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Analysis of Chlormequat in Pear and leaves of Pear using Liquid Chromatography/Mass Spectrometry.

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SUMMARY

In this report the method, the limited validation and the analysis of chlormequat in pear, and leaves of pear is described. The method used was modified from CEN-method CEN/TC 275/WG 4 N 134, March 2001, Determination of Chlormequat as chlormequat cation in non-fatty foods. The method was fully validated in flour according to EU SANCO 1805/2000, Rev. 1. as described in RIKILT Report 2002.505. After validation the method was recorded as RSV-A0911, Meel - Kwantitatieve Bepaling van Chloormequat - LC-MSMS. The method is partially validated in pear. The method is not validated in leaves of pear (because there is no MRL established) but the quality controls that were used in the analysis of chlormequat in pear were also used for the analysis of leaves of pear. The pear, and leaves of pear samples were derived from the Algemene Inspectiedienst (AID).

For leaves of pear no MRL is available. However, out of 84 samples of suspicious leaves of pear 15 samples were below the LOQ of 0.010 mg/kg, and 69 samples were within a range of 0.01 to 55 mg/kg.

Out of 38 samples of suspicious pears one sample was below the LOQ of 0.010 mg/kg, 12 samples were positive but below the MRL-value of 0.5 mg/kg, and 25 samples were within a range of 0.56 to 14 mg/kg, which is above the MRL-value of 0.5 mg/kg.

1 INTRODUCTION

Chlormequat chloride ($C_5H_{13}Cl_2N$, [2-chloroethyl]trimethylammonium chloride or CCC) is a quaternary ammonium plant growth regulator, introduced by American Cyanamid Co in USA and BASF AG in Germany (1966). It is a Gibberellin biosyntheses inhibitor used for producing sturdier plants, and increased flowering and harvest. Chlormequat is extensively used in growth control of cereals like wheat, rye, and oats, and to prevent fruit drop in fruits like pears and grapes (The Pesticide Manual, 1997). In the Netherlands the use of chlormequat is restricted to pears, cereals, and ornamental plants.
Recently concern has risen about the acute toxicity of chlormequat. Especially for infants and small children there might be a risk (SANCO 1139/2000 - MR final). Because of the concern about the toxicity of chlormequat, the MRL for pears has been lowered from 3.0 mg/kg to 0.05 mg/kg (Commission Directive 2000/42/EC). However recently a temporary MRL of 0.5 mg/kg (valid until 31 July 2003) was implemented by the European Commission (Commission Directive 2001/35/EC). No MRL is available for leaves of pear.
In 2001, samples of conventionally grown wheat, organically grown wheat, bread, spaghetti, leaves of pear, and pear were analysed at RIKILT. The results of the analysis in the conventionally grown wheat samples, the organically grown wheat samples, the bread samples, and the spaghetti samples were described in RIKILT Report 2002.505. The results of the analysis of chlormequat in pear and leaves of pear are described in this report. The leaves of pear samples result from investigations by the Algemene Inspectiedienst (AID). If the analysis of these leaves resulted in suspicious samples, the accompanying pear samples were also analysed.

2. METHOD DESCRIPTION

2.1 Summary

After addition of an internal standard (D4-chlormequat chloride) an aqueous methanolic extract is prepared by continuous shaking for 16 hours. The sample is centrifuged and the obtained extract is filtered and directly analysed by liquid chromatography with mass spectrometric detection using positive electrospray ionisation.
In the analysis of chlormequat in pear, and leaves of pear a gradient is introduced into the chromatography to clean the column, and to enhance the peak-shape and accordingly improve the signal to noise ratio, resulting in a lower detection limit.

2.2 Sample preparation

Approximately 10.00 g (± 0.10 g) of comminuted pear and leaves of pear is weighed accurately into a 50 ml extraction tube. Subsequently 100 µl of D4-chlormequat chloride solution (5 µg/ml) is added, followed by 25 ml of extraction fluid (methanol-water = 2-1 (v/v)). The samples are shaken overnight (150 rpm.) at room temperature and centrifuged the following morning at 1800 rcf. The extract is filtered through a 0.45 µm filter and stored in the refrigerator (max. 7°C) until injection into the LC-MSMS system.

