



Quick Method for the LC-MS/MS Analysis of Highly Polar Pesticides in Foods of Plant Origin involving a Common Extraction Step with Methanol

1. Scope and Short Description

A method is described for the analysis of polar, non-QuEChERS-amenable pesticides ($\log K_{ow} < -2$) in foods of plant origin such as fruits (including dried fruits), vegetables, cereals and processed products thereof.

Residues are extracted from the test portion following the addition of acidified methanol. The mixture is centrifuged, filtered and directly analyzed by LC-MS/MS. Quantification is performed with the help of isotopically labeled internal standards (analogues of the target analytes), which are added directly to the test portion at the beginning of the procedure.

2. Apparatus and Consumables

- Powerful sample processing equipment, for example Stephan UM 5
- 50 ml centrifuge tubes with screw caps, for example: a) 50 ml Teflon® centrifuge tubes with screw caps (e.g. Nalgene/Rochester, USA; Oak-ridge, article-no. 3114-0050) or b) disposable 50 ml centrifuge tubes (e.g. Sarstedt/Nümbrecht, Germany, 114x28 mm, PP, article-no. 62.548.004)
- Automatic pipettes, suitable for handling volumes of 10 to 100 μl , 200 to 1000 μl and 1 to 10 ml.
- 10 ml solvent-dispenser for methanol
- Centrifuge, suitable for the centrifuge tubes employed in the procedure and capable of achieving at least 2000 g
- Syringes, e.g. 2 or 5 mL disposable syringes
- Syringe filters, 0.45 μm pore size
- Injection vials, suitable for LC auto-sampler



3. Chemicals

Unless otherwise specified, use reagents of recognized analytical grade. Take every precaution to avoid possible contamination of water, solvents, sorbents, inorganic salts, etc.

- Methanol, HPLC quality
- Acetonitrile, HPLC quality
- Concentrated formic acid (> 95%)
- Citric acid monohydrate
- Dimethylamine (e.g. 40%, Fluka article-no. 38940)
- Ammonium formate
- Water (deionized)
- Pesticides or pesticide stock solutions
- Isotopically labeled standards (see Annex)

4. Disclaimer

This method refers to several trade name products and instruments which are commercially available and suitable for the described procedure. This information is given for the convenience of the users of this method and does not constitute an endorsement by the CRL of the products named.

The application of this method may involve hazardous materials, operations and equipment. It is the responsibility of the users of this method to establish appropriate safety and health practices prior to use.

5. Procedure

5.1. Sample preparation

To obtain representative test-portions from the laboratory sample, proceed as required by the respective regulations and guidelines. For fruits and vegetables cryogenic milling (e.g. using dry ice) is to be preferred.

In the case of dried fruits and similar commodities (< 30 % water content) the following procedure is proposed: Add 850 g of cold water to 500 g frozen dried fruits and homogenize the mixture (if possible by adding dry ice). 13.5 g of this homogenate will correspond to 5 g sample.



5.2. Extraction

- Weigh 10 g* of the comminuted sample into a 50 mL centrifuge tube.

***Note:** When analyzing dry samples such as grains, raisins or flour, the sample amount should be reduced to 5 g. Smaller test samples may be required for extract-rich commodities or commodities with high water absorbing capacity not allowing proper extraction. The amount of water to be added to the different dry commodities is shown in Table 9 in the Annex.

- Add 100 µL of isotopically labeled internal standard solution of the analyte(s)

Table 1: Exemplary concentrations of the isotopically labeled ISTDs:

Analyte	ISTD	Suggested Concentration of ISTD solution in µg/mL	Expected concentration of ISTD in final extract in µg/mL
Chlormequat	Chlormequat chloride D4	10-20	0.5-1
Mepiquat	Mepiquat iodide D3	10-20	0.5-1
Ethephon	Ethephon D4	10-20	0.5-1
Fosetyl-Al	Fosetyl-Al D15	10-20	0.5-1
Maleic acid hydrazide	Maleic acid hydrazide D2	20-200*	1-10
Daminozide	Daminozide D6	10-20	0.5-1
Glyphosate	Glyphosate ¹³ C ₂ ¹⁵ N	10-20	0.5-1
AMPA	AMPA ¹³ C ¹⁵ N	10-20	0.5-1

* Keep in mind that the ISTD may contain some amounts of non-labelled maleic hydrazide. Smaller amounts of ISTD added will reduce the added amount of maleic hydrazid to the sample. Having approximately the same concentration of ISTD in sample extract and calibration solutions is paramount.

