EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: Guazatine (a mixture of various oligomers with amino- and/or guanidine groups)
- **Commodities**: Citrus fruits
- **Method(s)**: Various tested (Influence of extraction solvent, pH, temperature, extraction time studied)
- **Instrumentation**: LC-MS/MS

Analysis of Guazatine in Food Products

Reported by: EURL-SRM
Version 1 (last update: 15.06.2018)

Background information / Initial Observations/Aims:

Guazatine is a non-systemic contact fungicide that is also used as a repellent. It consists of a complex mixture of different oligomers composed of octamethylene bridges connecting randomly guanylated primary and/or secondary amino groups.

With all guazatine components being strongly basic and highly polar, recovery rates by the citrate buffered QuEChERS method [QuEChERS EN15662], were extremely low. Recoveries using the QuPPe method [EURL-SRM-QuPPe}, which does not involve any partitioning step and is thus suitable for polar compounds, were surprisingly also very low. Besides the difficulties with achieving good recoveries the coverage of the full residue definition "*Guazatine (guazatine acetate, sum of components)*" is also challenging due to the complexity of the guazatine mixture. Even if one or a few marker compounds are quantified, extrapolations to guazatine (sum) is associated with uncertainty for a number of reasons including a) the reported fluctuations in the in the composition of the technical mixture used to produce guazatine-formulations; b) the potential differences in the degradation rates of the individual guazatine components within the crops; and c) the variable, and to a certain extent uncertain composition of the available analytical standards of guazatine mixtures.

In 1997 JMPR contemplated establishing an enforcement residue definition based on only one guazatine component ("*octane-1,8-diyldiguanidine (GG), expressed as octane-1,8-diyldiguanidine*"). It was concluded that the GG content should be multiplied by 3 for risk assessment purposes assuming that the content of GG is 30% of the total guazatine content [FAO 1998]. Finally Codex implemented CXLs in 1999 for citrus and cereals with the residue definition being simply "Guazatine".

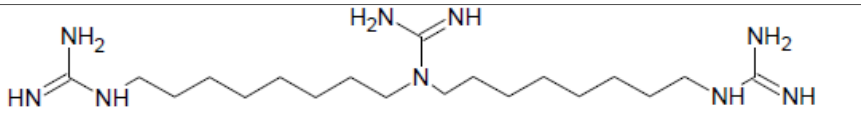
In 2007, Scordino et al. analyzed 77 citrus fruit samples of non-EU origin. 64% of these samples were found to contain guazatine at levels > 0.010 mg/kg [Scordino et al. 2007].

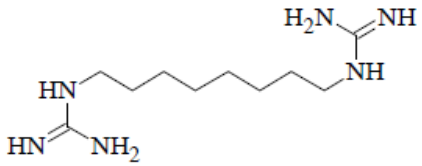
In 2014 an application for an EU import tolerance was submitted to cover the post-harvest treatments of citrus fruits with guazatine in South Africa. Thereafter the RMS (UK) proposed an MRL of 4 mg/kg. EFSA, however, expressed its reservations to establish an MRL due to uncertainties in the toxicological reference values, the insufficient elucidation of metabolic pathways in plants and livestock and the questionable validity of the supervised residue trials, which focused on only one single marker compound of the technical material (GG-diacetate). Furthermore, EFSA did not agree with the position of the applicant, that GG-diacetate and GGG-triacetate can be used to fully characterize the metabolism to all guazatine components in citrus fruits [EFSA 2014]. With Regulation 2015/1910/EU the MRLs for guazatine were lowered to the consensus LOQ of 0.05 mg/kg.

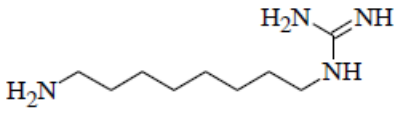
The present study aimed to develop a method allowing the analysis of the main guazatine components (preferably via LC-MS/MS) and to examine whether the introduction of a different residue definition based on the sum of a few selected marker compounds (with or without extrapolation to total guazatine) would be a feasible option from the analytical point of view. The present paper is an interim report of an ongoing study and will be periodically updated.

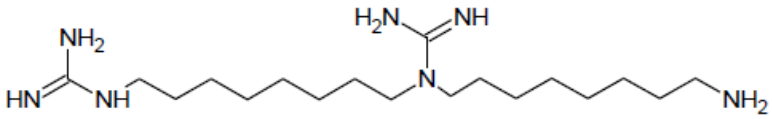
Facts at a glance:

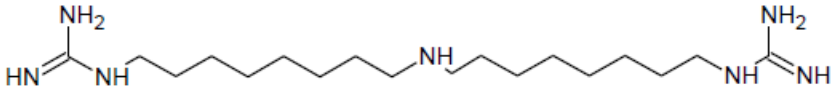
Guazatine	
Definition	The ISO common name of the active substance is '***Guazatine Acetates***' and is defined as a '*A mixture of the reaction products from polyamines, comprising mainly octamethylenediamine, Iminodi(octamethylene) diamine and octamethylenebis(imino-octamethylene)diamine, and cyanamide'. In its evaluation report FAO additionally provides the following definition: "Guazatine is a preparation of the triacetates of dimeric and trimeric guanidated octane-1,8-diyldiamine which also contains a range of oligomers and reaction products" [FAO 1998].* Guazatine is always manufactured in the form of the acetate salt, hence the identity should be stated as "guazatine acetates" [EFSA 2010]. Note: The approved common name „guazatine" was originally defined as applying to *1,1'iminodi-(octamethylene)diguanidine* (=iminoctatine = GNG; CAS: 108173-90-6), but it was later realized that the material marketed commercially is a reaction mixture.
Coding	A coding system is used for the compounds that make up guazatine, in which '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 [FAO 1998].
Typical composition	As guazatine acetates is a complex mixture of polymorphic compounds, the manufacturing process is such that there is **variability between production batches of guazatine acetates** [EFSA 2010]. A typical composition of free guazatine (not of guazatine acetates, the salts which are used in practice) is as follows: **GGG 30.6%, GG 29.5%, GN 9.8%, GGN 8.1%**, GGGG 5.1%, GNG 4.5%, GNN 1,7%, GNNG 1.4%, GGGN 1.4%, GGGGG 1.1%, NN 0.8%, NGN 0.8%, NNN <0.1%, Other tetramines 3.1%, other pentamines 1.4%, hexamines and above 0.6%. Overall: diamines 40%, triamines 46%, tetramines 11%, other 3%. Formulations are expressed as guazatine acetate, e.g., a 200 SL formulation contains 200 g/l of guazatine acetate or 133 g/l of guazatine. [EFSA 2010].
Mode of action and uses	Guazatine is a non-systemic contact fungicide, it also acts as a repellent. It is used for the **seed treatment of cereals** (wheat, rye, and triticale) as it controls a wide range of seed-borne diseases of cereals, e.g. seedling blight (fusarium spp.), glume blotch (septoria), common bunt (tilletia spp.), common root rot (helminthosporium) and smut (ustilago) [FAO 1998]. On **citrus fruit,** it controls sour rot (geotrichum candidum), green mould (penicillium digitatum) and blue mould (penicillium italicum). It is used in multiple ways: as a bulk dip after harvest, in the packing line as a spray and in washing installations to disinfect the process water [FAO 1998].
CAS No.	*[108173-90-6] for guazatine* ***(now belongs to iminoctatine GNG, see note above)*** [115044-19-4] for guazatine acetates
Part. coeff.	See individual components
Solubility	In water: >600 g/L at ~20°C (pH 4,7 and 9) for 70.6% (TK) [EFSA 2010]. In other solvents: Methanol 510 g/l, ethyl acetate <100 mg/l, n-hexane <100 mg/l) [EFSA 2010].
Dissociation constant	pKa1 = 10.5, pKa2 = 4.6 (70% TK) [EFSA 2010]. (see also individual components)
ADI	0.002 mg/kg bw/day for 100% guazatine acetates [EFSA 2010].
ARfD	0.0048 mg/kg bw for 100% guazatine acetates (pure active ingredient) mg/kg bw [EFSA 2010].
Registration Status and uses	**No longer authorized within the EU (**see Reg. (EC) 2008/934 on Commission Decision concerning the non-inclusion of guazatine in Annex I to Council Directive 91/414/EEC). A resubmission application for the inclusion of guazatine in Annex I was not successful. Without a further characterization of the technical material, it was not possible to conclude on the identity of the active substance. Guazatine used to be authorized in some EU countries as a fungicide for cereals and rape seed. It is still used overseas (e.g. RSA, Argentina) for the surface treatment of citrus fruits, the cultivation of sugar cane, melons and tomatoes. There has been an import tolerance application for guazatine in citrus by RSA. EFSA identified some data requirements which prevented to conclude on the consumer risk assessment and the evaluation of the import tolerance application was stopped [EFSA 2014].
Residue def. and MRLs	Through Reg. (EU) 2015/1910 (applies since:13/05/2016); the **EU MRL** for "Guazatine (guazatine acetate, sum of components)" is set at 0.05* for all commodities. **Codex Alimentarius** has established a guideline level for citrus fruits at 5 mg/kg but no full CXL was derived because of substantial data gaps that led to the withdrawal of the ADI [FAO 1998]. The MRL for citrus established in **South Africa** is 5mg/kg; the residue definition is set as free guazatine.

