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Short communication

Analysis of guazatine mixture by LC and LC–MS and antimycotic activity determination of principal components

Elena Dreassi [a,*], Alessandra Tania Zizzari [a], Silvia D'Arezzo [b], Paolo Visca [b,c], Maurizio Botta [a]

[a] *Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, via A. Moro 2, Siena I-53100, Italy*
[b] *Istituto Nazionale di Malattie Infettive "Lazzaro Spallanzani" I.R.C.C.S., via Portuense 292, Roma I-00149, Italy*
[c] *Dipartimento di Biologia, Università "Roma Tre", viale G. Marconi 446, Roma I-00146, Italy*

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Abstract

Guazatine is a non-systemic contact fungicide, a mixture of reaction products from polyamines, comprising mainly octa-methylenediamine, iminodi(octamethylene)diamine, octamethylenebis(imino-octamethylene) diamine and carbamonitrile. In this work, the analysis of guazatine mixture by LC and LC–MS has been treated for the first time. In the guazatine mixture diamine derivatives account for 40% of the constituents of guazatine, triamines for 46%, tetramines for 11% and other amine derivatives for 3%. The most abundant individual components are the fully guanidated triamine (GGG, 30.6%) and the fully guanidated diamine (GG, 29.5%) followed by the monoguanidated diamine (GN, 9.8%) and a diguanidated triamine (GGN, 8.1%). The identification and separation of main components of commercial guazatine was performed through a new LC–MS method. The separation was performed on an Alltima C_{18} column using linear gradient elution (formic acid in water and acetonitrile) with UV-detection at 200 nm and the identification was performed by ESI^+-mass spectrometry analysis. The main components (GN, GG, GNG, GGN, GGG and GGGG) were then purified and separated from the mixture. Antimycotic activity of guazatine derivatives was determined on different species and strains belonging to genus *Candida*. The results obtained suggest that GNG and GGGG components can further be developed in new antifungal compounds with high potential for the treatment of *Candida* infections.

Keywords: HPLC/UV/MS; Guazatine; *Candida*; Antimycotic activity; MICs

1. Introduction

Guazatine is a non-systemic contact fungicide which disturbs the membrane function of fungi [1]. WHO has classified guazatine as moderately hazardous with an oral LD_{50} value in rats of 280 mg/kg bw [1]. It is widely used in agriculture to control a wide range of seed-borne diseases of cereals. On citrus fruit, guazatine is used as a bulk dip after harvest and in the packing line as a spray [1].

Guazatine acetate, the salt that is used in practice, is a mixture of reaction products from polyamines, comprising mainly octa-methylenediamine, iminodi(octamethylene)diamine, octamethylenebis(imino-octamethylene) diamine, and carbamonitrile. A coding system 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 $H_2N–C(NH)NH–(CH_2)_8–NH–C(NH)–NH_2$ [1].

The guanidated diamines and triamines are the most abundant components of guazatine [2]. A typical composition of free guazatine is described in Table 1. It can be seen that diamine derivatives account for *ca*. 40% of the constituents of guazatine, triamines for *ca*. 46%, tetramines for *ca*. 11% and other amine derivatives for *ca*. 3%. The most abundant individual components are the fully guanidated triamine (GGG, 30.6%) and the fully guanidated diamine (GG, 29.5%), followed by the monoguanidated diamine (GN, 9.8%) and by a diguanidated triamine (GGN, 8.1%).

* Corresponding author. Tel.: +39 0577234321; fax: +39 0577234333.
E-mail address: dreassi@unisi.it (E. Dreassi).

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Table 1
Typical composition of free guazatine reported on International Portal on Food Safety, Animal and Plant Health [1]

Component	%
NN	0.8
GN	9.8
GG	29.5
NNN	<0.1
NGN	0.8
GNN	1.7
GGN	8.1
GNG	4.5
GGG	30.6
GNNG	1.4
GGGN	1.4
GGGG	5.1
Other tetramines	3.1
GGGGG	1.1
Other pentamines	1.4
Hexamines and above	0.6
Total	99.9

Nowadays, standard guazatine purity is determined by titration of the guanidinic groups. Until now, the only separation and identification of main components of guazatine has been obtained by GC after derivatization with hexafluoroacetylacetone [3]. In other works characterization of guazatine through MS-FAB is reported [4,5].