- Add 10 mL acidified methanol (methanol containing 1% concentrated formic acid).
- Close the tube and shake vigorously for 1 to 2 min. The resulting volume of the extract (taking into account the natural water content of the sample and the miscibility of water and methanol) is ca. **20 mL** (corresponding to **0.5 g sample per mL extract**).
- Centrifuge (e.g. 5 min at >2500 g)
- Transfer an aliquot of the extract into a syringe and filter it into an autosampler vial. Employ for LC-MS/MS analysis



5.3. Recovery experiments and preparation of blank extracts

Sample preparation for recovery experiments: Spike e.g. 100 µL (use small volumes!) of a pesticide standard solution to the blank-matrix portion* and proceed sample preparation as described for the test sample.

**Use the same sample amount as for the test sample!*

Preparation of blank extract for matrix matched calibrations: Take 10 g* of blank matrix test portion* (not containing any detectable residues of the analyte) and proceed sample preparation exactly the same way as described for the test sample. However, **DO NOT ADD ISTD**. Transfer sufficient aliquots of the blank extract to HPLC autosampler vials and proceed according to 5.4.

**Use the same sample amount as for the test sample!*

5.4. Preparation of Calibration Standards

An exemplary pipetting scheme for the preparation of a calibration solution is shown in Table 2.

Note: Where isotopically labeled ISTDs are used matrix-matched calibration is not essential as the ISTD matches for any matrix-related signal suppressions or enhancements. Where isotopically labeled ISTDs are not used matrix matching is essential.

Table 2: Pipetting scheme for the preparation of a calibration solution (exemplary)

	Matrix-matched		Solvent based
	using ISTD	without ISTD	
Blank extract or methanol with 1% formic acid	800 µL	900 µL	800 µL
ISTD solution (diluted): 1:20 dilution of the solution added to the test sample in step 5.2	100 µL*	-	100 µL*
Pesticide standard solution e.g. 0.5 µg/mL**	100 µL	100 µL	100 µL
Total volume	1000 µL	1000 µL	1000 µL

* Same volume as added in 5.2

** In this case the end-concentration of the pesticide in the calibration standard equals 0.05 µg/mL, which is equivalent to 0.1 mg/kg (when using 10 g test portions) or 0.2 mg/kg (when using 5 g test portions). It is of high importance to prepare the calibration solutions in a way that the ISTD-concentration approximately equals that of the final extracts of the test samples.



5.5. LC-MS/MS Measurement Conditions

Any suitable LC and MS/MS conditions may be used. Below you will find some proposals of instrument parameters.

Table 3: Proposed LC-MS/MS conditions for Ethephon, Glyphosat, AMPA* (metabolite of Glyphosate), Glufosinate, MPPA* (metabolite of Glufosinate)

Instrument parameters	Conditions
Ionization mode	ESI neg
Column**	Dionex IonPac AS 11 2 x 250 mm (P/N 44077)
Pre-column	Dionex IonPac AG11 2 x 50 mm (P/N 44079)
Eluent A	Water
Eluent B Make sure your solvent filters of the eluent can handle alkaline solvents.	1 mM citric acid in water adjusted to pH 11 with di-methylamine
Gradient	Flow: 0.3 mL/min Gradient: 100% A in 8 min to 50 or 100 % B hold B for about 7 min
Injection volume	20 µL (in case of analyzing only ethephon 5 µL may be enough -depending on the instrument)
Calibration solutions and levels	e.g. 0.05 or 0.1 µg/ISTD + one level near the LOD is recommended
Acquired mass transitions	AMPA 110/63 (target ion) AMPA 110/79 AMPA 110/81 Ethephon 143/107 (target ion) Ethephon 143/79 Ethephon 145/107 Glufosinate 180/136 Glufosinate 180/63 (target ion) Glufosinate 180/85 Glufosinate 180/95 Glyphosate 168/124 Glyphosate 168/150 Glyphosate 168/63 (target ion) Glyphosate 168/81 MPPA 151/63 (target ion) MPPA 151/107 MPPA 151/133 AMPA ¹³ C ¹⁵ N 112/63 (internal standard) Ethephon D4 147/111 (internal standard) Glyphosate ¹³ C ₂ ¹⁵ N 171/63 (internal standard)