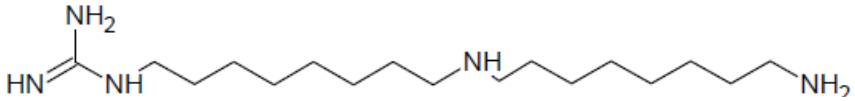
Guazatine component GGG (exemplary content in formulation 31 %; in standard 30%)	
Parameter	**Value**
Molecular formula	
Molar Mass	397.616 g/mol
Exact mass	397.364142 Da
CAS	?
IUPAC name	N,N-bis(8-carbamimidamidooctyl)guanidine
Other names	1,1-bis-(8-guanidin-1- yl-octyl)guanidine
pKa (calc. by chemicalize)	pK_{a1} = 12.8; pK_{a2} = 12.3; pK_{a3} = 11.8 (all very strongly basic) Predominantly triply protonated at pH <11.8 Predominantly non-ionized at pH > 12.8; >90% non-ionized at pH >13.8
LogD	**EFSA Peer Review** [EFSA2010]: -3.5 (pH 9); -4.4 (pH7); -4.6 (pH4) **Chemicalize** (computed): highest logD =1.75 at pH>14 (non-ionic form), pH 2-8 logD = -5.5

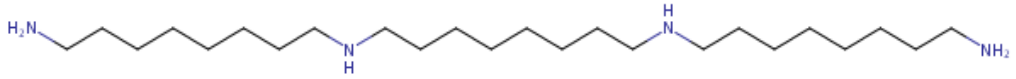
Guazatine component GG (exemplary content in formulation 30 %; in standard 10%)	
Parameter	**Value**
Molecular formula	
Molar Mass	228.344 g/mol
Exact mass	228.206245 Da
CAS	19010-48-1; 25303-05-3 (as dichloride salt)
IUPAC name	N-(8-carbamimidamidooctyl)guanidine
Other names	1-8-diguanidino-octane
pKa (calc. by chemicalize)	pK_{a1} = 12.6; pK_{a2} = 12.0 (both very strongly basic) Predominantly doubly protonated at pH <11.8 Predominantly non-ionized at pH > 12.7; >90% non-ionized at pH >13.6
LogD	**EFSA Peer Review** [EFSA2010]: -3.3 (pH 9); -3.1 (pH7); -3 (pH4) **Chemicalize** (computed): highest logD =0.27 at pH>14 (non-ionic form), pH 2-8 logD = -4.56

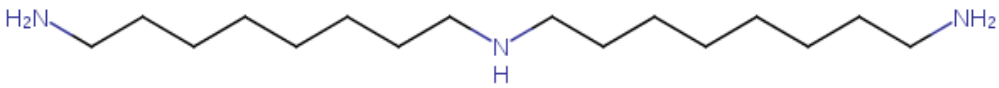
Guazatine component GN (exemplary content in formulation 10 %)	
Parameter	**Value**
Molecular formula	
Molar Mass	228.344 g/mol
Exact mass	228.206245 Da
CAS	?
IUPAC name	N-(8-aminooctyl)guanidine
Other names	1-amino-8-guanidin-1- yl-octane
pKa (calc. by chemicalize)	pK_{a1} = 12.3 at guanidine group; pK_{a2} = 10.2 at amino group (both very strongly basic) Predominantly doubly protonated at pH <10.2 Predominantly singly protonated at pH range from 10.2 to 12.2 Predominantly non-ionized at pH > 12.2; >90% non-ionized at pH >13.3
LogD	**EFSA Peer Review** [EFSA2010]: -3.2 (pH 9); -4.0 (pH7); -3.4 (pH4) **Chemicalize** (computed): highest logD =0.6 at pH>14 (non-ionic form), pH 2-6 logD = -4.8

Guazatine component GGN (exemplary content in formulation 8 %; in standard 14.5 %)	
Parameter	**Value**
Molecular formula	
Molar Mass	355.575 g/mol
Exact mass	355.342344 Da
CAS	?
IUPAC name	N-(8-aminooctyl)-N-(8-carbamimidamidooctyl)guanidine
Other names	1-(8-guanidin-1-yl-octyl)-1-(8-amino-octyl)guanidine
pKa (calc. by chemicalize)	pK_{a1} = 12.6 at guanidine group; pK_{a2} = 12.0 at guanidine group; pK_{a3} = 10.2 at amino group (all very strongly basic) Predominantly triply protonated at pH <10.2 Predominantly doubly protonated at pH range from 10.2 to 11.9 (both guanidine groups) Predominantly non-ionized at pH > 12.7; >90% non-ionized at pH >13.6
LogD	**EFSA Peer Review** [EFSA2010]: -3.4 (pH 9); -4.6 (pH7); -4.3 (pH4) **Chemicalize** (computed): highest logD =2.1 at pH>14 (non-ionic form), pH 1-6 logD = -5.8

Guazatine component GNG (exemplary content in formulation 4.5 %; in standard 0.28 %)	
Parameter	**Value**
Molecular formula	
Molar Mass	355.575 g/mol
Exact mass	355.342344 Da
CAS	79956-56-2,108173-90-6,13516-27-3
IUPAC name	N-{8-[(8-carbamimidamidooctyl)amino]octyl}guanidine
Other names	1,1-bis-(8- aminooctyl)guanidine 2-[8-[8-(diaminomethylideneamino)octylamino]octyl]guanidine Iminoctatine
pKa (calc. by chemicalize)	pK_{a1} = 12.6 at guanidine group; pK_{a2} = 12.0 at guanidine group; pK_{a3} = 10.7 at amino group (all very strongly basic) Predominantly triply protonated at pH <10.7 Predominantly doubly protonated at pH range from 10.7 to 11.8 (both guanidine groups) Predominantly non-ionized at pH > 12.7; >90% non-ionized at pH >13.6
LogD (calc. by chemicalize)	Highest logD =2.3 at pH>14 (non-ionic form), pH 1-6 logD = -5.8

Guazatine component GNN (exemplary content in formulation 1.7 %; in standard ?)	
Parameter	**Value**
Molecular formula	
Molar Mass	313.534 g/mol
Exact mass	313.320546 Da
CAS	?
IUPAC name	N-{8-[(8-aminooctyl)amino]octyl}guanidine
Other names	1-{8-[(8- aminooctyl)amino]oct yl}guanidine
pKa (calc. by chemicalize)	pK_{a1} = 12.3 at guanidine group; pK_{a2} = 10.8 at secondary amino group; pK_{a3} = 10.1 at terminal amino group (all very strongly basic) Predominantly triply protonated at pH <10 Predominantly singly protonated at pH range from 11 to 12.3 (guanidine group) Predominantly non-ionized at pH > 12.3; >90% non-ionized at pH >13.3
LogD (calc. by chemicalize)	Highest logD =2.59 at pH>14 (non-ionic form), pH 1-6 logD = -6.1

Guazatine component NNNN (exemplary content in formulation <0.1 %; in standard 7 %)	
Parameter	**Value**
Molecular formula	
Molar Mass	398.724 g/mol
Exact mass	398.434848 Da
CAS	15518-46-4
IUPAC name	N'-[8-(8-aminooctylamino)octyl]octane-1,8-diamine
Other names	N,N'-Bis(8-aminooctyl)-1,8-octanediamine
pKa (calc. by chemicalize)	pK_{a1} and K_{a2}= 11.2 and 10.3 at secondary amino groups; pK_{a3} and pK_{a4} = 10.7 and 9.8 at terminal amino groups (all very strongly basic) Predominantly quadriply protonated at pH <9.6 Predominantly non-ionized at pH > 11.3; >90% non-ionized at pH >12.2
LogD (calc. by chemicalize)	highest logD =4.9 at pH>12 (non-ionic form), pH <6 logD = -7.5

Guazatine component NNN (exemplary content in formulation <0.1 %; in standard ?)	
Parameter	**Value**
Molecular formula	
Molar Mass	271.493 g/mol
Exact mass	271.29900 Da
CAS	39202-36-3
IUPAC name	bis(8-aminooctyl)amine
Other names	N-(8-Aminooctyl)octane-1,8-diamine; 1,8-Octanediamine,N-(8-aminooctyl); N-(8-aminooctyl)-1,8-octanediamine; dioctamethylene triamine; 1,17-diamino-9-azaheptadecane; BIS(8-AMINOOCTYL)AMINE; iminodi(octamethylene)diamine
pKa (calc. by chemicalize)	pK_{a1} = 11.0 at secondary amino group ; pK_{a2} and pK_{a3} = 10.4 and 10.0 at terminal amino groups (all very strongly basic) Predominantly triply protonated at pH <9.7 Predominantly non-ionized at pH > 11.3; >90% non-ionized at pH >12
LogD	Chemicalize: highest logD (logP) 2.92 at pH>11.8 (non-ionic form), pH <6 logD = -6.3 ACD-Labs: pH5.5 -2.36; logP 3.11

Materials and instrumentation (exemplary[1]):

Guazatine acetate standard was purchased from Sigma/Aldrich (PESTANAL® Product: 37915; Batch: SZBE090XV). According to the company's certificate the standard contained 38% water (Karl Fischer) and 20.6% acetates (HPLC) and was analyzed via HPLC-UV (@210 nm), with the peak signals between 2 and 47 minutes being considered to represent 100% of the guazatine. A number of peaks within the chromatogram were allocated to specific guazatine components with the share of each of their signals to the total signal area of guazatine being as follows: GG (9.8%); GGG (30.2%) GGN (14.5%); GNG (0.28%); NNNNN (5.9%); NNNN (7.2%). Other ("non identified") compounds corresponded to 44.1% of the total signal.

A **stock solution** of the above standard was prepared in methanol at 1 mg/ml. This stock solution was diluted 5-fold in methanol to obtain a **working standard** at 200µg/mL, which was used for spiking experiments. This working standard was further diluted 100-fold in methanol to obtain a **second working standard** at 2µg/mL, which was used to prepare calibration standards at 0.1µg/mL. Considering the 38% water content and the 20.6% acetate content in the purchased standard, the total concentration of guazatine in the 0.1µg/mL calibration standards was calculated as 0.0414 µg/mL. **All solutions were prepared in plastic bottles as the guazatine components tend to interact with glass surfaces.** Following the Relana proposal [Relana 2016], the assumption was made that the peak allocations by the manufacturer are correct and that the UV signal areas are proportional to the concentration of each of the allocated components, with the concentration of individual identified components in the calibration standard calculating as follows: GGG 0.0124986 µg/mL; GG 0.00405 µg/mL; GGN 0.00601 µg/mL; GNG 0.00011 µg/mL, NNNN 0.002981; NNNNN 0.001615. According to the Relana® approach, using the assumptions above, the sum of four Guazatine Indicator Components (GIC) GGG, GG, GGN and GNG can be extrapolated to 'Guazatine acetate (sum of components) by multiplying with the so called ''Realana factor' of 2.733. For the second working standard the GIC concentration adds up to 0.022685 µg/mL and the concentration of 'Guazatine acetate (sum of components)' calculates to 0.062 µg /mL.