2.3 LC-MSMS

The analysis of chlormequat in pear and leaves of pear is carried out on a Micromass Quattro Ultima LC-MSMS. Chromatography is performed at 30°C on a Shodex RS-Pak DE-613 column (6 x 150 mm) equipped with a Shodex RS-Pak DS-G pre-column. The mobile phase is constituted out of solvent A (5mM ammoniumacetate in methanol-water (9-1 (v/v))) and solvent B (5mM ammoniumacetate in methanol-water (1-1 (v/v))). The applied gradient is starting at 50% A/50% B and is linearly changed to 70% A/30% B in 7.5 minutes. Finally the starting composition of the gradient is linearly restored in 2.5 minutes where it is kept for 5 minutes preceding the next injection.
Ten µl of sample or standard is injected at a flow of 0.75 ml/min, and the sample is introduced into the source with a 1:3 split (75 % of the eluent is discarded).
MSMS is performed with the needle voltage set to + 2.70V, and the cone voltage to 50V. The source temperature is set to 120°C, and the desolvation temperature to 300°C. The cone gas flow is set to 205 l N_2/hr, and the desolvation gas flow to 507 l N_2/hr. The ion-energy of the first and second quadrupole is 1.0V. Quantitation data of the native component are acquired for the following transitions: m/z 122.2 → m/z 58, and m/z 124.2 → m/z 58. Quantitation data of the D4-chlormequat internal standard are acquired for the following transitions: m/z 126.2 → m/z 58, and m/z 128.2 → m/z 58. All transitions are measured with 0.25 sec dwell time, 30 eV collision energy, and a tolerance of ± 0.5 mass units. Argon was used as collision gas. The results are processed by the MassLynx NT software, ver. 3.3.

3.VALIDATION IN SAMPLES OF PEAR.

3.1 Introduction

The method described in RSV-A0911, "Meel - Kwantitatieve bepaling van chloormequat - LC-MSMS" was fully validated for wheatflour (as described in RIKILT Report 2002.505). In wheatflour the following performance characteristics were verified: the trueness, the recovery, the repeatability, and the within-laboratory reproducibility. The analytical limits detection capability (CCβ), decision limit (CCα), detection limit (LOD), quantification limit (LOQ), and ruggedness were also calculated and/or reviewed. Furthermore the linearity of the calibration curves was calculated.
The LOQ is the concentration level where the signal-to-noise (S/N) ratio = 6. The calculated value is the average of the S/N-ratio ratio at m/z = 122.2 -> 58 (as calculated by RMS-method present in the MassLynx software) of six different blank pear or leaves of pear samples, fortified at 0.010 mg/kg.
To show the applicability of the analytical method to pears a limited validation is performed in which the linearity of the calibration curves, the recovery at LOQ level (0.010 mg/kg) and at five times LOQ level (0.050 mg/kg), and the signal-to-noise ratio (s/n) was determined at LOQ level. As there is no maximum residue limit (MRL) for the amount of chlormequat in leaves of pear no validation was performed, but the quality controls that are being used in the analysis of chlormequat in pear are also used for the analysis of leaves of pear.

3.2 Description of the limited validation procedure

A calibration curve in methanol (range: 0.0, 2.0, 5.0, 10.0, 25.0, and 50.0 ng/ml) was constructed. Furthermore a series of blank pear samples was spiked at 0.0, 0.01, and 0.05 mg/kg, and finally the s/n-ratio was determined in the 0.01 mg/kg samples. The experiments were performed in three different series.