GC after derivatization with hexafluoroacetylacetone was used also for the determination of the guazatine residues in agricultural crops [6,7]. Many of the non-published studies involve the hydrolysis of residues to bis(8-amino-octyl)amine (NNN), and its determination by GC either directly or after derivatization. Better results were achieved by using the marker GG, one of the major guazatine components, for quantification. This method incorporates a correction factor because GG represents only 30% of the total guazatine [2].

Therefore, the development of a new method of analysis and characterization without derivatization is of great interest. For the set up of a new analytical method that provide quantification of the residues, it will be necessary to have a standard of the principal component of the guazatine mixture. In this work we found a method by which it is possible to obtain, through chromatographic analysis, the main diamines and triamines and a tetramine (GN, GG, GNG, GGN, GGG and GGGG) that cover more than 87% of the total contents of the mixture.

Fungicide action of aliphatic amines has been known for a long time; guanidine compounds are strongly adsorbed by fungi membrane and are potent antifungal agents [8–10]. *Candida* infections are an important cause of morbidity and mortality, especially among immunodeficient patients. The recommended therapy relies primarily on azoles, like fluconazole. However, the widespread clinical use of these agents has resulted in measurable rates of acquired or innate fungal resistance in *Candida* species [11–13]. Moreover, many of the currently available drugs are loosing their therapeutic efficacy against new or re-emerging fungi because of the rapid development of resistance. These problems have exacerbated the need to develop new effective antifungal agents.

Aim of this work was to develop an LC method for identification and purification of main components of the commercial mixture of guazatine. The antimycotic activity of its principal components has also been defined.

2. Experimental

2.1. LC-MS analysis

2.1.1. Materials and reagents

Technical guazatine acetate salt was purchased from Rhone-Poulench Agro Italia (L'Aquila, Italy), and the guazatine acetate Pestanal® standard was from Riedel-de Haen. Pure fluconazole (molecular mass 306.28 Da; Pfizer Italia, Roma, IT) was provided by the Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy.

All the solvents and reagents were from Sigma–Aldrich Srl. (Milan, IT). Milli-Q quality water (Millipore, Milford, MA, USA) was used.

2.2. Instrumentation and method conditions

2.2.1. Equipment and LC–MS conditions

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, an UV detector, and an 1100 MSD model VL benchtop mass spectrometer with API–ES interface. The UV detector was set at 200 nm.

The Agilent 1100 series MSD single-quadrupole instrument was equipped with the orthogonal spray API (Agilent Technologies, Palo Alto, CA). Nitrogen was used as nebulizer gas and drying gas (350 °C). The LC–API–MS determination was performed by operating the MSD in the positive ion mode. Mass spectra were acquired over the scan range m/z 100–1500 using a step size of 0.1 μm. 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. For the fragmentation study the fragmentor voltage was set in the range 70–200 V.

2.2.2. Chromatographic separation

The chromatographic separation was performed on a Alltima C_{18} column (250 mm × 10 mm; 5 μm) (Alltech Italia Srl., Sedriano, Milan, Italy). Technical guazatine was dissolved in 0.2% (v/v) formic acid in water (2.0 mg/ml) and injected (200 μl) after filtration.

The separation was performed by using linear gradient elution 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 the flow rate of 3.5 ml/min.

After the UV detector an aliquot of the eluent (400 μl/min) was directed to MSD for spectra analysis.

The fractions containing the principal compounds of the mixture (GN, GG, GNG, GGN, GGG and GGGG) were collected and the solvent was evaporated under vacuum.

2.2.3. Identity and purity determination

The identity and purity of each compound were evaluated by LC–MS analysis on the basis of spectra analysis and retention times. The chromatographic separation was performed on the same column used for the separation. Each single isolated compound was dissolved in 0.2% (v/v) formic acid in water (10 μg/ml) and injected (20 μl) after filtration. The UV detector and the MSD parameters are listed above.