Guazatine formulation Kenopel 200 SL (by Adama, South Africa) containing ca 19.2 % (w/w) guazatine acetates, was kindly provided by Labor Friedle/Tegernheim. This was diluted with water to obtain a solution containing ca. 1000 µg/mL guazatine acetates, which was used for the dipping. Prof. Horacio Heinzen and Prof. Veronica Cesio from the Universidad Catholica de Montevideo / Uruguay also kindly provided a formulation with a double concentration but a similar ratio of the four main components.

Wax emulsions "Citrosol A" (based on partly oxidized polyethylene emulsified in ammonia-containing water) and "Citrosol A Cámara" (based on partly oxidized polyethylene and shellac emulsified in ammonia-containing water) were kindly provided by Citrosol S.A. (Valencia/Spain).

Acetone was purchased from Carl Roth GmbH&Co. KG, Germany

For other QuEChERS reagents and consumables please refer to EN15662 [QuEChERS EN15662]

[1] Disclaimer: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

For other QuPPe related information please refer to the QuPPe protocol [EURL-QuPPe].

Choice of internal standard

Various compounds were tested as to their suitability as internal standards. Thiabendazol, carbendazim and imazalil were selected as candidates as they exhibit basic properties, which is also the case for the guazatine components. All three compounds showed high recovery rates (92-94%) using a one-phase extraction method in which the final extract was composed of ca. 66% water (including sample water), 33% of acetone, and 0.17% of Formic acid (v/v). In LC-MS/MS analysis (see conditions there), imazalil eluted much later than the guazatine components whereas thiabendazole and carbendazime eluted at a similar retention time range. Thiabendazole showed a relatively strong peak tailing, thus carbenadazim D4 was finally considered as a suitable internal standard, mainly for the correction of volume deviations. Matrix effects were eliminated by the use of matrix-matched calibrations. At a later stage 1,6-Bis(guanidino)hexane (GG-C6) was also introduced and showed similar analytical behavior to the guazatine component GG (GG-C8). GG-C6 thus qualifiesg as an internal standard for GG.

Instrumental Analysis

The LC- and MS/MS settings used are shown in Table 1 and the mass transitions used in Table 2. The mass transitions based on multiply charged parent ions gave the most intensive signals and were thus chosen for data acquisition. In such cases daughter m/z values were larger than parent m/z values.

Table 1: Instrumentation details

LC	WATERS Acquity UPLC / WATERS I-class with FTN sample manager		
MS/MS	SCIEX API 4000 QTrap / API 5500QTrap, run **in ESI positive mode**		
Column	Acquity BEH C18, 2.1x100 mm, 1.7 µm		
Pre-column	Acquity BEH C18, 2.1x5 mm, 1.7 µm		
Mobile Phase	A: 0,2% formic acid in purified water (5% methanol) B: 0,2% formic acid in methanol		
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Mobile Phase B** (%)
	0	98	2
	3.5	50	50
	4	10	90
	6	10	90
	6.1	98	2
Equilibration Time	5 min		
Run Time	11 min		
Flow	0.4 mL min^{-1}		
Injection volume	2 µL, partial loop with needle overfill		
Column temperature	40°C		

Table 2: MRM details (ESI-pos. mode using Sciex API 4000 QTrap):

Compound	MW	Intensity ranking	Parent Ion	Q 1	Q 3	DP	CE	CXP
GG-C6 6-Bis (guanidino) hexane (ISTD)	**200**	1	$[M+2H]^{2+}$	101.128	142	46	17	8
		2	$[M+2H]^{2+}$	101.128	125	46	15	6
		3	$[M+H]^{+}$	201.215	159.2	66	21	8
Carbendazim D4 (ISTD)			$[M+H]^{+}$	196.099	164.1	61	27	8
GG 1,8-diguanidin-1-yl-octane	**228**	2	$[M+2H]^{2+}$	115.186	127.9	51	21	6
		2	$[M+2H]^{2+}$	115.186	153.2	51	15	8
		1	$[M+2H]^{2+}$	115.186	170.1	51	17	10
		3	$[M+2H]^{2+}$	115.186	212.1	51	11	12
GGG 1,1-bis(8-guanidin-1-yl-octane		Poor sensitivity	$[M+H]^{+}$	398.5	356.4	126	29	4
	397	Poor sensitivity	$[M+H]^{+}$	398.5	314.3	126	39	18
		Poor sensitivity	$[M+H]^{+}$	398.5	322.3	126	45	16
		1	$[M+2H]^{2+}$	199.9	178.7	66	17	8
		4	$[M+2H]^{2+}$	199.9	157.7	66	21	8
		5	$[M+2H]^{2+}$	199.9	128.1	66	33	6
		1	$[M+3H]^{3+}$	133.6	170	51	17	8
		3	$[M+3H]^{3+}$	133.6	128	51	19	6
		2	$[M+3H]^{3+}$	133.6	119.4	51	13	6
GNG 1,1'-(iminodioctane-8,1-diyl) diguanidine = Iminoctadine	**355**	6	$[M+2H]^{2+}$	178.9	157.6	71	15	8
		4	$[M+2H]^{2+}$	178.9	187.2	71	21	10
		5	$[M+2H]^{2+}$	178.9	297.3	71	19	4
		1	$[M+3H]^{3+}$	119.6	128	41	17	8
		2	$[M+3H]^{3+}$	119.6	170.2	41	13	8
		3	$[M+3H]^{3+}$	119.6	187.2	41	15	4
GGN 1-(8-guanidin-1-yl-octyl)-1-(8-amino-octyl)guanidine	**355**	8	$[M+2H]^{2+}$	178.9	128.1	71	31	6
		2	$[M+2H]^{2+}$	178.9	157.6	71	15	8
		3	$[M+2H]^{2+}$	178.9	170.2	71	17	8
		4	$[M+2H]^{2+}$	178.9	187.2	71	21	10
		7	$[M+2H]^{2+}$	178.9	297.3	71	19	4
		5	$[M+3H]^{3+}$	119.6	128	41	17	8
		1	$[M+3H]^{3+}$	119.6	170.2	41	13	8
		6	$[M+3H]^{3+}$	119.6	187.2	41	15	4

Note: For measurements on an **API 5500 instrument** it is recommended to **increase the DP values by 20.**

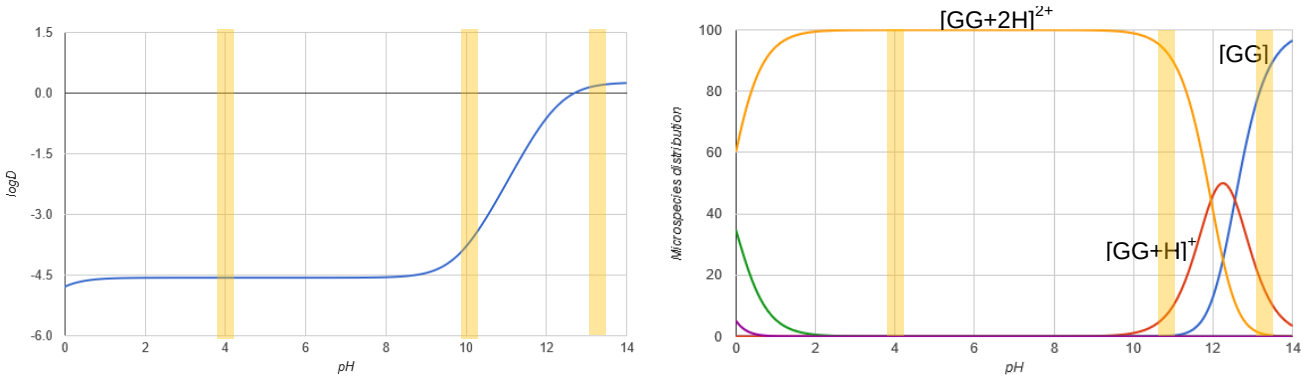
Experiments and Observations

Recovery rates using different extraction methods

As shown in Table 3 recovery rates using the citrate buffered QuEChERS (extraction pH ca. 4) were low, which was expected as the guazatine components are multiply protonated at this pH. Extractions at pH ~11 by applying the QuEChERS approach for nicotine [EURL-Nicotine], did not improve the situation, as the compounds are still predominantly protonated and thus highly polar at this pH, see

Figure 1. Unexpectedly, recoveries only marginally increased when using QuEChERS extractions at a pH of 13.5.

Figure 1: LogD and ratio of the various forms of GG plotted against the pH (computed by Chemicalize).



Surprisingly, the QuPPe method [EURL-QuPPe], involving extraction at a water/methanol ratio of 50% and a formic acid concentration of 0.5 % in the extractant also resulted in poor recovery rates. Following indications in literature [Dreassi et al. 2007a and 2007b an extraction solvent based on water, acetone and formic acid without any partitioning step was also tested. Table 3 shows recovery rates of GG using the different extraction approaches.

Table 3: Recovery rates achieved for GG and GGG in lemon matrix, using different methods (n=2 each)

Method	Sample weight	Extractant volume	Extractant composition	Notes	Mean Recovery in %	
					GG	GGG
Two-phase extraction						
QuEChERS (EN15662)	10 g	10 mL	Acetonitrile	Extraction **pH ~4**	**0.8**	**4**
QuEChERS	10 g	10 mL	Acetonitrile	Extraction **pH ~11** adjusted with NaOH, partitioning with MgSO4 / NaCl (4:1) without citrate salts	**1.9**	**10**
QuEChERS	10 g	10 mL	Acetonitrile	Extraction **pH ~13.5**, adjusted with NaOH, partitioning with 1g NaCl without MgSO4 or citrate salts	**1.9**	**14**
One-phase extraction						
QuPPe (Wa50-Me50-FA0.5)*	10 g	10 mL	Methanol with 1% FA	Methanol:water ratio 1:1, 0.5% formic acid	**1.1**	**8**
Wa66-Ac33-FA0.17 *	10 g	10 mL	Acetone+ Water (w. 1% FA) 1+2	10 g sample + 10 mL extraction solvent	**76**	**78**

*** Approximate solvent composition in the extract: Water 50%, Methanol 50%, Formic acid 0.5% ;**
**** Approximate solvent composition in the extract: Water 66%, Acetone 33%, FA 0.17% (v/v);**
(in both cases assuming a water content of 10 mL in 10g sample).