^x AMPA: Aminomethylphosphonic acid; MPPA: 3-Methylphosphinicopropionic acid

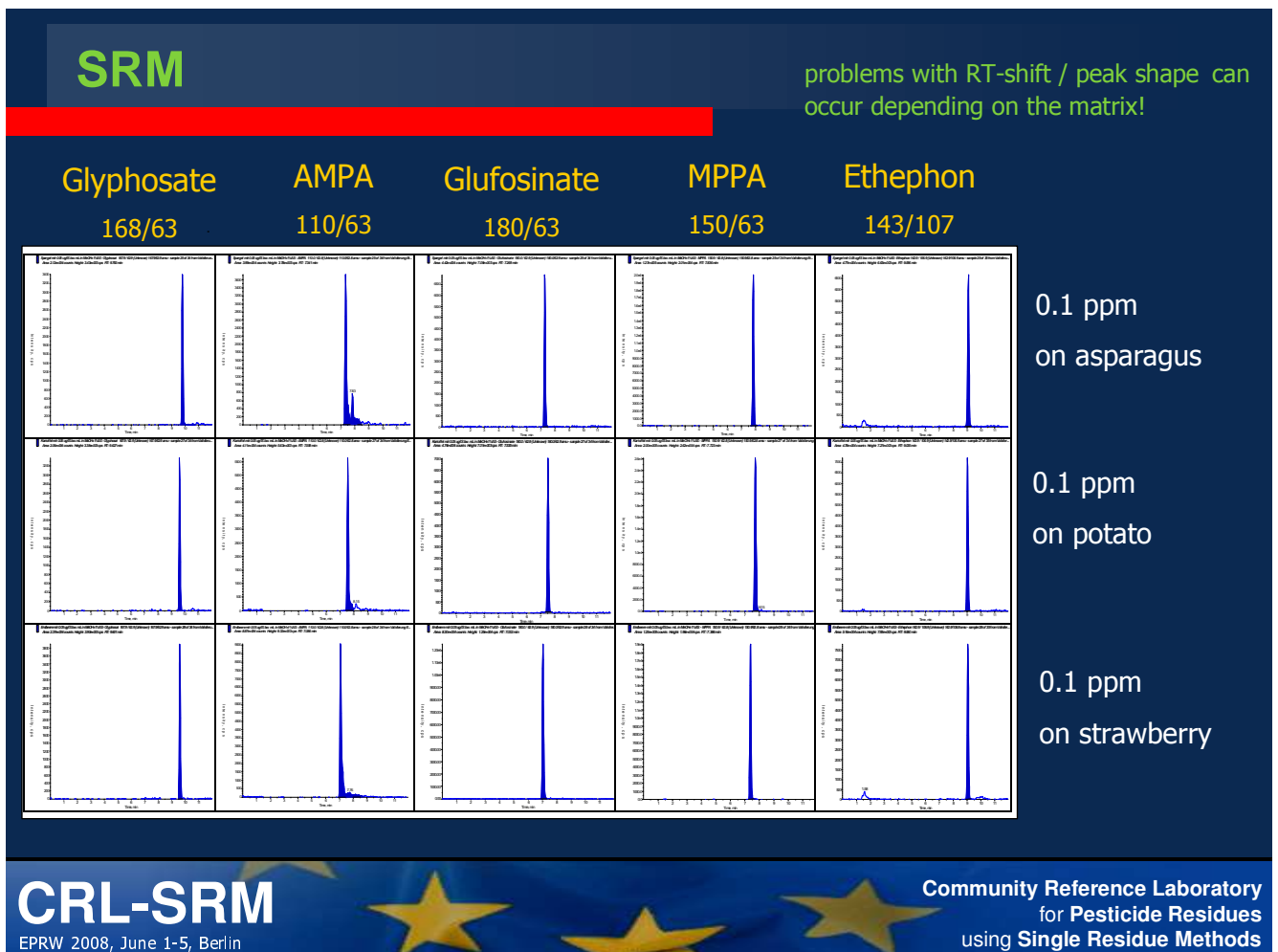


****Column Care:**

To extend the lifetime of the column, it is recommended to change the guard column regularly. Additionally, the column should be flushed from time to time with 30 mM NaOH for cleaning. If many samples are analyzed per sequence, this cleaning procedure may be necessary after every sequence to extend the lifetime of the column. **The NaOH solution has to go directly into a waste bottle and MUST NOT reach the ion source of the MS!!**

Especially for AMPA, MPPA and glufosinate it is important to have a column with a good performance. Otherwise peak shapes worsen considerably and substantial retention time shifts occur depending on the matrix.