2.3. Antimycotic activity

2.3.1. Materials and methods

2.3.1.1. Organisms. A total of eight clinical isolates belonging to clinically relevant *Candida* species were tested, including three strains of *Candida albicans*, two of *Candida parapsilosis*, and one each of *Candida krusei*, *Candida glabrata* and *Candida tropicalis*. All clinical isolates were obtained from respiratory specimens, with each strain representing a single isolate from a patient. Strains had previously been identified by standard morphological, cultural and biochemical tests [13] at the Istituto Nazionale di Malattie Infettive "Lazzaro Spallanzani", Rome, Italy. In addition, *C. albicans* ATCC60193, *C. krusei* ATCC14243, and *C. parapsilosis* ATCC34136 were purchased from the American Type Culture Collection (Manassas, VA, USA) and used as reference strains in each set of experiments. Strain were stored in Sabouraud dextrose agar (Oxoid Italia SpA, Garbagnate Milanese, IT) slants at room temperature.

2.3.2. Determination of minimal inhibitory concentrations (MICs)

The reference microdilution method proposed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) was used for determination of MICs of antifungal agents [14].

Antifungal compounds were prepared as 8 mM stock solutions in dimethyl sulfoxide (Sigma–Aldrich Srl., Milan, IT), and stored frozen at −80 °C until used.

2.3.2.1. Assay medium. Assay medium was RPMI 1604 without sodium bicarbonate and with L-glutamine, buffered at pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS, Sigma–Aldrich Srl., Milan, IT) and supplemented with 2% (w/v) glucose. The medium was prepared as double-strength solution and sterilized by filtration, then diluted 1:2 (v/v) with the fungal inoculum in sterile distilled water.

2.3.2.2. Preparation of fungal inocula. The yeast isolates were grown on Sabouraud dextrose agar (Oxoid Italia SpA, Garbagnate Milanese, IT) for 48 h at 37 °C before testing. Inocula were prepared by combining in sterile distilled water five distinct colonies of >1 mm diameter for each culture. This suspension was adjusted to a turbidity of 0.5 McFarland in a PhoenixSpec photometer (Becton Dickinson, Franklin Lakes, NJ, USA), then diluted 1:10 (v/v) in sterile distilled water to achieve a yeast concentration of (1–5) × 10^5 colony forming units (cfu)/ml.

2.3.2.3. Susceptibility testing. Sterile plastic microtitration plates containing 96 flat-bottomed wells were used (Costar, purchased by Sigma–Aldrich Srl., Milan, IT). The plates were loaded with 100 μl/well of serial two-fold dilutions of the antifungal drugs in assay medium. Two drug-free medium wells as sterility and growth controls were used. The trays were inoculated with 100 μl/well of the fungal inoculum, with the exception of sterility control wells which were supplemented with 100 μl of sterile water. The final suspension contained between 0.5 × 10^5 and 2.5 × 10^5 cfu/ml. The range of concentrations tested for each drug was 1.25–80 μM. Microtitration plates were incubated at 37 °C for 24 h for determination of MIC values.

2.3.2.4. Spectrophotometric endpoint determination. Microtitration plates were agitated using a microtitration plate shaker before reading to ensure uniform turbidity of the fungal suspension. Yeast growth was determined by measuring the absorbance at 450 nm with a microtitration plate spectrophotometric reader (ETI-System Fast Reader, Sorin Biomedica S.p.A., Vicenza, IT). The value of the sterility control (*i.e.*, the uninoculated well) was subtracted from reading of the rest of the wells. The MIC_{50} endpoint was defined as the lowest drug concentration resulting in a growth reduction of ≥50% (as spectrophotometrically determined) compared with the growth of the control.

3. Results and discussion

3.1. LC–MS analysis

Mass spectra of the technical mixture of guazatine and standard guazatine are recorded and significant differences were not detected.

An example of a mass spectrum obtained by a direct injection of sample of guazatine standard is reported in Fig. 1. Signals from principal mixture components (Table 1), pseudomolecolar signals and double and triple charged signals, are detected.