Using the above-mentioned extractant composition, with 33% acetone, poor chromatographic behavior of guazatine was noticed. It was therefore decided to check how alternative extractant compositions

impact chromatographic performance and recovery rates. As shown in Table 4, reducing the acetone content from 33.3 to 16.6% had only little impact on the recovery rates of GG, GGG, and GNG/GGN. The addition of methanol, however, had a notably negative impact. GGN and GNG, which share many mass-transitions, were not separated well chromatographically and were therefore quantified as a sum in this experiment. As GNG is a very minor component, the summing of GNG and GGN was considered acceptable for such orientational experiments.

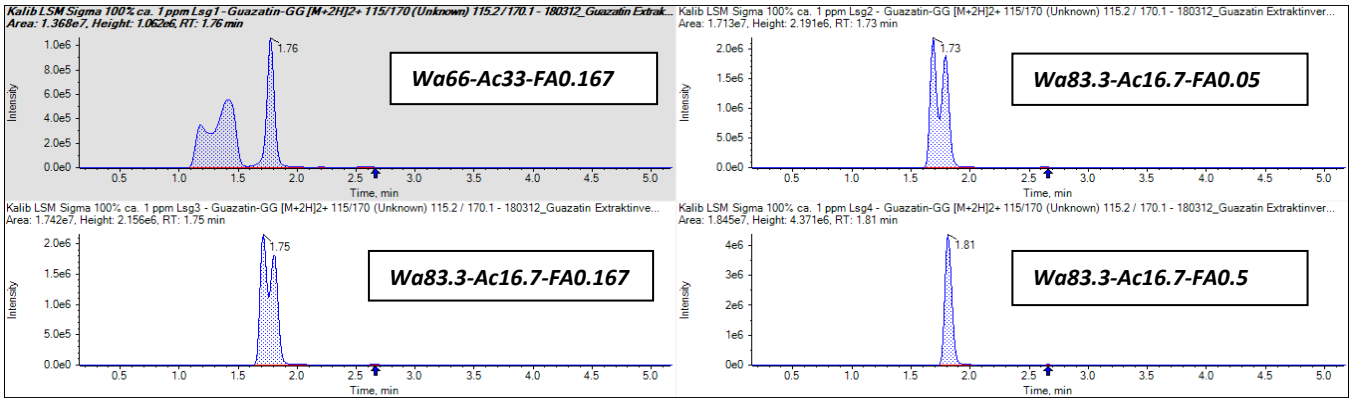
Table 4: Recovery rates of GG, GGG and GGB/GNG from grapefruit using different methods; spiking level 0.83 mg/kg guazatine acetates (corresponding to 0.081, 0.25, 0.12 and 0.0023 mg/kg of GG, GGG, GGN and GNG respectively).

Method code (see table 3 for coding explanation)	**Sample weight**	**Extract tract-ant vol.**	**Extractant composition**	**Recovery rates in % (n=2)**		
				GG	**GGG**	**GGN/GNG**
Wa66-Ac33-FA0.083	10 g	10 mL	Water 0,5%FA + Acetone (1+2)	86	88	86
Wa66-Ac33-FA0.166	10 g	10 mL	Water 1%FA + Acetone (1+2)	82	85	81
Wa66-Ac33-FA0.5	10 g	10 mL	Water 3%FA + Acetone (1+2)	91	96	92
Wa75-Ac25-FA0.25	10 g	10 mL	Water 1%FA + Acetone (1+1)	93	93	96
Wa83.3-Ac17.7-FA0.33	10 g	10 mL	Water 1%FA + Acetone (2+1)	93	97	88
Wa66-Ac17.7-Me17.7-FA0.167	10 g	10 mL	Water 1%FA + Acetone + Methanol (1+1+1)	61	73	79

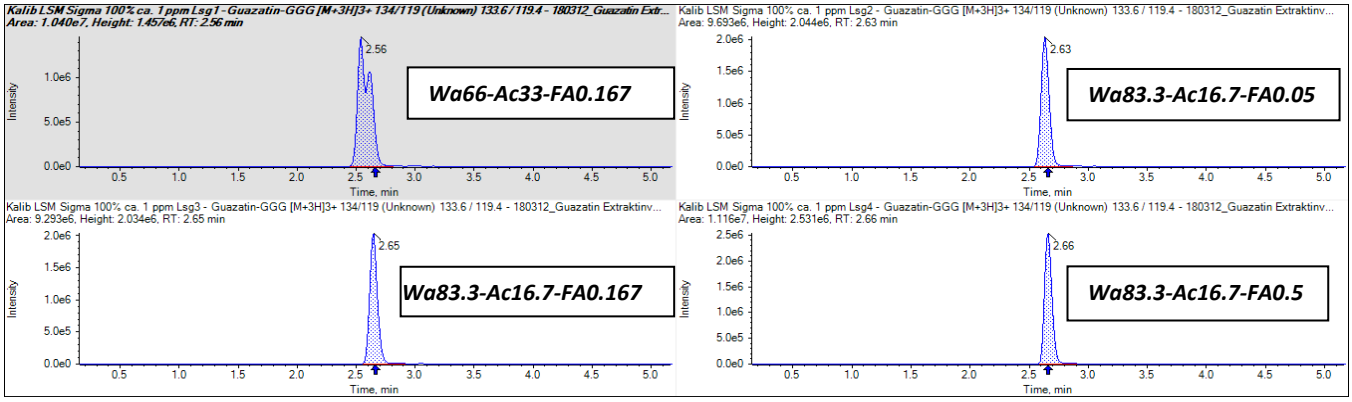
Varying the water/acetone ratio from 2:1 (66 vs 33%) to ca. 5:1 (83.3 vs 17.7%) and the formic acid content between 0.083 and 0.5% had only marginal impact on the recovery rates of spiked guazatine. The impact on the chromatographic behavior of the guazatine components was, however, very pronounced. As shown in Figure 2 both low acetone content and higher acidity had a positive impact on the peak shapes of the compounds. In general, lowering the acetone content and increasing acidity of the injected extracts positively impacted the peak form of guazatine components.

Figure 2: Exemplary chromatograms of GG, GGG, and GNG/GGN obtained by injecting differently composed grapefruit extracts spiked with 0.062 µg/mL guazatine acetates. Matrix content 0.5 g /mL, measured by AB-Sciex 5500 and a Waters-I-class UPLC. Impact of acetone and formic acid content in the injected extracts on the peak form of guazatine components.

a) GG



b) GGG



c) GNG/GGN

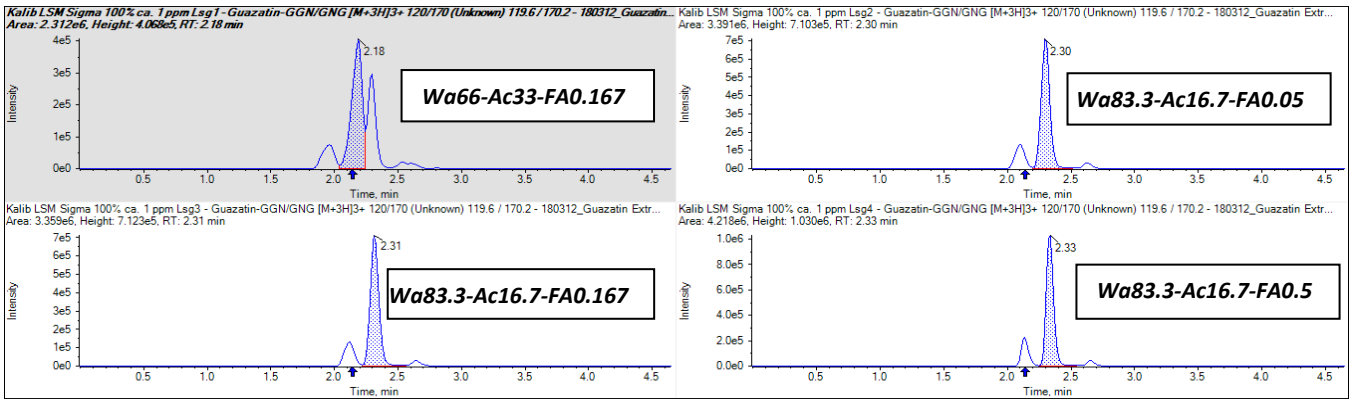


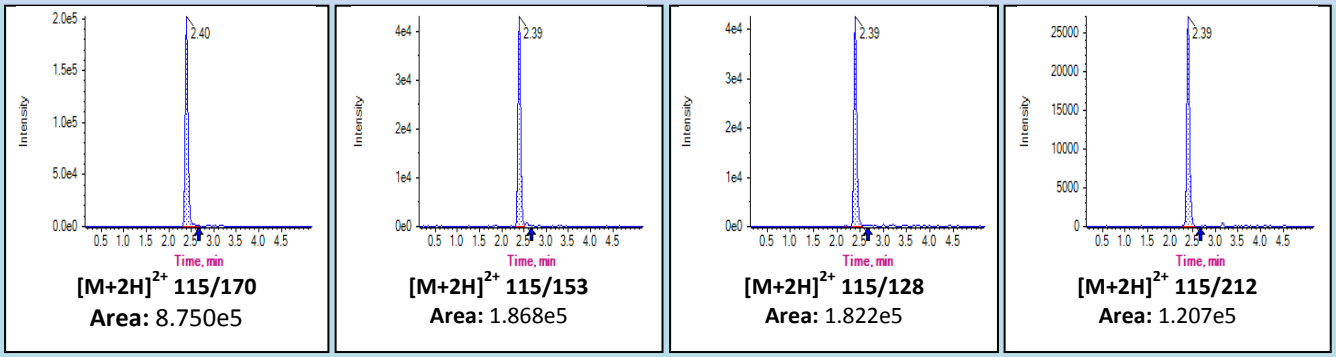
Figure 3 shows some exemplary chromatograms of GG, GGG, GGN and GNG obtained when injecting blank-orange-based standard with an Wa83.3-Ac16.7-FA0.5 composition. GNG and GGN separated well in this case. Through in-source fragmentation GGG gives a signal at mass transition 179/187, which is common to GGN and GNG, but chromatographic separation was fortunately sufficient.