3.3 Results of the validation, starting criteria

According to RSV-A0911, "Meel - Kwantitatieve bepaling van chloormequat - LC-MSMS" several acceptance criteria have to be met to start the measurement of the samples. The criteria are determined with the calibration curve in methanol. The criteria are:

- the repeatability of the system,
- the sensitivity of the system (calculated from a reference solution containing 2.0 ng/ml chlormequat),
- the linearity of the calibration curves,
- the difference in slope between the different calibration curves of each series of measurement.

All criteria were met in each series (see Table 1.).

Table 1. Quality control parameters of the three validation series.

	Limit	1st Series	2nd Series	3rd Series
Repeatability	CV < 5 % (n=3)	2.7%	2.2%	1.5%
Sensitivity	s/n > 6	19	19	19
1st cal. Curve R^2	> 0.995	0.9987	0.9991	0.9993
a(1st) <-> a(2nd)	< 15 %	4.2%	0.1%	3.9%

3.4 Results of the validation, quality controls in pear

The calibration curve in methanol (y=Ax+B with correlation coefficient R^2) is linear across the concentration range (R^2 > 0.995). The trueness of the fortified samples is given. The fortified samples are within the range of 102-117%. Although this is partly outside of the limit imposed by SANCO 1805/2000, Rev. 1., which dictates 80-110%, the results are accepted because of the low level of fortification compared to the MRL. The signal to noise ratio (s/n) is calculated for six fortified samples at 0.01 mg/kg. A minimum of 6 is obligatory but the lowest value found is 19 which confirms that the requested LOQ (0.010 mg/kg) is applicable to pear (see Table 2.).

Table 2: Validation parameters in pear

	Limit	1st Series	2nd Series	3rd Series
Calibration curve in methanol				
A		0.0661	0.0689	0.0688
B		-0.0299	-0.0177	-0.0086
R^2	> 0.995	0.9987	0.9991	0.9993
Trueness fortified samples				
0.01 mg/kg	80-110%	110.8%	111.0%	114.9%
0.05 mg/kg	80-110%	102.4%	116.9%	116.2%
Sensitivity[1)]	s/n > 6	54/34	42/46	28/19

1) = at 0.01 mg/kg; n=2 per series.

4. RESULTS OF CHLORMEQUAT ANALYSIS IN PEAR AND LEAVES OF PEAR

4.1 Summary

The method for the analysis of chlormequat in pear and leaves of pear is described in 2. Method description.
The pear and leaves of pear samples were analysed in seven runs. After measurement of 38 pear samples, one sample was below the LOQ of 0.010 mg/kg, 12 samples were positive but below the MRL-value of 0.5 mg/kg, and 25 samples were within a range of 0.56 to 14 mg/kg, which is above the MRL-value of 0.5 mg/kg.
After measurement of 84 leaves of pear samples, 15 samples were below the LOQ of 0.010 mg/kg, and 69 samples were within a range of 0.01 to 55 mg/kg.

4.2 Introduction

The analysis of chlormequat in 38 pear and 84 leaves of pear samples was performed according to a modified RSV-A0911, a validated LC-MSMS method. The modification was necessary to be able to lower the LOQ from 0.070 mg/kg to 0.010 mg/kg. The modifications consisted of increasing the amount of sample (10.0 g. instead of 2.5 g.) and the introduction of a gradient in the HPLC-chromatography, resulting in an improved peak-shape and, consequently, an improved signal to noise ratio. Finally the amount of the internal standard D4-chlormequat was reduced to fit in with the lower LOQ. The method RSV-A0911 is classified as quantitative according to SANCO 1805/2000, Rev. 1, and is modified from CEN-method CEN/TC 275/WG 4 N 134, March 2001, Determination of chlormequat as chlormequat cation in non-fatty foods.