Figure 1: Typical chromatograms of Glyphosate, AMPA, Glufosinate, MPPA and Ethephon



**Table 4:** Proposed LC-MS/MS conditions for Fosetyl-AI and Maleic Hydrazide

Instrument parameters			
Ionization mode	ESI neg		
Column	Obelisc R 2.1 x 150mm 5µm 100 Å (SIELC; OR-21.150.0510)		
Pre-column	Obelisc R 2.1 x 10mm 5 µm (SIELC; OR-21.G.0510)		
Eluent A	50 mmol NH ₄ -formate in water + 0.1 % formic acid use brown glass bottles		
Eluent B	Acetonitrile		
Gradient	%A	Flow [mL/min]	Time [min]
	3	0.3	0
	10	0.3	6
	70	0.5	15
	70	0.5	18
	3	0.5	18.1
	3	0.5	28
Injection volume	5 µL		
Calibration solutions and levels	e.g. 0.05 or 0.1 µg/ml + one level near the LOD is recommended For maleic hydrazide (MH) a level of 1 µg/ml may be useful as well, due to high residue levels; consider that MH is typi- cally only relevant for potatoes and <i>Alliaceae</i>		
Acquired mass transitions	Fosetyl-AI 109/81 (target ion) Fosetyl-AI 109/63 Maleic hydrazide 111/82 (target ion) Maleic hydrazide 111/42 Maleic hydrazide 111/55 (Maleic hydrazide 111/83) Fosetyl-AI D15 (ISTD) 114/82 Maleic hydrazide D2 (ISTD) 113/42		

Figure 3: Typical chromatograms of Fosetyl-AI

			Recovery test on strawberry 0.1 mg/kg = 0.05 µg/mL
			Fosetyl-AI solvent calib. 0.005 µg/mL = 0.01 mg/kg
			Fosetyl-AI solvent calib. 0.05 µg/mL = 0.1 mg/kg
Fosetyl-AI 109 / 81	Fosetyl-AI 109 / 63	Fosetyl-AI D15 114 / 82 (ISTD)	

Figure 4: Typical chromatograms of Maleic Hydrazide

				Maleic hydrazide solvent calib. = 0.05 µg/ml
				onion sample 0.5 g/ml n.n.
				onion sample 0.5 g/ml ~ 0.1 mg/kg Maleic hydrazide
				onion sample 0.5 g/ml ~ 4 mg/kg Maleic hydrazide
Maleic hydrazide D2 113/42 (ISTD)	Maleic hydrazide 111 / 82 (target ion)	Maleic hydrazide 111 / 55	Maleic hydrazide 111 / 42	

** It should be kept in mind that the isotopically labeled maleic hydrazide (MH) may contain small amounts of MH (in this case less than 0.2%). The influence of this impurity on quantitation is typically low because of the small amount of added impurity (in this case corresponding to less than 0.005 mg MH/kg sample following addition of 20 µg ISTD to 10 g sample) is small compared to the typically high MH-levels encountered in treated



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samples and because of the fact that the calibration solutions also contain ISTD (blank value) which causes a shift of the calibration curve and corrects quantification. In case of samples with low MRLs (e.g. baby food) or organic samples, care should be taken in quantification. Analysis of the sample without ISTD added will help to identify false positives. Keeping the concentration of the ISTD solution employed low will reduce these effects.

Table 5: Proposed LC-MS/MS conditions Chlormequat, Mepiquat, Daminozide and Trimethylsulfonium-Cation (counterion of Glyphosate)

Instrument parameters	Conditions
Ionisation mode	ESI pos
Column	Obelisc R 2.1 x 150mm 5µm 100 Å (SIELC; OR-21.150.0510); 40°C
Pre-column	Obelisc R 2.1 x 10mm 5 µm (SIELC; OR-21.G.0510)
Eluent A	20 mmol NH ₄ -formate in water (pH 3) Use brown glass bottles
Eluent B	Acetonitrile
Gradient	Flow:: 0.4 mL/min Gradient: 20% A in 4 min to 90 % A
Injection volume	5 µL
Calibration solutions and levels	e.g. 0.05 or 0.1 µg/IS + one level near the LOD is recommended
Acquired mass transitions	Chlormequat 122/58 (target ion) Chlormequat 122/63 Chlormequat 124/58 Mepiquat 114/98 (target ion) Mepiquat 114/58 Trimethylsulfonium-cation 77/62 (target ion) Trimethylsulfonium-cation 77/47 Daminozide 161/143 (target ion) Daminozide 161/101 Daminozide 161/61 Daminozide 161/115 Chlormequat D4 (ISTD) 126/58 Mepiquat D3 (ISTD) 117/101 Daminozide D6 (ISTD) 167/149

