on the basis of the guideline and $^1/_2$ guideline values established by Codex for GG. [b] Spiking concentration corresponding to 0.2 mg/kg of guazatine; the various compounds were added at the same average percentage of the formulation. [c] Average of five trials. [d] Coefficient of variation.

established by the law. The best results for chromatographic separation were obtained by using a semipreparative column, while reverse phase analytical or ionic exchange columns did not provide single component separation (data not shown). The identification of each peak was achieved by a fragmentation study, which occurs by loss of ammonia, cyanamide, or guanidine, and by cleavage of the various carbon−carbon or carbon−nitrogen bonds to give a series of ions. At low fragmentation energies, the doubly and triply charged cations prevailed. The chemical structures, chromatographic profiles, and LC/ESI/MS spectra of GN, GG, GNG, GGN, GGG, and GGGG standards are reported in **Figures 1** and **2**.

To verify the performance of the LC/MS analysis, the intraday and interday precisions were evaluated by carrying out five daily replicate analyses of a standard solution of each compound and by injecting the same solution for five consecutive days. The obtained CV% values ranged from 1.4 to 2.4% and from 2.2 to 4.3% for intraday and interday precisions, respectively.

The specificity of the developed method was evaluated by analyzing other widely used fungicides. Thiabendazole and imazalil were chosen in order to verify if possible interferences could be detected. The ability of the method to distinguish between the analyte and the other compounds was verified by analyzing the standard solution of the investigated compounds in the presence of tiabendazole and imazalil. The spiked samples (prepared as described) with the addition of tiabendazole and imazalil (0.05 mg/kg) were also analyzed. No interferences were detected (retention time (Rt) for thiabendazole and imazalil, 16.02 and 22.20 min, respectively).

Calibration curves were obtained by analyzing spiked samples at six different concentrations in the range reported in **Table 1**. The analytical LC/MS response was linear in the calibration range, with correlation coefficients (R^2) greater than 0.990. For quantification, the base peak of the mass spectrum was used (a fragment ion for GN at *m*/*z* 170 originating from the ammonia loss (−17 amu) $[M+H-NH_2]^+$, a fragment ion for GGN at *m*/*z*

