SLOVENSKI STANDARD
SIST EN 15637:2009

01-april-2009

Hrana rastlinskega izvora - Določanje ostankov pesticidov z uporabo LC-MS/MS za ekstrakcijo z metanolom in čiščenjem z diatomejsko zemljo

Foods of plant origin - Determination of pesticide residues using LC-MS/MS following methanol extraction and clean-up using diatomaceous earth

Pflanzliche Lebensmittel - LC-MS/MS-Verfahren zur Bestimmung von Pestizidrückständen mit Methanolextraktion und Reinigung an Diatomeenerde

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Aliments d'origine végétale - Détermination des pesticides par LC-MS/MS après extraction méthanolique et purification sur terre de diatomées

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Ta slovenski standard je istoveten z: **EN 15637:2008**

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

SIST EN 15637:2009 **en,fr,de**

2003-01.Slovenski inštitut za standardizacijo. Razmnoževanje celote ali delov tega standarda ni dovoljeno.

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Foods of plant origin - Determination of pesticide residues using LC-MS/MS following methanol extraction and clean-up using diatomaceous earth

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SIST EN 15637:2009

Contents

Page

iTeh STANDARD PREVIEW
(standards.iteh.ai)
SIST EN 15637:2009
https://standards.iteh.ai/catalog/standards/sist/0cd93c63-424f-407d-a7e4-69541e821510/sist-en-15637-2009

SIST EN 15637:2009

Foreword

This document (EN 15637:2008) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2009, and conflicting national standards shall be withdrawn at the latest by May 2009.

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SIST EN 15637:2009
https://standards.iteh.ai/catalog/standards/sist/0cd93c63-424f-407d-a7e4-69541e821510/sist-en-15637-2009

SIST EN 15637:2009

1 Scope

This European Standard describes a method for the analysis of pesticide residues in foods of plant origin, such as fruits, vegetables, cereals, nuts as well as processed products including dried fruits. The method has been collaboratively studied on a large number of commodity/pesticide combinations.

2 Principle

The sample is extracted with methanol after addition of some water. After partition into dichloromethane the organic phase is evaporated and the residue is reconstituted with methanol. Quantification of pesticide residues is performed by liquid chromatography with tandem mass spectrometric detection, using electrospray ionisation. To achieve the required selectivity the mass spectrometer is operated in the selected reaction monitoring mode (SRM).

3 Reagents

3.1 General and safety considerations

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

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3.2 Ammonium formate

3.3 Sodium chloride

SIST EN 15637:2009
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3.4 Water, HPLC quality

3.5 Dichloromethane, for residue analysis

3.6 Methanol, HPLC quality

3.7 Internal Standard (ISTD) solutions in methanol, ρ = 10 µg/ml to 50 µg/ml[1)]

Table 1 shows a list of potential internal standards that may be used in this method. The concentrations listed refer to the ISTD solutions that should be added at the first extraction step (5.2) and to standard solutions.

Table 1: Potential internal standards (ISTDs) or quality control (QC) standards

Name of the compound	Log P (octanol-water partion coefficient)	Chlorine atoms	Concentration C_{ISTD} µg/ml
Triphenyl phosphate	4,59	-	20
Tris-(1,3-dichlorisopropyl)-phosphate	3,65	6	50
Bis-nitrophenyl urea (nicarbazin)	3,76	-	10

1) ρ = mass concentration

SIST EN 15637:2009

3.8 Pesticide stock solutions

Prepare individual stock solutions of analytical standards at concentrations that are sufficiently high to allow the preparation of complex pesticide mixtures. The solvent used should not negatively influence the stability of the pesticides employed.

NOTE Usually, store stock solutions at ≤ -18 °C. Check the stability of stock solutions during storage regularly. In some cases the addition of acids or bases can be helpful to enhance stability and extend the acceptable storage period.

3.9 Pesticide mixtures

Because of the broad applicability of this method and due to the partly divergent pH-stability of pesticides, analyte mixtures of different composition can be needed. These are prepared by mixing together defined volumes of the required analyte stock solutions (3.8) and appropriately diluting them with methanol. The analyte concentrations in this mixture should be sufficient to allow the preparation of the required matrix matched standards (see 3.10.3) with moderate dilution of the blank sample extract (e.g. less than 20 %).