The separation and identification of the main components of guazatine was performed through LC–MS analysis. We searched for better conditions to obtain single component separation trough LC–MS analysis. RP analytical or ionic exchange columns did not provide good separation (data not shown), while better results were obtained using a semipreparative column. The chromatographic profile obtained for a sample of technical guazatine is reported in Fig. 2.

The ESI analysis of these compounds was not previously described in any work so each compound has been identified trough mass spectra after fragmentation studies using the spectra obtained with various fragmentor energies. Fragmentation of the monoprotonated bases 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 reported in Fig. 3. At low fragmentation energy the doubly and triple charged cations prevailed.

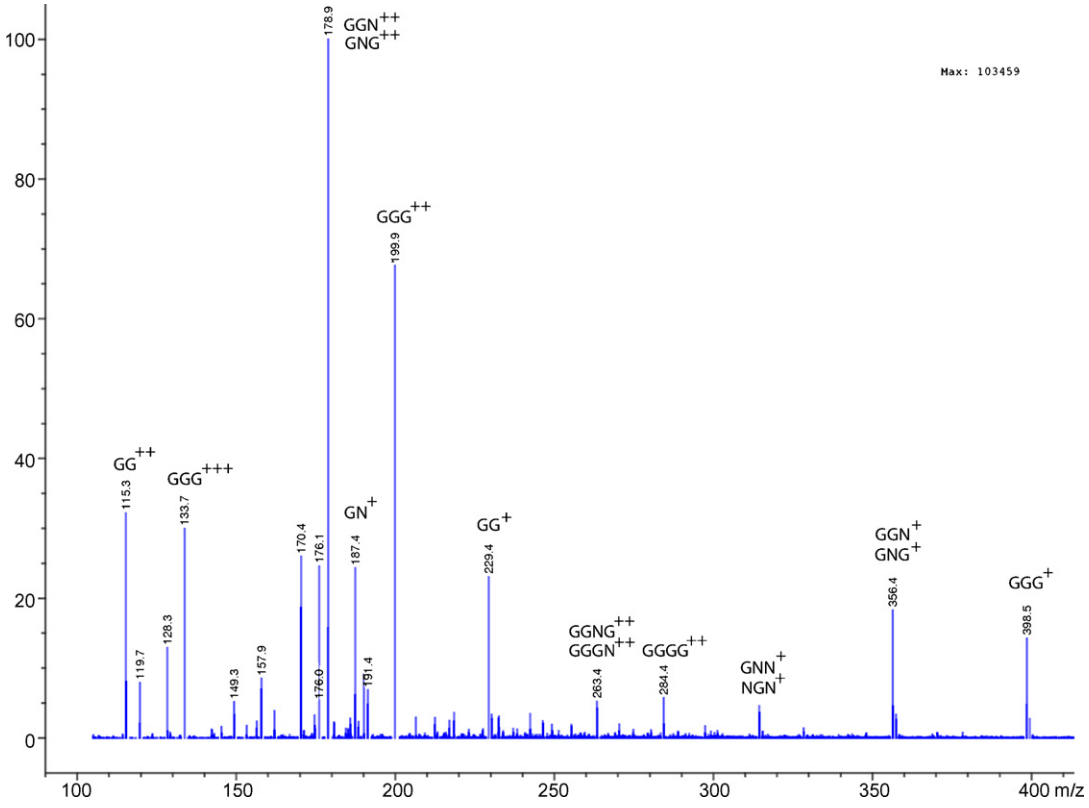

Fig. 1. Mass spectrum obtained by direct injection of a sample of standard guazatine in the conditions described in Section 2.