Table 6: Proposed alternative LC-MS/MS conditions for Chlormequat and Mepiquat

Instrument parameters	Conditions
Column:	MonoChrom MS 100x2 mm; 5 µm (Varian); at 40°C
Eluent A:	5 mmol/L NH ₄ -acetate + 0.1% acetic acid
Eluent B:	Acetonitrile
Gradient:	5% A in 2 min to 95% A
Flow rate:	0.4 mL/min
Injection volume:	5 µL



6. Performance

Results of 5-fold analyses using matrix matched calibrations (for more information see method validation database at www.crl-pesticides-datapool).

Table 7: Overview of Validation Data

Pesticide	Mean Recovery [%]	Variation-coefficient [%]	Spike Level [mg/kg]	Matrix	ISTD
Chlormequat-Cation (MRM 122/58)	97	4.6	0.1	Wheat Flour	Chlormequat D4
Mepiquat-Cation (MRM 114/98)	102	4.9	0.1	Wheat Flour	Mepiquat D3
Trimethylsulfonium-Cation (MRM 77/62)	104	1.9	0.1	Wheat Flour	-
Glyphosate (MRM 168/63)	124	7.6	0.1	Wheat Flour	Glyphosate ¹³ C ₂ ¹⁵ N
AMPA (MRM 110/63)	119	5.7	0.1	Wheat Flour	AMPA ¹³ C ¹⁵ N
Glufosinate (MRM 180/63)	85	4.6	0.1	Wheat Flour	-
MPPA (MRM 151/63)	85	1.3	0.1	Wheat Flour	-
Ethephon (MRM 143/107)	85	5.9	0.1	Wheat Flour	-
Fosetyl-Al (MRM 109/81)	95	4.1	0.1	Strawberry	Fosetyl-Al D15
Maleic Hydrazide (MRM 111/82)	106	3.7	0.1	Onion	Maleic Hydrazide D2
Daminozide (MRM 161/143)	101	2.8	0.1	Wheat Flour	Daminozide D6

7. References

Anastassiades, M and Mack, D (2008); New Developments in the Analysis of Pesticides Typically not Covered by Multiresidue Methods; European Pesticide Residue Workshop, EPRW 2008, Berlin, oral presentation O1, Book of Abstracts

Alder L. and Startin J. R. (2005); Determination of Chlormequat and Mepiquat in Foods by Liquid Chromatography/Mass Spectrometry or Liquid Chromatography/Tandem Mass Spectrometry: Interlaboratory Study; Journal of AOAC International Vol. 88, No. 6: 1762-1776

Vahl, M. et al. (1998); Analysis of Chlormequat residues in grain using liquid chromatography-mass spectrometry (LC-MS/MS); Fresenius J Anal Chem 361:817-820

**Annex:****Table 8:** Isotopically labeled internal standards available from Dr. Ehrenstorfer GmbH:

Name	Company	Article-No.	Conc.	Amount	Price*
Aminomethyl phosphonic acid (AMPA) ¹³ C ¹⁵ N	Dr. Ehrenstorfer	XA10205100WA	100 ng/μL	1.1 mL	280
Captan D6	Dr. Ehrenstorfer	XA10960100AC	100 ng/μL	1.1 mL	138
Chlormequat chloride 1,1,2,2-D4	Dr. Ehrenstorfer	X11340100DO	100 ng/μL	10 mL	310
	Dr. Ehrenstorfer	XA11340100DO	100 ng/μL	1.1 mL	73
Daminozide D6	Dr. Ehrenstorfer	XA11960100AL	100 ng/μL	1.1 mL	89
Diquat dibromide D4 monohydrate	Dr. Ehrenstorfer	XA12960010DO	100 ng/μL	1.1 mL	84
Ethephon D4 (2-Chloroethyl-1,1,2,2-D4)	Dr. Ehrenstorfer	XA13230100AC	100 ng/μL	1.1 mL	128
Ethylene thiourea D4	Dr. Ehrenstorfer	C13330100		50 mg certified	310
Fosetyl-aluminium D15	Dr. Ehrenstorfer	CA13940010		10 mg certified	380
Glyphosate 1,2- ¹³ C ₂ ¹⁵ N	Dr. Ehrenstorfer	XA14050100WA	100 ng/μL	1.1 mL	330
Maleic hydrazide D2	Dr. Ehrenstorfer	C14730100		10 mg certified	230
Mepiquat iodide D3 (methyl D3)	Dr. Ehrenstorfer	X14880100DO	100 ng/μL	10 mL	410
	Dr. Ehrenstorfer	XA14880100DO	100 ng/μL	1.1 mL	68