Usually, store pesticide mixtures at ≤ -18 °C. Since the stability of the pesticides in the mixture may be lower than in stock solutions, stability has to be checked regularly. In some cases the addition of acids or bases can be helpful to enhance stability and extend acceptable storage times.

3.10 Standard solutions

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3.10.1 Standard solutions prepared in pure solvent (solvent-based standards)

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Solvent-based standards are prepared by mixing a certain volume of methanol with known amounts of pesticide mixtures (3.9). The preparation of multiple standards of different pesticide concentration is useful to cover a broad concentration range.

SIST EN 15637:2009
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NOTE An analyte concentration of 1 µg/ml correlates to a residue level of 0,4 mg/kg when a 10 g sample is employed (e.g. samples with water content > 30 %) or 0,8 mg/kg when a 5 g sample is employed (e.g. cereals).

3.10.2 Standard solutions with internal standard prepared in pure solvent

Solvent-based standards with ISTD are prepared by mixing a certain volume of methanol with known amounts of pesticide mixtures (3.9) and a fixed volume of internal standard solution (3.7). The volume used shall result in that concentration of ISTD which is obtained in the final extracts after sample extraction and clean-up (see 5.2 and 5.3). The concentration of internal standard in the final extract (C_{ISTD}^{sample}) can be calculated using Equation (1). The preparation of multiple standards of different pesticide concentration but with constant ISTD concentration is useful to cover a broad concentration range.

$$C_{ISTD}^{sample} = \frac{V_{ISTD} \times C_{ISTD} \times (V_2 - V_1) \times V_3}{V_2 \times V_{ex} \times V_{end}} \quad (1)$$

where:

V_{ISTD} is the volume of internal standard solution (3.7) added to the test portion;

C_{ISTD} is the concentration of internal standard solution (3.7);

V_1 is the volume of NaCl solution (2,5 ml);

V_2 is the volume of measuring flask used in 5.2 (10 ml);

SIST EN 15637:2009

V_3 is the volume used for solid supported liquid/liquid extraction (5 ml);

V_{ex} is the total volume of extraction solvents and natural water (30 ml);

V_{end} is the final volume of extract obtained after clean-up (0,5 ml).

NOTE The internal standard may correct for deviations from the correct extraction volume, a wrong estimate of water content of samples, losses of methanol during preparation of the final extract and fluctuations of instrument sensitivity during a batch of measurements. However, validation results in Annex B were obtained without internal standards.

3.10.3 Standard solutions prepared in blank matrix extracts (matrix-matched standards)

Prepare matrix-matched standards in the same way as the solvent-based standards, however, instead of pure methanol use extracts of blank samples (prepared as described in 5.2, but without ISTD addition). To minimize errors caused by matrix induced effects during chromatography, it is best to choose similar commodities (e.g. apple for apple samples, carrots for carrot samples, etc.).

The stability of pesticide in matrix-matched standards may be lower than that of standards in pure acetonitrile and has to be checked more thoroughly.

3.11 5 ml cartridge for solid supported liquid/liquid extraction, 5 ml sample volume, diatomaceous earth, for example ChemElut CE 1005[2)]

3.12 20 ml cartridge for solid supported liquid/liquid extraction, 20 ml sample volume, diatomaceous earth, for example ChemElut CE 1020[2)]

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4 Apparatus

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Usual laboratory apparatus and, in particular, the following:

4.1 Carving board and knife, for chopping up food samples for analysis

4.2 Homogenizer or high speed blender, fitted with jar

4.3 Laboratory balance

4.4 Measuring flasks, 10 ml and 20 ml

4.5 Ultrasonic bath

4.6 Centrifuge tubes, 80 ml

4.7 Centrifuge, capable of producing a relative centrifugal force (RCF) of at least 3000 *g* (at the bottom of the tube)

4.8 Round bottom flasks, 50 ml and 250 ml

4.9 Glass syringe, minimum volume 2 ml

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2) ChemElut is a product supplied by Varian, Inc. (Palo Alto, CA, USA). This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