Figure 3: Exemplary chromatograms of GG, GGG, GGN and GNG obtained by injecting orange based calibration standard containing 0.062 µg/mL guazatine acetates. Matrix content 0.5 g /mL, extract composition: ***Wa83.3-Ac16.7-FA0.5***, Measured by ABSciex API 5500

a) **GG**

GG-cation share 9.8%

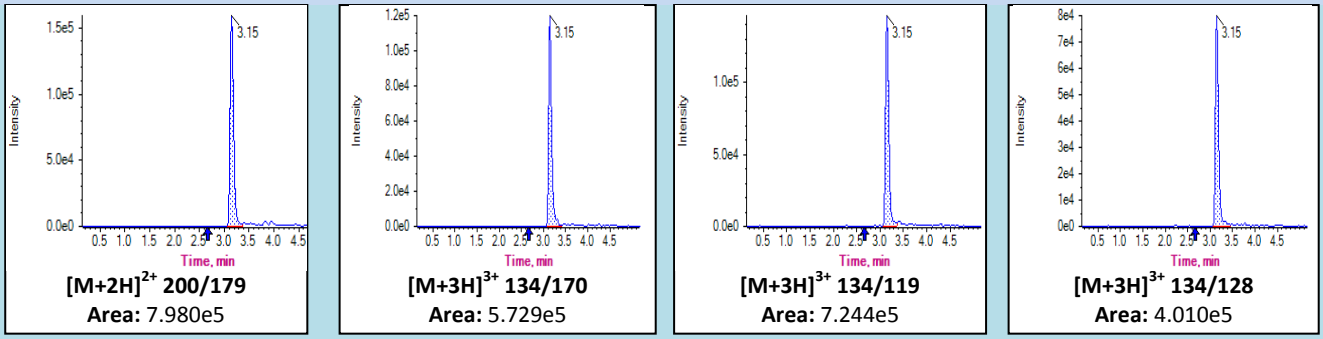
GG-cation conc.: 0.008 mg/kg = 0,004 µg/mL



b) **GGG**

GGG-cation share 30.2%

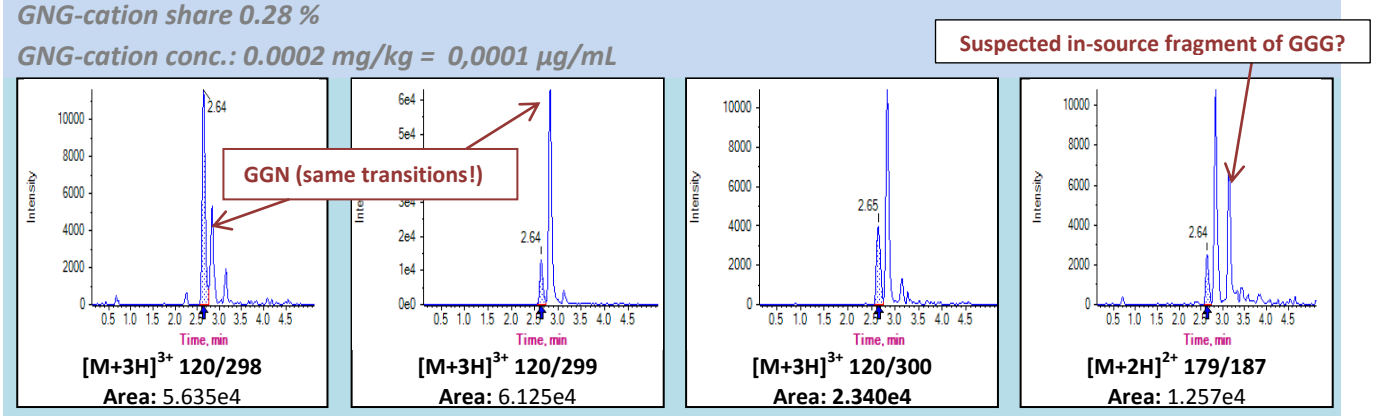
GGG-cation conc.: 0.025 mg/kg = 0,0125 µg/mL



c) **GNG**

GNG-cation share 0.28 %

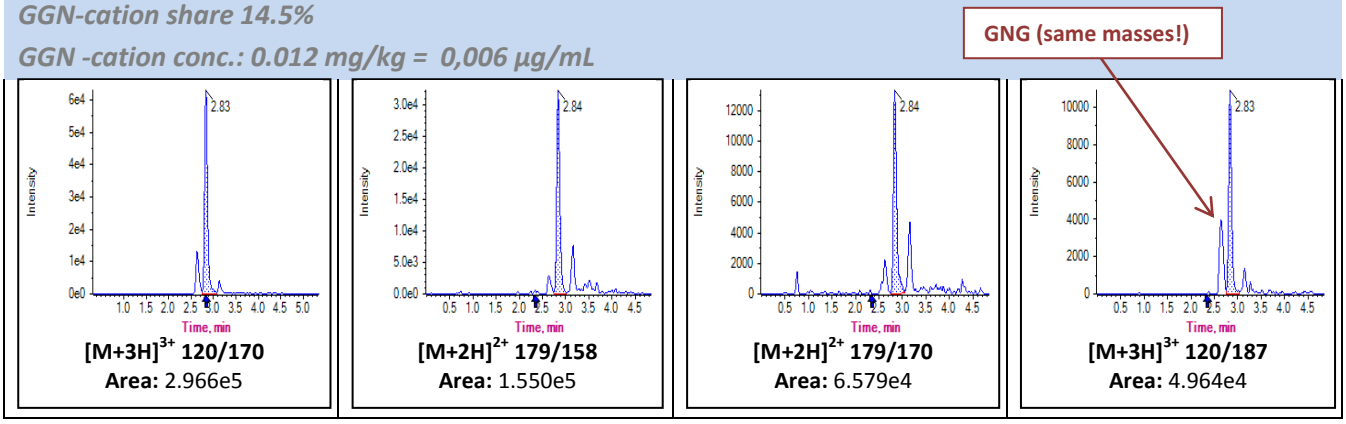
GNG-cation conc.: 0.0002 mg/kg = 0,0001 µg/mL



d) **GGN**

GGN-cation share 14.5%

GGN -cation conc.: 0.012 mg/kg = 0,006 µg/mL



Treatment of Citrus Fruits with Guazatine to Simulate Incurred Residues

Lowering the acetone content of the extraction solvent has had a positive impact on the chromatographic performance (see Figure 2), and no major effect on the recovery rates of spiked guazatine, but there was still a concern whether this measure would compromise extraction efficiency of incurred guazatine by weakening the ability of the extractant to dissolve or at least soak into the wax layer, covering or incorporating guazatine residues. In lack of suitable material containing incurred guazatine residues, it was decided to perform a guazatine treatment under conditions roughly resembling treatments in industrial packing plants.

In practice, post-harvest guazatine treatment of citrus fruits is conducted in different ways, e.g. by spraying the fruits with, or dipping the fruits into an aqueous guazatine solution (containing e.g. 0.05 to 0.2 kg a.i./hl) or dipping the fruits into guazatine containing wax-emulsions (containing e.g. 0.3 kg ai/hl). Aqueous treatment may be followed by a wax treatment. Guazatine is furthermore used to sanitize water, tanks and belts in packing stations [FAO 1998].

For the treatment, organic lemons and oranges were purchased from the local market. In total 24 units of each oranges and lemons were used and divided into 6 groups of 4 units each. Table 5 gives an overview of the treatments of each group.

For the application of guazatine the citrus fruit units were first spiked with 200 µL of an aqueous dilution of a guazatine formulation (Kenopel), containing ca. 1000 µg/mL guazatine acetates. This was done using a pipette that was able to dispense solvent in multiple defined (in this case 10 µL) portions. Each 10 µL portion was applied on a different spot on the fruit surface. The amount spiked corresponded to ca. 200 µg guazatine acetates per fruit. With the fruits weighing on average around 200 g the treatment resulted in a concentration of ca. 1 mg/kg guazatine acetates. The guazatine-spiked fruits were left to dry before further processing, i.e. waxing or homogenization. For the waxing the fruits were dipped into a commercial wax emulsion (Citrosol). Two types of wax-emulsions which are both used in citrus packing plants, were used for the treatment: a) “Citrosol A” (an aqueous emulsion of partly oxidized polyethylene, containing a substantial amount of ammonia and other ingredients, e.g. surfactants); and b) “Citrosol A Cámara” (which is similar to “Citrosol A” but additionally contains shellac, which gives the fruits a shiny appearance). Both wax-emulsions were kindly provided by Citrosol S.A. (Valencia/Spain). Following wax-treatment the fruits were left to dry out for ca. 8h. Figure 4 illustrates the setup of the lemon and orange treatments with guazatine and wax.
For the homogenization, the fruits of each group were first cut coarsely, then placed in a freezer overnight, and finally cryogenically milled with dry ice, as it is routinely done at CVUA Stuttgart. Due to other tasks, the homogenates were, however, not extracted immediately but after several months.

***Table 5**: Guazatine and wax treatment scheme of lemons and oranges*

Treatment Group		**Guazatine spiked onto surface?**	**Dipped in Wax Emulsion?**	
			Citrosol A (PE Wax)	**Citrosol A Cámara (PE + Shellac)**
Group A	**Blank**	No	No	No
Group B		No	**Yes**	No
Group C		No	No	**Yes**
Group D	**Spiked**	**Yes**	No	No
Group E		**Yes**	**Yes**	No
Group F		**Yes**	No	**Yes**

***Figure 4:** Setup for the treatment*

Impact of Extraction Conditions on the Extractability of Incurred Guazatine Components

Aiming to comprehend how the extraction conditions (solvent composition, initial sample temperature, shaking time) influence the extractability of the various guazatine components, several experiments were conducted using the above-mentioned laboratory-treated lemon and orange samples containing "quasi-incurred" guazatine residues.

Influence of acid content on recovery rates: To start with, recovery experiments were conducted using blank orange homogenates (group A, B and C). These were spiked with the guazatine working standard at 200 µg/mL standard. Considering the 38% water content and the 20.6% acetate content the spiking level calculates to 0.83 mg/kg guazatine acetates. This corresponds to an assumed concentration of 0.081, 0.25, 0.12 and 0.0023 mg/kg for GG, GGG, GGN and GNG respectively. Calibration was matrix-matched using similarly derived extracts of the respective blank orange homogenates (A, B and C). All homogenates were used in a frozen state and extracted for 5 minutes. The recovery rates achieved are shown in Table 6. As can be seen in Table 6, increasing the acid content had overall a positive impact on the recovery rates.