* December 2008

Table 9: Water content of selected foods and amount of water, which has to be added to test sample prior to extraction

Commodity group	Commodity	Typical water content g/100 g	Amount of water added to 10 g test portion g	Amount of water added to 5 g test portion g	Remarks
Fruits					
Citrus fruits	citrus juices	90			
	grapefruit	90			
	lemon/lime	85			
	orange	85			
	tangerine	90			
Pome fruit	apple	85			
	apple, dried	30		8.5 (see 5.1)	Weigh 13.5 g rehydratized homogenate
	apple sauce	80			
	apple juice	90			
	pear	85			
	quince	85			
Stone fruit	apricot	85			
	Apricot, dried	30		8.5 (see 5.1)	Weigh 13.5 g rehydratized homogenate
	apricot nectar	85			
	cherry	85			
	mirabelle	80			
	nectarine	85			
	peach	90			
	Peach, dried	20		8.5 (see 5.1)	Weigh 13.5 g rehydratized homogenate
	plum	85			
	plum. dried	20		8.5 (see 5.1)	Weigh 13.5 g rehydratized homogenate
Soft and small fruits	blackberry	85			
	blueberry	85			
	currant	85			
	elderberry	80			
	gooseberry	90			



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Commodity group	Commodity	Typical water content g/100 g	Amount of water added to 10 g test portion g	Amount of water added to 5 g test portion g	Remarks
	grapes	80			
	raspberry	85			
	raisin	20		8.5 (see 5.1)	Weigh 13.5 g rehydrated homogenate
	strawberry	90			
	pineapple	85			
Other fruits	banana	75	2.5		
	fig, dired	20		8.5 (see 5.1)	Weigh 13.5 g rehydrated homogenate
	kiwi	85			
	mango	80			
	papaya	90			
	papaya	90			
Vegetables					
Root and tuber vegetables	beetroot	90			
	carrot	90			
	celeriac	90			
	horseradish	75	2.5		
	parsley root	90			
	radish	95			
	black salsify	80			
	potato	80			
	garlic	60		7.0	
Leek plants	onion	90			
	leek	85			
	shallot	80			
	chive	85			
	aubergine	90			
Fruiting vegetables	cucumber	95			
	melon	90			
	pepper, sweet	90			
	pumpkin	95			



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Commodity group	Commodity	Typical water content g/100 g	Amount of water added to 10 g test portion g	Amount of water added to 5 g test portion g	Remarks
	tomato	95			
	zucchini (courgette)	95			
	broccoli	90			
Cabbage	brussels sprouts	85			
	cauliflower	90			
	chinese cabbage	95			
	kale	90			
	kohlrabi	90			
	red cabbage	90			
	savoy cabbage	90			
	white cabbage	90			
	lettuce varieties	95			
Leafy vegetables and herbs	endive	95			
	cress	90			
	lamb's lettuce	85			
	parsley	80			
	rucola	85			
	spinach	90			
	asparagus	95			
Stem vegetables	celery	95			
	leek	85			
	rhubarb	95			
	artichokes	85			
	beans, peas, lentils (dried)	<10		10	
Legumes	beans (fresh)	75	2.5		
	beans (fresh)	75	2.5		



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Commodity group	Commodity	Typical water content g/100 g	Amount of water added to 10 g test portion g	Amount of water added to 5 g test portion g	Remarks
Miscellaneous					
Cereals	cereals (grain. flour. etc.)	10		10	
High Extract commodities	coffee beans	<10		10 (use 2 g test portion if extract-rich)	
	tea	<10			
	dry herbs and spices	<10			
Other	mushrooms	90			
	wine	90			

**Table 10:** Document History:

Action	When?	Changes introduced	Version
Development of Method by the CRL-SRM	2006-2008		-
Presentation of method at the EPRW in Berlin (oral presentation plus poster)	Jun. 2008		-
Drafting of the present document	Nov.-Dec. 2008		V 1
Placing of the present document in CRL-Website	Jan. 2009		V 1