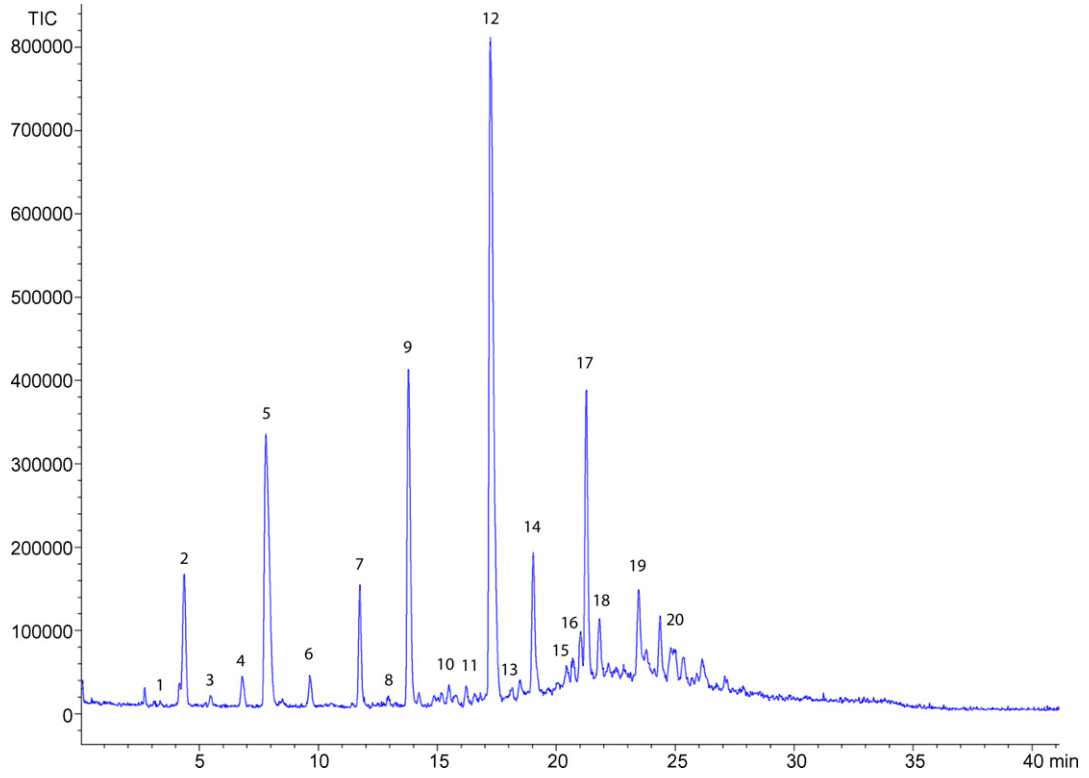

Fig. 2. Chromatographic profile obtained for a sample of commercial guazatine (350 μg/ml) by LC–MS. (1) NN; (2) GN; (3) NNN; (4) GNN; (5) GG; (6) NGN; (7) GNG; (8) GGNN; (9) GGN; (10) GNGN; (11) GNNG; (12) GGG; (13) GGNG; (14) GGGN; (15) GGNGG; (16) GGGNG; (17) GGGG; (18) GGGGN; (19) GGGGG; (20) GGGGGG.