SIST EN 15637:2009

4.10 Microliter syringes, for sample fortification

4.11 Rotary evaporator, with temperature-controlled water bath

4.12 Syringe filters, 0,45 µm pore size, 4 mm diameter, polytetrafluoroethylene (PTFE) membrane

4.13 Glass vials and caps, 1,8 ml volume, suitable for an autosampler

4.14 LC-MS/MS system, triple quadrupole mass spectrometer with electrospray interface

5 Procedure

5.1 Preparation and storage of the samples

5.1.1 General

Sample processing and storage procedures should be demonstrated to have no significant effect on the residues present in the test sample (sometimes also called “analytical sample”). Processing should also ensure that the test sample is homogeneous enough so that sub-sampling variability is acceptable. If a single analytical portion is unlikely to be representative of the test sample, larger or replicate portions shall be analysed, to provide a better estimate of the true value. The degree of comminution supports a quantitative residue extraction.

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5.1.2 Laboratory sample

SIST EN 15637:2009
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A laboratory sample that is wholly or extensively spoiled or degraded should not be analysed. When possible, prepare laboratory samples immediately after arrival and in any event, before any significant physical or chemical changes have taken place. If a laboratory sample cannot be prepared without delay, it should be stored under appropriate conditions to keep it fresh and to avoid deterioration. Generally, laboratory samples should not be stored longer than 3 days before preparation. Dried or similarly processed samples should be analysed within their stated shelf life.

5.1.3 Partly-prepared test sample

For preparation of the partly-prepared test sample take only the portion of the laboratory sample to which the maximum residue level applies. No further plant-parts may be removed.

The reduction of the laboratory sample shall be carried out in such a way that representative portions are obtained (e. g. by sub-division into four and selection of opposite quarters). For samples of small units (e. g. small fruits such as berries, legumes, cereals), the sample shall be thoroughly mixed before weighing out the partly-prepared test sample. When the samples are made up of larger units, take wedge-shaped sections (e. g. melons) or cross sections (e. g. cucumbers) that include the skin (outer surface) from each unit [1].

5.1.4 Test sample

From each partly-prepared test sample, any parts that would cause difficulties with the homogenisation process should be removed. In the case of stone fruits, the stones shall be removed. A record of the plant-parts that have been removed shall be kept. Precautions should be taken to avoid any losses of juice or flesh. This is the test sample. Calculation of the residue shall be based on the mass of the original test sample (including the stones).

Where the homogeneity of the test sample is not sufficient or the extraction of residues may be significantly compromised due to large particle sizes, intensive comminution should be performed using appropriate means. This is possible at ambient temperature, if separation of flesh and juice or degradation of target

SIST EN 15637:2009

pesticides does not occur to a significant extent. Comminution of samples in a frozen state can significantly reduce losses of chemically labile analytes and usually results in smaller particle sizes and thus achieve a higher degree of homogeneity. Cutting the samples coarsely (e. g. 3 cm x 3 cm) with a knife and putting them into the freezer (e. g. -18 °C overnight) prior to comminution facilitates processing. Processing can be also assisted and improved by cryogenic milling (using dry ice or liquid nitrogen) by keeping the temperature below 0 °C. Especially in the case of fruits and vegetables, cryogenic milling is much more effective at homogenising commodities that have tough skins (e. g. tomatoes or grapes) compared to milling at ambient temperature. Given the fact that non-systemic pesticides often predominantly occur on the skin, cryogenic milling significantly reduces sub-sampling variability. When processing test samples at low temperatures, condensation caused by high humidity should be avoided. Residual carbon dioxide should be allowed to sufficiently dissipate so that its contribution to weigh of the sample will be negligible.

5.1.5 Test portion

Individual test portions each sufficient for one analysis are taken from the comminuted test sample. These test portions should be analysed immediately. If test portions cannot be analysed directly, the test sample or the test portions shall be frozen until required. If test portions are taken from test samples after being stored frozen, the test samples shall be mixed before taking test portions to ensure that homogeneity has been re-established.

5.2 Extraction

Transfer a representative test portion of m_A = 10 g into a centrifuge tube (4.6). For dry sample materials like cereal products, weigh a homogenised portion of 5 g (m_A) into the centrifuge tube. Add sufficient water, that a total volume (added and natural) of 10 ml water is obtained. For typical water contents of crops and cereals, see Table 2. In the case of dry sample materials wait 10 min after addition of water. Add 20 ml of methanol (3.6) to the mixture and homogenise for 2 min using the high speed blender (4.2). Take at least 10 ml of the resulting extract of 30 ml (= V_{ex}) and centrifuge at approximately 3000 *g*. Pipette 2,5 ml of NaCl solution (20 %, w/w) *(=* V_1*)* into a 10 ml measuring flask *(=* V_2*)* (4.4), fill up to the mark with supernatant of centrifugation and mix.