Table 6: *Recovery rates of GG, GGG and GGB/GNG from spiked oranges that were differently treated using extraction methods with variable acetone, water and formic acid content. Measured by API5500Q-2/Waters I-class*

Extraction Method*	Initial Temp.	Shaking time	Group A without wax			Group B PE wax			Group C PE wax + shellac		
			GG	GGG	GGN	GG	GGG	GGN	GG	GGG	GGN
			Recovery rates in % (average of n=2)								
Wa83-Ac18-FA0.05	Deeply frozen	5 min	78	53	58	77	53	65	78	50	65
Wa83-Ac18-FA0.17	Deeply frozen	5 min	74	68	72	82	64	74	78	65	82
Wa83-Ac18-FA0.5	Deeply frozen	5 min	100	74	85	92	81	85	87	79	95

***** The volume percentages of water, acetone and formic acid stated in the method refer to the composition of the extraction mixture considering the water content in the 10 g sample portion (assumed to be 10 mL)

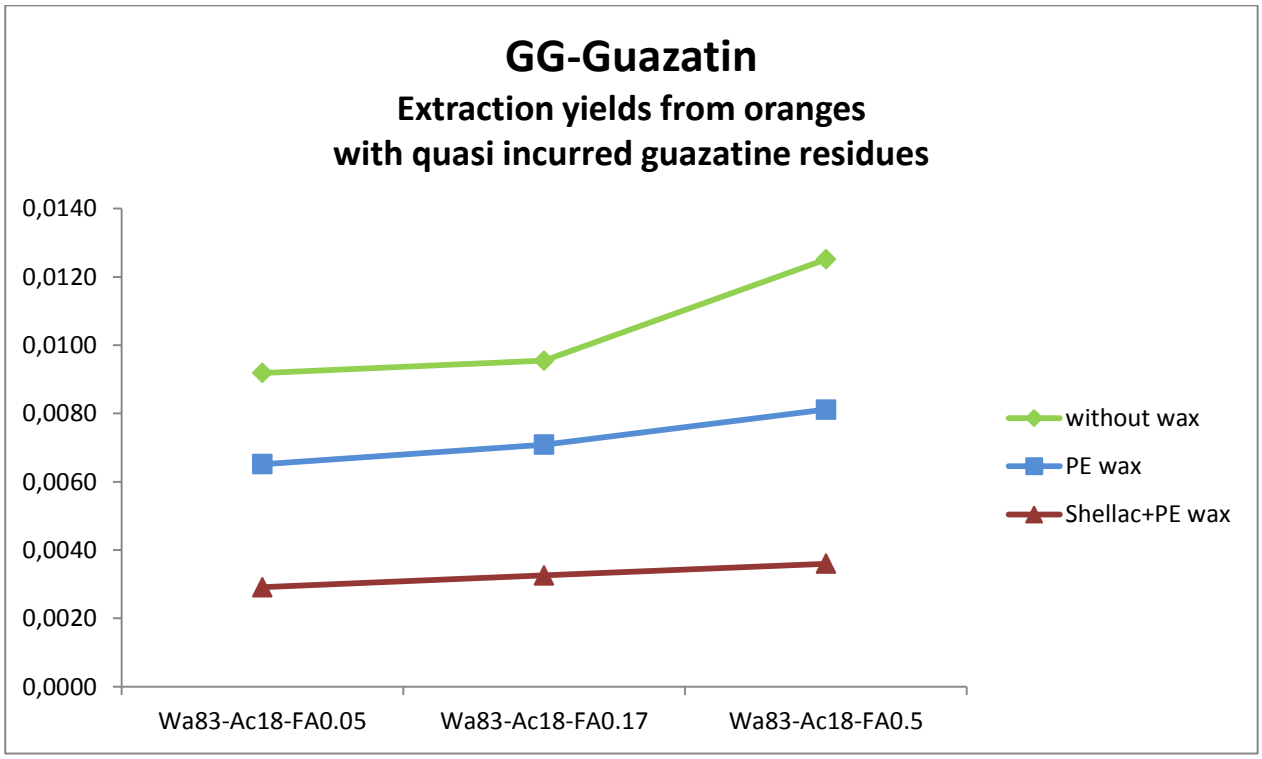
Quantifier masses:

- GG $[M+2H]^{2+}$ 115/170
- GGG $[M+2H]^{2+}$ 134/119
- GNG $[M+3H]^{3+}$ 120/299 (= Iminocatidine)
- GGN $[M+3H]^{3+}$ 120/299

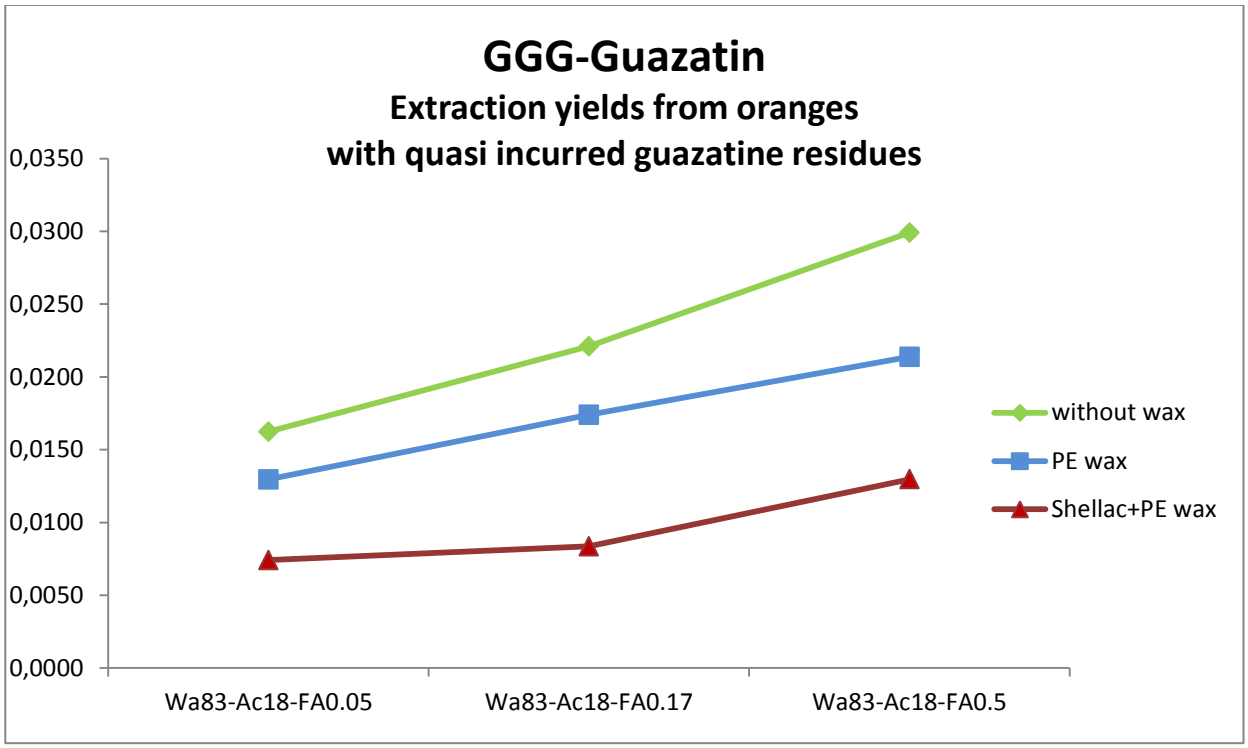
Influence of acid content on extraction yields of quasi incurred guazatine components: In parallel, it was checked whether the formic acid content in the extraction solution influences the extractability of the various "quasi incurred" guazatine components. For this, guazatine-spiked orange homogenates of group D (no wax treatment), group E (PE wax) and group F (PE + shellac wax) were extracted using various extraction solvents differing in their formic acid content. Also here, all homogenates were used in a deep frozen state and extracted for 5 minutes. As shown in Figure 5a and b, the yields of GG and GGG markedly increased by increasing the acidity of the extraction solution. Furthermore, samples treated with guazatine only, showed higher yields than samples additionally covered by PE-wax or PE+shellac wax, with the latter showing the lowest yields. This trend was suspected, as waxes form barriers hindering the accessibility of residues incorporated in or covered by them. It could not be ruled out, however, that the differently treated samples also contained different concentrations of guazatine, e.g. due to a wash-off of guazatine during the wax-dipping process or due to differences in the degradation behavior of guazatine during the long interval between treatment and extraction. Further experiments are planned to elucidate this aspect.

Figure 5: Impact of acidity on the extraction yields of a) GG and b) GGG from guazatine-treated orange samples; Calibrated against standards prepared from similarly extracted blank extracts; average values of n=2,

a) GG



b) GGG



Influence of initial temperature and shaking time: Interested to further comprehend how the extractability of guazatine residues is influenced by solvent composition, initial sample temperature and shaking time, several experiments were conducted using the Group F orange sample (treated with guazatine and "Citrosol A Cámara" wax containing shellac). These shellac-treated fruits were considered as the worst case scenario as regards the extractability of residues, as the shellac-containing wax seems to form the strongest barrier for the capture the extraction solvent.

As can be seen in Table 7, when employing sample homogenates at frozen condition, extraction yields of all tested guazatine components notably increased when extending extraction time from 5 to 60 min, with the highest increase being noted for GG. Employing the sample homogenate at ambient temperature enabled a faster extraction (higher yields) than when the homogenate was employed frozen. Heating the sample/extraction solvent mixture up to 60°C followed by a 5 min shaking step, additionally increased extraction yields. The results furthermore reconfirm that increasing the acetone content in the extraction solution (e.g. from 17 to 33%) has a positive effect on the extractability of the guazatine components and this benefit becomes more pronounced when extending the extraction time. An acetone content of only 17% in the extractant is obviously too low for the extractant to penetrate the wax. From the results of these experiments it became obvious that the extractability of guazatine residues is challenging, at least when applied in combination with wax, and that more experiments would be needed to reach a plateau in the extraction yield.