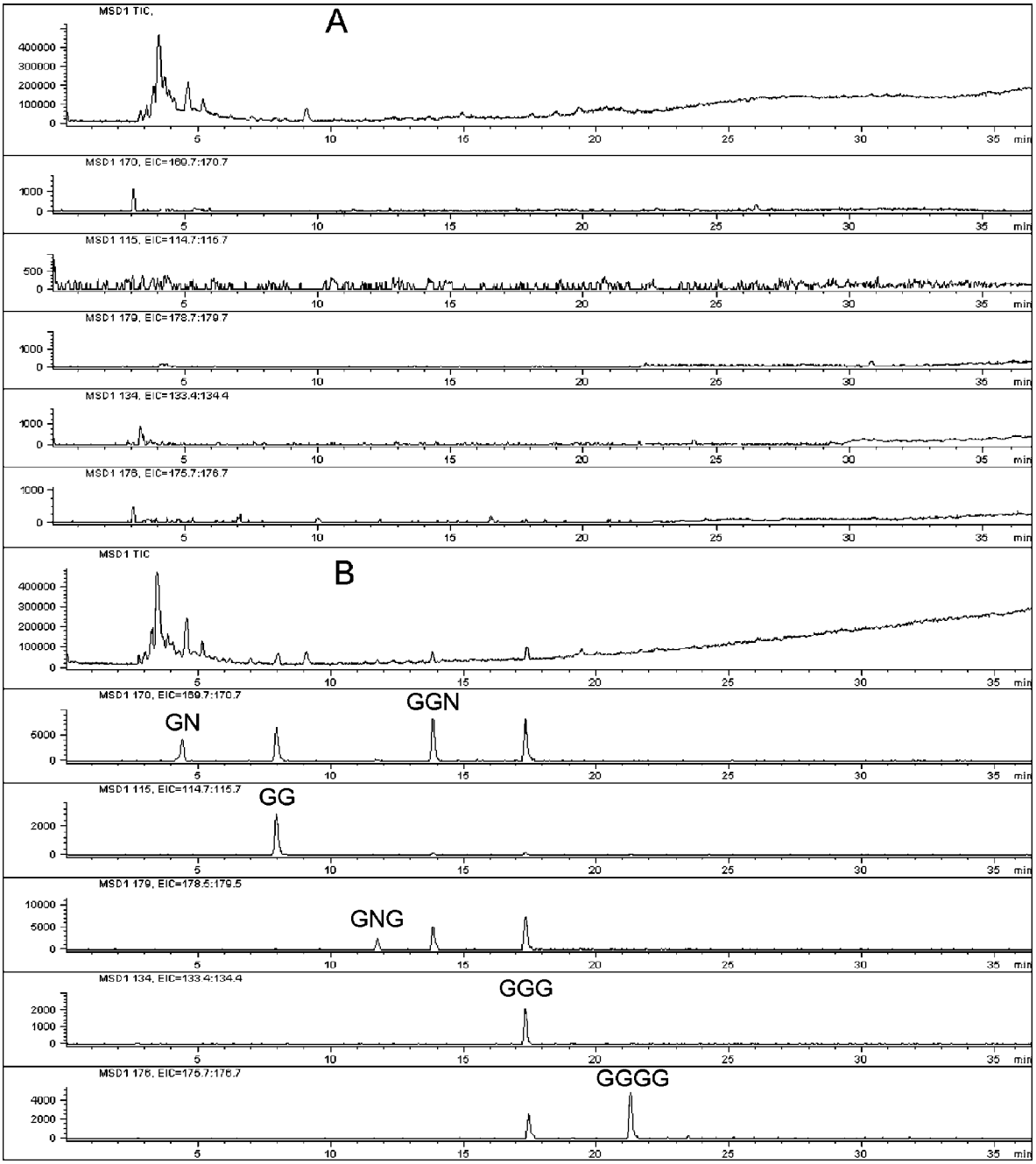

Figure 3. LC-ESI-MS chromatograms of a control sample (**A**) and a spiked hard wheat sample (GN, GG, GNG, GGN, GGG, and GGGG standards at the quantification limits) (**B**).

170 $[M+H-C_9H_{22}N_4]^+$, the doubly charged ion for GG (*m*/*z* 115) $[M+2H]^{2+}$ and GNG (*m*/*z* 179) $[M+2H]^{2+}$, triply charged for GGG (*m*/*z* 134) $[M+3H]^{3+}$ and the doubly charged ion of a fragment for GGGG (*m*/*z* 176) $[M+2H-CH_2N_2]^{2+}$.

Figures 3 and **4** show the chromatographic profile obtained for both spiked matrices (GN, GG, GNG, GGN, GGG, and GGGG standards at the quantification limits). The analysis of untreated samples were also reported (control).

For the extraction of samples, the direct application of the method published previously (*8*) did not give recovery values better than 50%. Optimization of the experimental conditions, in this study, yielded better results in terms of mean recovery. For the recovery experiments, maize and hard wheat samples were fortified with various compounds at 0.05 and 0.025 mg/kg. The spiking concentrations were selected on the basis of the guideline and $^1/_2$ guideline values established for GG. The various compounds were also added at the same average percentage at which they are present in the formulation to have a spiking concentration corresponding to 0.2 mg/kg of guazatine (temporary MRL proposed by EU) (*14*). Recovery data for all of the samples analyzed and the corresponding CVs values are listed in **Table 2**. The average recoveries for the six compounds at 0.05 mg/kg ranged from 81 to 86%, with the CVs ranging from 0.9 to 5.5% ($n = 5$). At 0.025 mg/kg, the recoveries ranged from 78 to 87%, with the CVs ranging from 0.8 to 6.3% ($n = 5$). The recovery and the CV values were not influenced by the kind of matrix or by the spiked concentration because significant differences were not found (t-test, $P > 0.05$). The recovery tests of maize samples fortified with the various compounds at 0.05