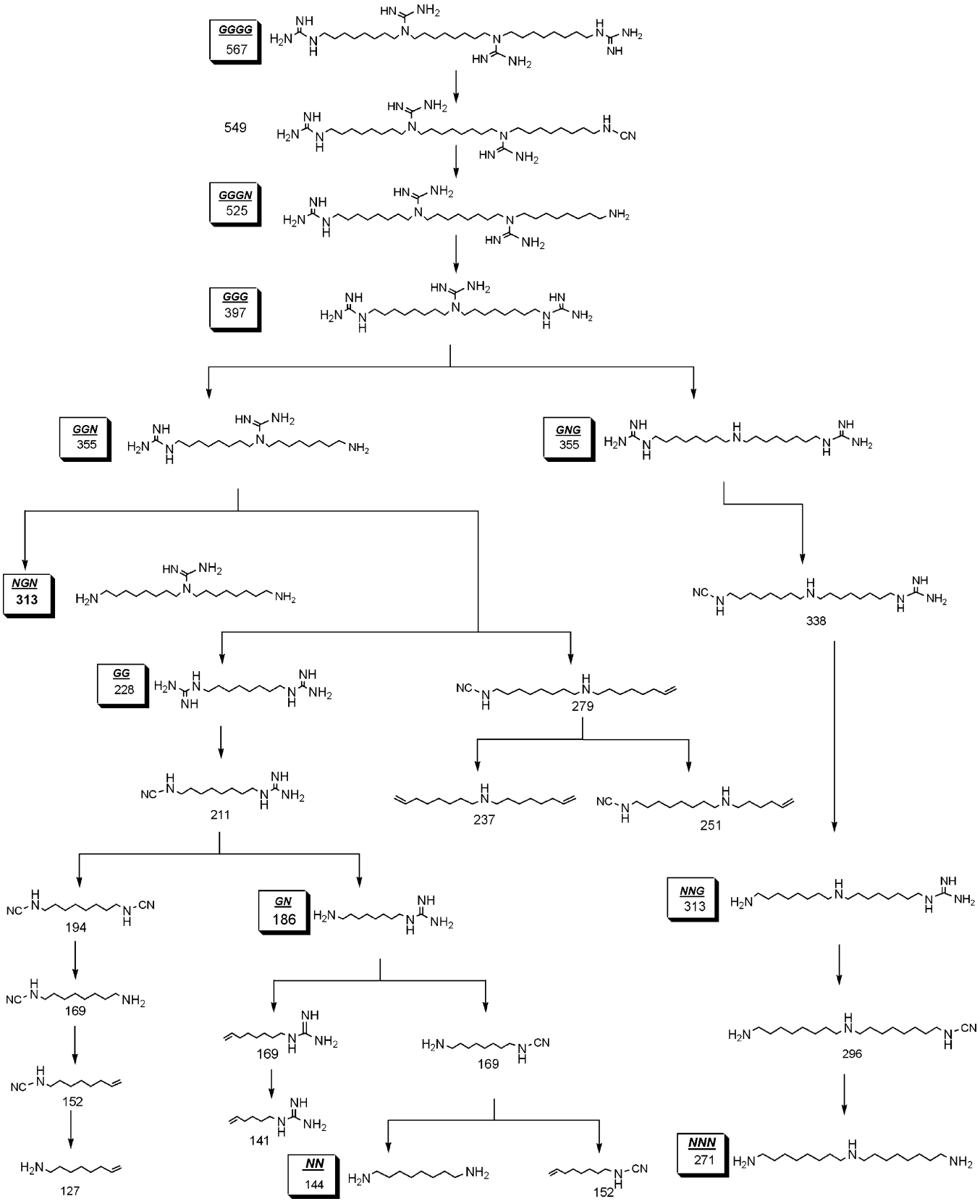

Fig. 3. Fragmentation pathways for the principal component of guazatine.

Table 2
Principal ions used for the identification of the various guazatine components

Compound	Molecular mass	Rt (min)	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+4H]^{4+}$	Major fragment ions, *m/z*											
NN	144	3.3	145.3				128.3											
GN	186	4.4	187.4				128.3	142.3		170.4	153.3							
GG	228	7.8	229.4	115.3			128.3	142.3	145.3	170.4	153.3	187.4	212.3					
NNN	271	5.5					128.3		145.3						272.4	297.4		
GNN	313	6.8	314.5	157.9			128.3		145.3	170.4		187.4			272.4	297.4		
NGN	313	9.6	314.5	157.9			128.3		145.3		153.3	187.4		238.4	272.4	297.4		
GNG	355	11.7	356.4	178.9			128.3	142.3	145.3	170.4					272.4	297.4		
GGN	355	13.8	356.4	178.9			128.3	142.3	145.3	170.4	153.3	187.4	212.3	238.4	272.4	297.4	314.5	
GGG	397	17.2	398.5	199.9	133.7		128.3	142.3	145.3	170.4	153.3	187.4	212.3	238.4	272.4	297.4	314.5	
GGNN	482	13.1	483.6	242.2	161.7													
GNGN	482	15.3	483.6	242.2	161.7													
GNNG	482	16.6	483.6	242.2	161.7													
GGNG	524	18.1	525.5	263.5	176.0	132.5								238.4				
GGGN	524	19.0	525.5	263.5	176.0	132.5	128.3	142.3		170.4	153.3	187.4	212.3		272.4	297.4	314.5	398.5
GGGG	566	21.2	567.5	284.4	191.1	142.9	128.3	142.3	145.3	170.4	153.3	187.4	212.3	238.4	272.4	297.4	314.5	
GGNGG	693	20.3	694.7	348.0	232.4	174.7	128.3											
GGGNG	693	20.7	694.7	348.0	232.4	174.7	128.3										314.5	
GGGGN	693	21.8	694.7	348.0	232.4	174.7	128.3			170.4								
GGGGG	735	23.4	736.7	369.8	246.5	185.2	128.3	142.3				187.4						
GGGGGG	904	24.8		453.3	302.4	227.1												