Of concern was, however, the fact that the extraction yields of the guazatine components were overall very low compared to the guazatine amounts originally spiked to the fruits. It could, however, not be clarified at

this stage whether this underestimation was solely due to a hindered extraction in combination and/or slow extraction kinetics, or whether degradation of guazatine during the long storage period also played a role. In any case it was concluded that there was a need to check more closely the influence of temperature and extraction times.

Table 7: Impact of various conditions *(extract composition, initial sample temperature and shaking time) on the extraction yields of GG, GGG and GGB/GNG from oranges previously treated with guazatine and wax containing PE-Wax and shellac. Measured by API 4000/Waters Acquity UPLC; Average of (n=2) in µg/kg*

Method	Initial Temp	Incubation prior to shaking	Shaking time	GG	GGG	GGN/GNG (Peaks not well separated!)
Wa66-Ac33-FA0.5	Frozen	none	**5 min**	**1.95**	**8.14**	**5.23**
			30 min	**2.29**	**14.77**	**5.61**
			60 min	**3.03**	**15.57**	**6.67**
	Ambient	*none*	*5 min*	*Not conducted*	*Not conducted*	*Not conducted*
		30 min at 60°C	*5 min*	*Not conducted*	*Not conducted*	*Not conducted*
Wa83-Ac17-FA0.5	**Frozen**	**none**	**5 min**	**2.76**	**11.20**	**4.62**
			30 min	**2.97**	**11.15**	**5.17**
			60 min	**3.03**	**11.32**	**5.45**
	Ambient	**none**	**5 min**	**3.03**	**11.68**	**5.45**
		30 min at 60°C	**5 min**	**3.95**	**14.00**	**6.75**
Wa83-Ac17-FA1	**Frozen**	**none**	**5 min**	**2.68**	**8.96**	**5.17**
			30 min	**3.07**	**8.66**	**5.01**
			60 min	**3.26**	**10.10**	**4.98**
	Ambient	*none*	*5 min*	*Not conducted*	*Not conducted*	*Not conducted*
		30 min at 60°C	*5 min*	*Not conducted*	*Not conducted*	*Not conducted*

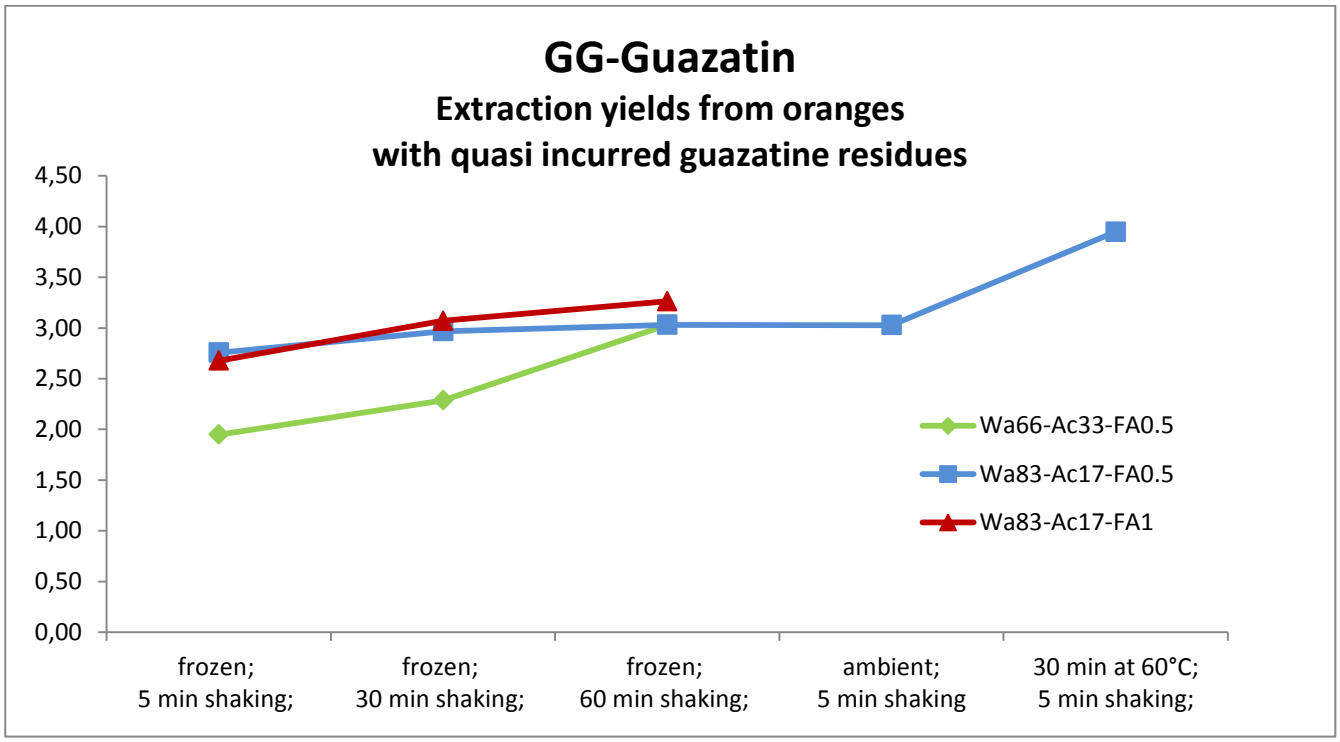
***** The volume percentages of water, acetone and formic acid stated in the method are rounded and calculated assuming that the water content within the sample is 10 mL

Quantifier masses:

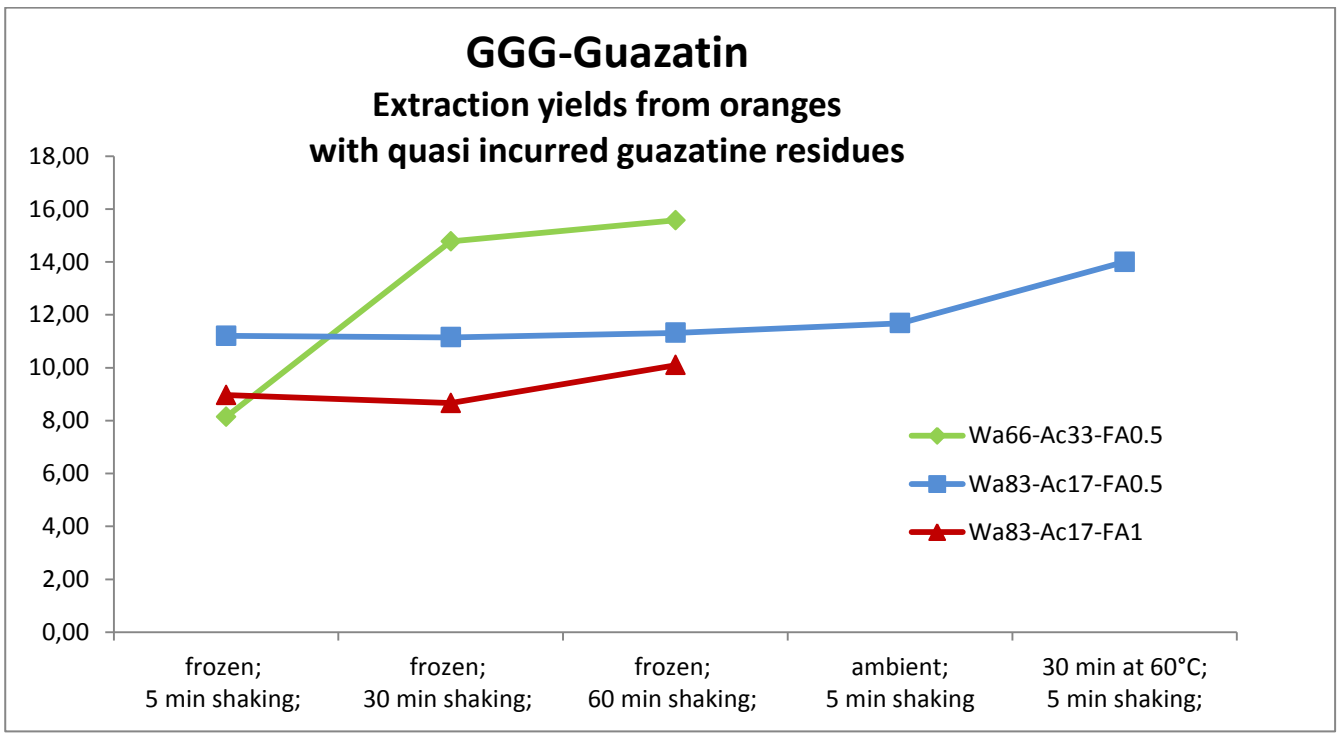
- GG $[M+2H]^{2+}$ 115/170
- GGG $[M+2H]^{2+}$ 134/119
- GNG $[M+3H]^{3+}$ 120/299 (= Iminocatidine)
- GGN $[M+3H]^{3+}$ 120/299

Figure 6: Impact of initial sample temperature and shaking time on the extraction yields of a) GG and b) GGG from guazatine-treated orange samples; Calibrated against standards prepared from similarly extracted blank extracts; average values of n=2,

a) GG



b) GGG



In a follow-up experiment, the impact of extraction time was studied when employing the sample in a slightly frozen state and extracting them with an extractant containing 33% acetone. As shown in Table 8, employing the samples in a slightly frozen rather than a deep frozen condition facilitated extraction, with the extraction yields at 15 min extractions being as high as those achieved when extracting a deep frozen sample for one hour (see results of previous experiment in Table 7). Interestingly, the extraction yields did not increase further when extending the extraction time to 120 min. An additional slight increase was noted when the sample was heated at 80°C for 15 min (static extraction) prior to the shaking step.