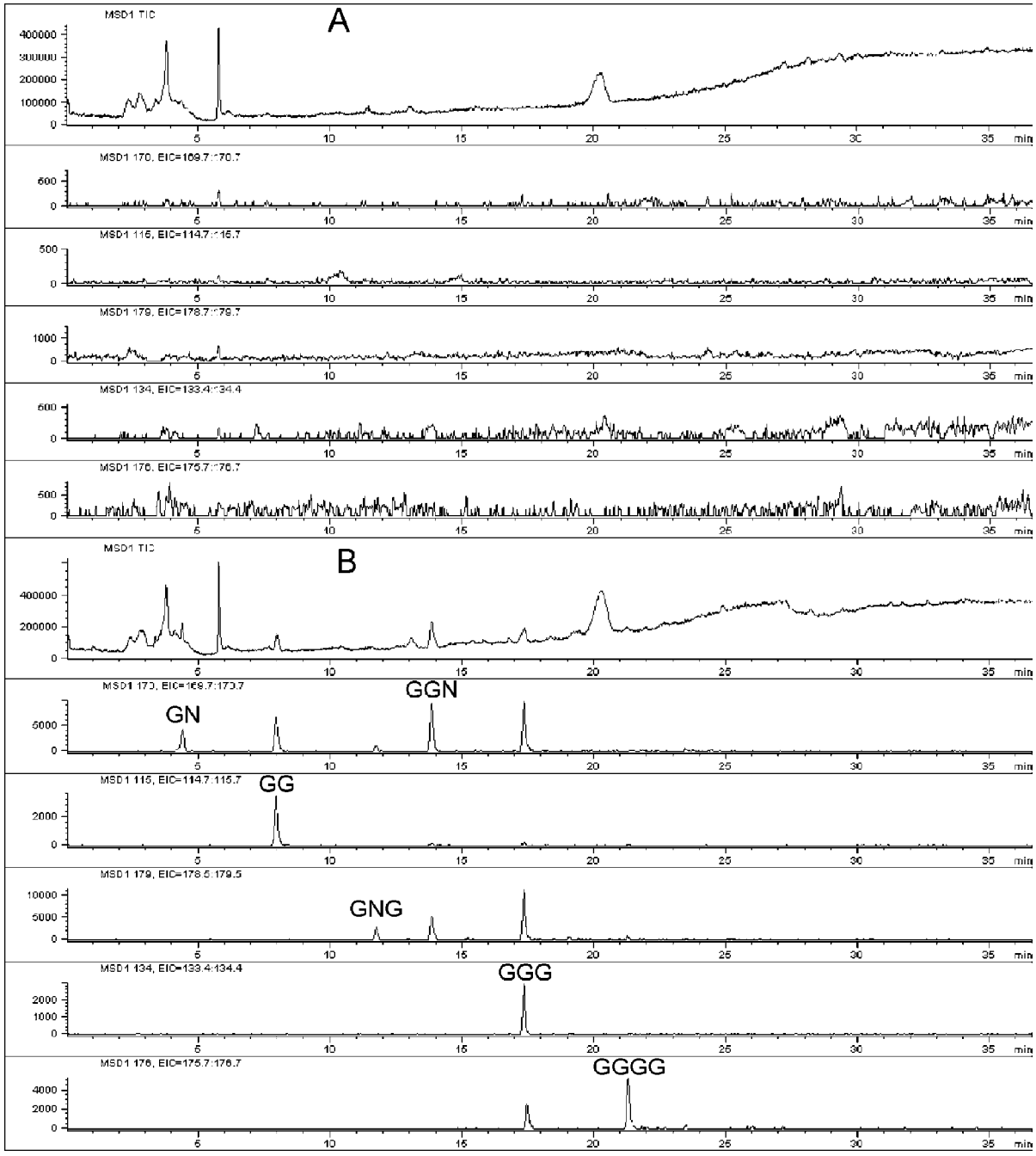

Figure 4. LC-ESI-MS chromatograms of a control sample (**A**) and a spiked maize sample (GN, GG, GNG, GGN, GGG, and GGGG standards at the quantification limits) (**B**).

mg/kg were carried out on three different days over two weeks. The CVs of the various average recoveries ranged within 6.2%.

The limits of quantification have been estimated to be 0.020, 0.004, 0.002, 0.002, 0.004, and 0.002 mg/kg, respectively, for GN, GG, GNG, GGN, GGG, and GGGG in maize and hard wheat (S/N ratio >10). The limit for GG has been estimated to be lower than the guideline established by the Codex Alimentarius Commission of the FAO (residue of GG in cereal crops 0.05 mg/kg) (*13*).

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