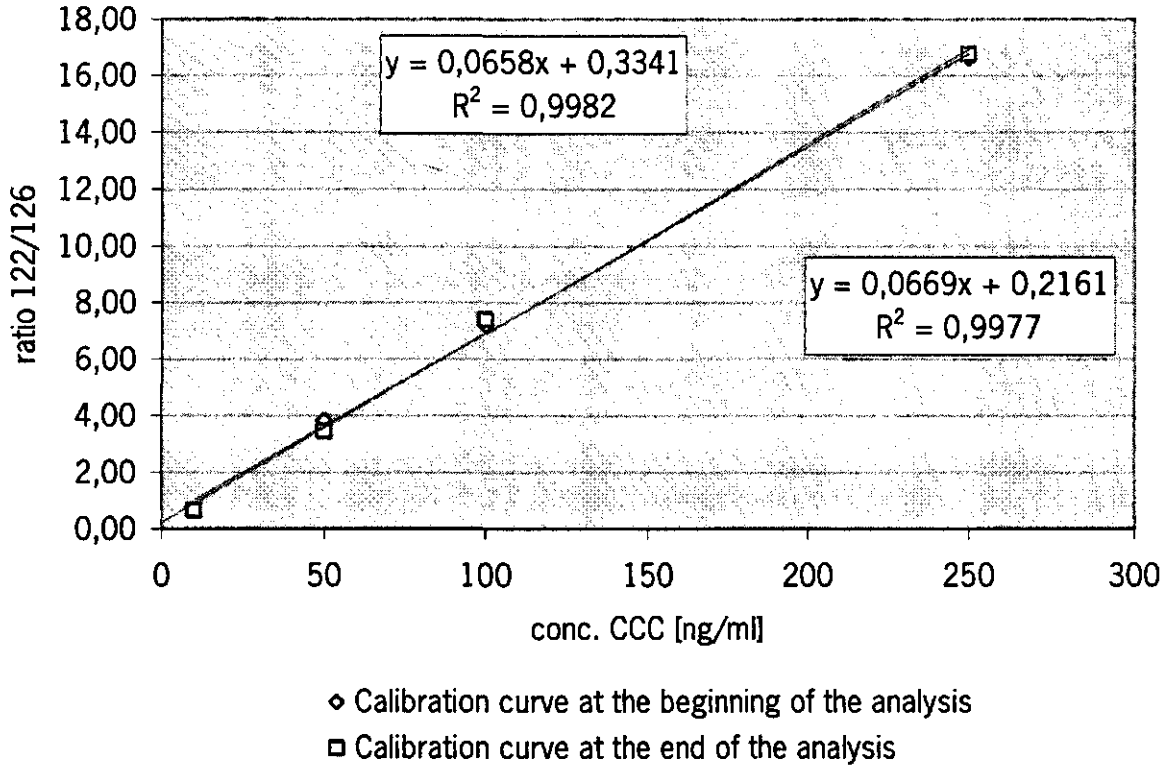
4.3 Procedure

The extraction and analysis procedure are described in detail in chapter 2. Method description.
The 38 pear samples and 84 samples of leaves of pear are divided into seven series. Each series of samples was extended with several quality control (QC)-samples, and other controls according to the following scheme:

1. A calibration curve in methanol, range: 0.00, 0.01, 0.05, 0.10, 0.25, and 2.50 µg/ml;
2. A blank pear or leaves of pear extract without addition of D4-chlormequat chloride;
3. The extraction mixture without addition of D4-chlormequat chloride;
4. The internal standard solution containing D4-chlormequat chloride;
5. Several fortified blanks, range: 0.0 (1x), 0.05 (2x), and 0,50 mg/kg (2x).

All samples contained D4-chlormequat chloride as internal standard, except when explicitly mentioned.
Typical calibration curves are represented in figure 1. The highest point (2.50 µg/ml) is omitted from the curve because of a better visibility. However, the curve including this point had a $R^2 >$ 0.9999.
After item 1., and 5.,(as described in the above mentioned scheme) and after sets of 10 samples a blank solvent (methanol) was injected to check for carry-over in the LC-MSMS system.
The internal standard D4-chlormequat chloride contained approximately 1% native chlormequat. This equals approximately 0.5 µg/kg in pear or leaves of pear (calculation based on 100% recovery) and is twenty times below the LOQ (0.010 mg/kg). Furthermore the benefits of the use of an isotope internal standard are evident and at MRL-level (0.5 mg/kg) the influence is negligible.