It should be noted, that heating at 80°C caused an overpressure with one of the falcon tubes experiencing a leak. **EXTRACTIONS AT 80°C ARE THEREFORE NOT RECOMMENDED WITH THE ABOVE SOLVENT COMPOSITION!!**

Table 8: Impact of various conditions *(extract composition, initial sample temperature and shaking time) on the extraction yields of GG, GGG and GGB/GNG from oranges previously treated with guazatine and wax containing PE-Wax and shellac. Measured by API 5500/Waters Acquity UPLC; Average of (n=3) in µg/kg*

Method	Initial Temp.	Incubation prior to shaking	Shaking time	GG	GGG	GGN/GNG
Wa66-Ac33-FA0.5	slightly frozen	none	15 min	3.0	13.5	6.4
			30 min	3.0	12.8	5.4
			60 min	3.2	13.0	6.1
			120 min	3.0	14.0	5.7
	slightly frozen	5 min at 80°C	5 min	3.6	13.8	5.5
		15 min at 80°C		3.9	16.0	6.2

***** The volume percentages of water, acetone and formic acid stated in the method are rounded and calculated assuming that the water content within the sample is 10 mL

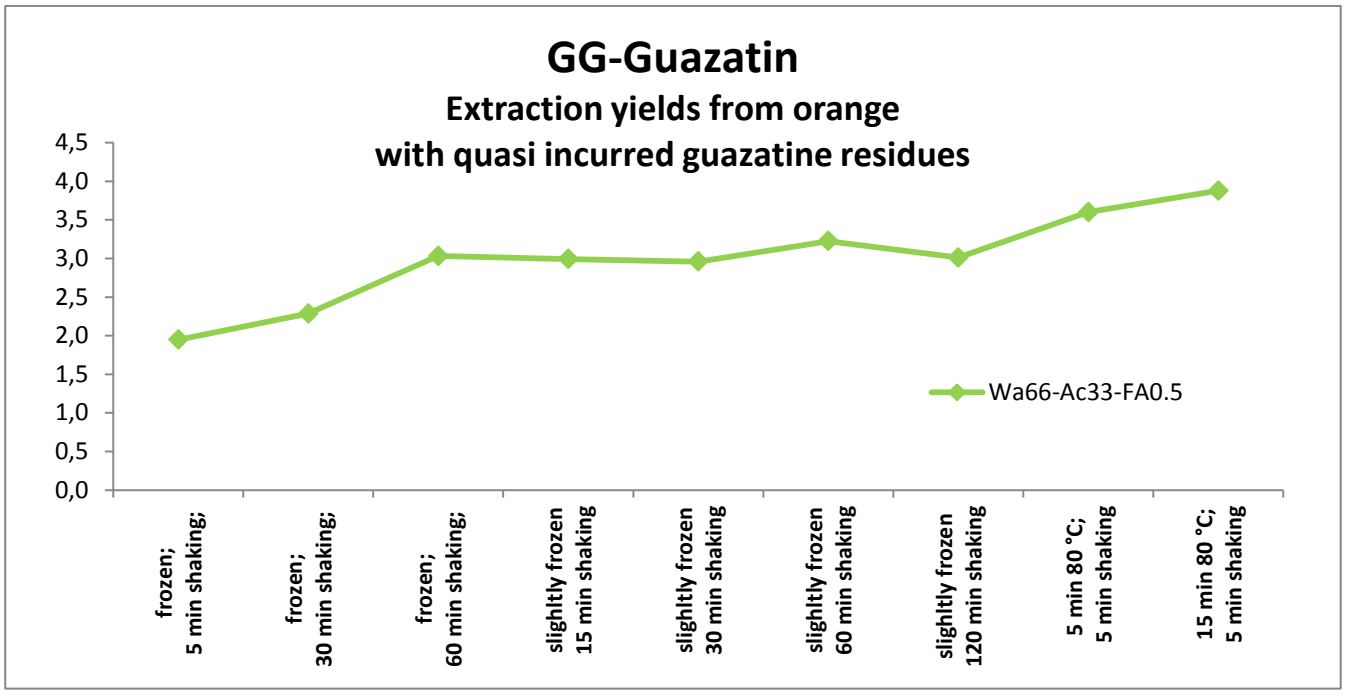
Quantifier masses:

- GG $[M+2H]^{2+}$ 115/170
- GGG $[M+2H]^{2+}$ 134/119
- GNG $[M+3H]^{3+}$ 120/299 (= Iminocatidine)
- GGN $[M+3H]^{3+}$ 120/299

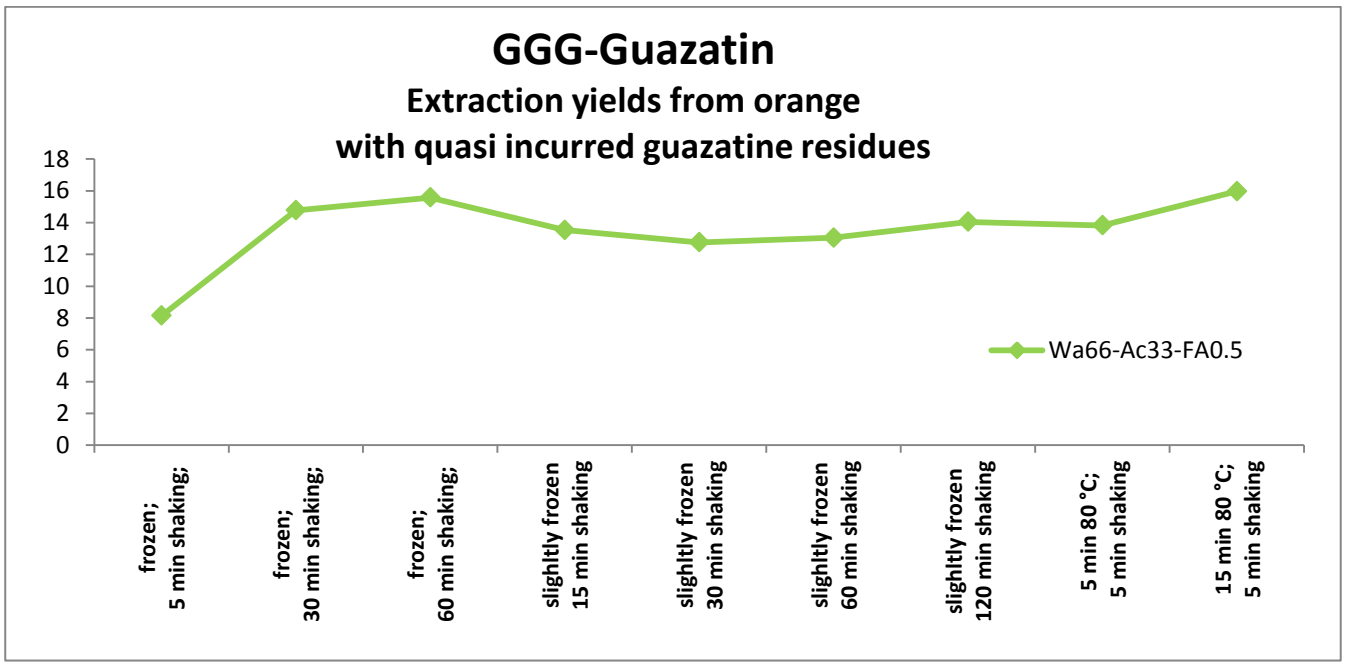
Figure 7 gives an overview on the impact of various extraction conditions of the yields of GG and GGG. In general it can be recognized that higher acetone content is beneficial for the extractability of the guazatine components from the wax-treated oranges. Higher temperatures were also beneficial for the extraction yields.
In parallel some experiments were conducted to study the solubility behavior of pure shellac. Shellac showed a generally very poor solubility in water acetone mixtures (Ac66-Wa33-FA1 vs. Ac33-Wa66-FA1), with solubility increasing at higher acetone content and higher temperature. This confirms the above results.

Figure 7: Overview of extraction yields for incurred guazatine using different extraction conditions (data from various experiments described above were mixed in this figure).

a) GG



b) GGG



Discussion and Conclusions:

The present document shows an interim status of the EURL-SRM studies on guazatine. Overall, the analysis of guazatine proved to be quite challenging. One of the reasons for this was the wording of the current EU residue definition, which refers to the sum of guazatine components, despite guazatine being a complex, variably composed, and not sufficiently defined, mixture. A residue definition based on individual marker compounds (e.g. GGG, GG and GGN) without a need to extrapolate to the sum, would have been preferable to circumvent the problem with the variable guazatine composition, but the non-availability of analytical standards of individual components (even of the most prominent ones) still requires the use of guazatine mixtures for quantification. The mixtures, however, do not necessarily match with the composition of technical guazatine used by farmers and the composition of the residues left in food.

Following the Relana® approach this study mainly focused on the quantification of 4 guazatine marker components (GG, GGG, GNG and GGN), which were quantified using commercially available standard of the guazatine mixture. To enable quantification this approach makes the assumption that the relative share of the components within the guazatine mixture is proportional to the relative intensities of their LC-UV signals in a chromatogram provided by the standard provider. Using a factor the summed concentration of the abovementioned four components is then extrapolated to the total guazatine acetate concentration. This approach was introduced by Relana® out of the need to circumvent the practical difficulties in quantifying guazatine (sum) and with the aim to establish a harmonized analytical procedure so that different laboratories can achieve comparable. This approach, however, does not guarantee that the quantification of guazatine (sum) is accurate. For example, the share of the 4 guazatine components (as cations) reported by the applicant for an exemplary formulation was 73.5% [FAO 1998], whereas the assumed share of these components in a currently available standard of the guazatine mixture is 54.8% (based on LC-UV measurements).

In LC-MS/MS analysis the guazatine components compounds behave much differently compared to other pesticides. The most abundant ions are for example doubly or triply charged of resulting in m/z values of parent ions being smaller than the m/z values of their respective daughter ions. Initial experiments with QuEChERS, at different pH levels, and QuPPe showed low recovery rates. Satisfactory recovery rates between 81 and 96 % for spiked residues were, however, achieved using a 1-phase extraction procedure involving the addition of acetone and formic acid to the samples. Higher formic acid and lower acetone contents positively impacted RP-LC-chromatography, but at the same time lower acetone contents proved detrimental as regards the extraction of incurred residues. In general, the extractability of residues proved very challenging. Raising extraction temperatures further increased extraction yields of incurred residues. More experiments are, however, needed to optimize the extractability of incurred residues.

Literature:

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History

Action	When	Version
Conduction of experiments	June 2015 – December 2017	
Method placed on-line	June 2018	V1