Table 3
Antimycotic activity of purified guazatine components on 11 yeast strains representative of clinically relevant *Candida* species

Candida strain	MIC_{50} (μM)[a]							
	GN	GG	GNG	GGN	GGG	GGGG	Guazatine mixture	Fluconazole
Candida albicans ATCC60193	>80	>80	80	>80	5	>80	40	0.8
C. albicans 4T	>80	>80	80	80	5	>80	20	209.0
C. albicans 53T	>80	>80	40	80	20	>80	40	418.0
C. albicans 15T	>80	>80	80	80	10	80	80	209.0
Candida krusei ATCC14243	>80	>80	20	40	10	80	20	209.0
C. krusei 193T	>80	>80	20	40	40	40	20	418.0
Candida parapsilosis ATCC34136	>80	>80	>80	>80	>80	>80	>80	6.5
C. parapsilosis 64E	>80	>80	40	40	>80	80	20	3.2
C. parapsilosis 81E	>80	>80	20	20	>80	40	20	13.0
Candida glabrata 70E	>80	>80	40	80	>80	>80	40	209.0
Candida tropicalis 86E	>80	40	1.25	20	1.25	10	10	52.2

[a] MIC_{50} values were obtained by spectrophotometric endpoint determination, as outlined in Section 2.

In the chromatographic analysis the identity of each compound was determined on the basis of principal ions reported in Table 2.

Trough UV profiles and mass spectra we isolated the main components from the guazatine mixture (GG, GN, GNG, GGN, GGG e GGGG). Purity of these compounds (in each case more than 94%) has been determined trough chromatographic analysis.

3.2. Antimycotic activity determination

The purified main components from commercial guazatine were tested against selected *Candida* species. Values obtained for the commercial mixture and fluconazole are reported for comparison (Table 3). The guazatine mixture showed potent antifungal activity on all but one *Candida* strains, with MIC_{50} values ranging between 10 and 80 μM. The activity of isolated compounds was highly variable. GN, GG and, to a lesser extent, GGGG and GGN showed modest activity within the concentration range tested. Conversely, GNG and GGG, showed an overall good activity and MIC_{50} values that were in most cases lower than to those of guazatine and fluconazole, taken as the reference drugs. GNG and GGG were particularly effective against fluconazole-resistant clinical isolates of *C. albicans*, *C. krusei*, *C. glabrata* and *C. tropicalis*. Notably, *C. parapsilosis* ATCC34136 was the only strain which resisted to ≥80 μM of all drugs tested, except fluconazole to which it was highly susceptible.

4. Conclusions

The use of a complex mixture such as guazatine presents a problem in choosing a method for residue analysis. It is not considered practical to attempt the determination of all the components, and therefore another alternative analysis method is necessary.

The choice of a major component as a 'marker', with the inclusion of a correction factor to give the total residue still has it's limitations considering the variety of the components present.

With the determination of the main components of guazatine, it would be possible to have a more accurate estimate than with the simple determination of only GG.

The results obtained with this chromatographic analysis open new perspective in the residue control of this fungicide in food products. In fact, the MRL for guazatine residues in food is 5 mg/kg expressed as guazatine. LC–MS allows the determination of the principal components of the mixture with a great sensibility (below 1.0 μg/ml) and specificity.

Both guazatine and some of its main components (particularly GNG and GGG) showed potent anti-*Candida* activity, superior to that of the commercial fluconazole, an antifungal agent widely used in current clinical practice. The low toxicity of guazatine and the overall high activity of the GGG component, which accounts for 30.6% of the commercial mixture, points in direction of future development of this antifungal lead.

Acknowledgement

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