Figure 1. Chlormequat calibration curves.



4.4 Acceptation criteria

The results of the different checks for pear and leaves of pear are given in Table 3.
The blank pear extract, the leaves of pear extract, the extraction mixture (methanol/water = 2/1 v/v), and the internal standard solution containing D4-chlormequat chloride showed no signs of contamination.
The blank solvent (methanol), injected at different moments in each series, showed no sign of carry-over between the injections.

4.5 Results of the samples

The results of the analysis of 84 leaves of pear samples are given in Table 4. The results of the analysis of 38 pear samples are given in Table 5.

Table 3. Quality Control results pear and leaves of pear.

	Limit	28-8-01	4-9-01	10-9-01	12-9-01	13-9-01	17-9-01	19-9-01
Repeatability	CV < 5%	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Sensitivity	s/n > 100	Passed	Passed	Passed	Passed	Passed	Passed	Passed
1st cal. Curve								
A		0.0683	0.0658	0.0669	0.0702	0.0776	0.0755	0.0725
B		-0.0893	0.3341	-0.0609	0.0885	-0.1368	-0.1181	0.0852
R^2	> 0.995	0.9995	0.9982	0.9998	0.9990	0.9996	0.9993	0.9997
2nd cal. Curve								
A		0.0636	0.0669	0.0660	0.0683	0.0723	0.0729	0.0736
B		0.0139	0.2161	0.1119	0.1113	0.0166	0.1746	0.2251
R^2	> 0.995	0.9997	0.9977	1.0000	0.9998	0.9999	0.9999	0.9999
a(1st) <-> a(2nd)	< 15%	6.7%	1.7%	1.3%	2.7%	6.8%	3.4%	1.5%
Recovery	> 50%	34-48%	25-88%	22-37%	32-36%	24-82%	32-111%	29-48%
Retention time stability	95-105%	98-101%	96-105%	100-101%	100-101%	100-101%	99-101%	99-101%
Isotope ratio	75-125%	95-114%	99-107%	91-104%	95-111%	89-100%	95-108%	95-108%
Trueness								
0.00 mg/kg	< 0.010 mg/kg	<0.01 mg/kg	<0.01 mg/kg	<0.01 mg/kg	<0.01 mg/kg	<0.01 mg/kg	<0.01 mg/kg	<0.01 mg/kg
0.05 mg/kg	80-110%	125%	101%	107%	114%	100%	87%	88%
0.50 mg/kg	80-110%	113%	112%	94%	99%	91%	99%	99%

Table 4. Results of chlormequat analysis in leaves of pear.

Rikilt number	Chlormequat (mg/kg)
41399	< LOQ*
41400	0.03
41435	0.10
41663	0.06
41664	55
41665	0.47
41666	1.5
41667	2.9
41668	0.14
41792	0.56
41793	0.52
41853	< LOQ*
41854	0.04
41953	< LOQ*
41954	0.07
41955	0.21
42057	0.05
42417	0.87
42418	< LOQ*
42419	0.14
42528	< LOQ*
42553	0.11
42554	< LOQ*
42614	0.24
42615	16
42616	0.63
42617	31
42618	8.1
42619	0.26
42620	< LOQ*
42644	23
42645	0.72
42646	0.42
42647	0.45
42648	0.04
42649	0.63
42650	0.11
42651	0.40
42652	0.08
42653	< LOQ*
42710	0.30
42711	0.04

Rikilt number	Chlormequat (mg/kg)
42712	0.09
42741	< LOQ*
42742	0.01
42749	1.75
42756	0.17
42786	0.73
42787	0.09
42788	0.02
42932	0.11
42933	< LOQ*
42934	0.03
42935	< LOQ*
42936	< LOQ*
43033	0.02
43034	2.0
43035	0.18
43036	2.2
43037	0.02
43722	0.54
43723	0.84
43724	1.9
43725	0.44
43726	0.09
43727	0.71
43728	0.38
43729	0.13
43730	< LOQ*
43731	< LOQ*
43732	0.41
43754	0.11
43755	0.18
43756	0.86
43757	< LOQ*
43914	0.06
43915	0.28
43916	0.08
44777	0.24
44778	0.25
45672	0.18
45673	0.01
45855	0.04
45856	0.06

* LOQ = 0.01 mg/kg.

Table 5. Results of chlormequat analysis in pear

Rikilt number	Chlormequat (mg/kg)	Species
42009	< LOQ*	Conference
42010	0.62	Conference
42011	0.37	Conference
42012	2.0	Doyenné du comice
42013	0.67	Conference
42014	0.67	Conference
42015	2.3	Doyenné du comice
42016	0.58	Conference
42017	0.74	Conference
42018	0.56	Conference
42019	2.1	Doyenné du comice
42020	0.89	Conference
42021	0.44	Conference
42022	0.74	Conference
42023	0.42	Conference
42024	0.26	Triumph
42025	0.02	Gieser wildeman
42026	2.5	Doyenné du comice
42027	4.2	Doyenné du comice
42028	12	Conference
42029	14	Conference
42030	11	Doyenné du comice
42031	0.47	Gieser wildeman
42919	0.85	Unknown
42920	0.65	Unknown
42921	0.98	Unknown
42922	0.07	Unknown
42923	0.08	Unknown
42924	0.09	Unknown
42997	0.45	Unknown
42998	0.25	Unknown
42999	1.8	Unknown
43000	1.3	Unknown
43001	1.1	Unknown
43002	1.7	Unknown
43003	0.77	Unknown
43004	1.4	Unknown
43733	0.13	Unknown

* LOQ = 0.01 mg/kg

4.6 Discussion pear and leaves of pear samples

All criteria were within the prescribed limits with two exceptions.
The trueness in series 1 (28-8-2001, 125% and 113%), series 2 (4-9-2001, 112%), and series 4 (12-9-2001, 114%) lies outside the range imposed by SANCO 1805/2000, Rev. 1. Because of the small deviation the results were nevertheless accepted.
The recovery of chlormequat from leaves of pear was considerably lower (typically 22-48%) than the recovery of chlormequat from pear (typically 80-111%). This was probably caused by the presence of interfering compounds in the matrix, leading to suppression of the ionisation. Because of the use of D4-chlormequat as internal standard the trueness was within acceptable limits, and the results were accepted. In future samples of leaves of pear the calibration curves will be constructed in blank extract. Furthermore the clean-up of the samples will be reviewed.
After measurement of 38 pear samples, one sample was below the LOQ of 0.010 mg/kg, 12 samples were positive but below the MRL-value of 0.5 mg/kg, and 25 samples were within a range of 0.56 to 14 mg/kg, which was above the MRL-value of 0.5 mg/kg.
Analysis of 84 leaves of pear samples showed that 15 samples were below the LOQ of 0.010 mg/kg, and 69 samples were within a range of 0.01 to 55 mg/kg.

5. CONCLUSION

Results of the limited validation in pear show that the fully validated analytical method for analysis of chlormequat in wheatflour is applicable to pear and leaves of pear. However, care must be taken in analysing leaves of pear as there is considerable interference, leading to suppression of the ionisation. As Chlormequat-D4 is used as internal standard the results are reliable as is shown in the results of the trueness.
Based on the results of the analysis of chlormequat in leaves of pear (69 samples within a range of 0.01 to 55 mg/kg), several fruit growers were visited again by the AID in order to obtain samples of pear. The leaves of pear proved to be an excellent marker for selection of selecting suspicious pears. Out of 38 suspicious samples of pear 12 samples were positive but below the MRL-value of 0.5 mg/kg, and 25 samples proved to be above the MRL-value of 0.5 mg/kg (ranging from 0.56 to 14 mg/kg). Only one sample was below the LOQ of 0.010 mg/kg.

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