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SIST EN 15637:2009

As an option an internal standard can be used additionally. In that case add a small volume (<1 % of V_{ex}) of internal standard solution (=V_{ISTD}) to the test portion after addition of 20 ml of methanol.

SIST EN 15637:2009

Table 2 — Water content of selected foods and amount of water, which have to be added

Food group	Food	Typical water content g/100 g	Amount of water added to 10 g of test portion g	Amount of water added to 5 g of test portion g
Fruits				
Citrus fruits	citrus juices	90	1,0	
	grapefruit	90	1,0	
	lemon	90	1,0	
	orange	85	1,5	
	orange peel	75	2,5	
	tangerine	90	1,0	
Pome fruit	apple	85	1,5	
	apple, dried	30		8,5
	apple sauce	80	2,0	
	apple juice	90	1,0	
	pear	85	1,5	
	quince	85	1,5	
Stone fruit	apricot	85	1,5	
	apricot, dried	30		8,5
	apricot nectar	85	1,5	
	cherry	85	1,5	
	mirabelle	80	2,0	
	nectarine	85	1,5	
	peach	90	1,0	
	peach, dried	20		9,0
	plum	85	1,5	
	plum, dried	20		9,0
Soft and small fruits	blackberry	85	1,5	
	blueberry	85	1,5	
	currant	85	1,5	
	elderberry	80	2,0	
	gooseberry	90	1,0	
	grapes	80	2,0	
	raspberry	85	1,5	
	raisin	20		9,0
	strawberry	90	1,0	
Other fruits	pineapple	85	1,5	
	banana	75	2,5	

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SIST EN 15637:2009
https://standards.iteh.ai/catalog/standards/sist/0cd93c63-424f-407d-a7e4-69541e821510/sist-en-15637-2009

SIST EN 15637:2009

Table 2 *(continued)*

Food group	Food	Typical water content g/100 g	Amount of water added to 10 g of test portion g	Amount of water added to 5 g of test portion g
	fig, dried	20		9,0
	kiwi	85	1,5	
	mango	80	2,0	
	papaya	90	1,0	
Vegetables				
Root and tuber vegetables	beetroot	90	1,0	
	carrot	90	1,0	
	celeriac	90	1,0	
	horseradish	75	2,5	
	parsley root	90	1,0	
	radish	95	0,5	
	scorzonera (black salsify)	80	2,0	
	shallot	80	2,0	
Onions	garlic	60		7,0
	onion	90	1,0	
Fruiting vegetables	aubergine	90	1,0	
	cucumber	95	0,5	
	melon	90	1,0	
	pepper, sweet	90	1,0	
	pumpkin	95	0,5	
	tomato	95	0,5	
	zucchini (courgette)	95	0,5	
Cabbage	broccoli	90	1,0	
	Brussels sprouts	85	1,5	
	cauliflower	90	1,0	
	Chinese cabbage	95	0,5	
	kale	90	1,0	
	kohlrabi	90	1,0	
	red cabbage	90	1,0	
	savoy cabbage	90	1,0	
	white cabbage	90	1,0	

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SIST EN 15637:2009

Table 2 *(continued)*

Food group	Food	Typical water content g/100 g	Amount of water added to 10 g of test portion g	Amount of water added to 5 g of test portion g
Leafy vegetables and	butterhead lettuce	95	0,5	
herbs	chive	85	1,5	
	cress	90	1,0	
	endive	95	0,5	
	iceberg lettuce	95	0,5	
	lamb's lettuce	85	1,5	
	parsley	80	2,0	
	spinach	90	1,0	
	witloof chicory	95	0,5	
Stem vegetables	artichokes	85	1,5	
	asparagus	95	0,5	
	celery	95	0,5	
	leek	85	1,5	
	rhubarb	95	0,5	
Beans, peas (fresh)	beans	90	1,0	
	peas with pods	80	2,0	
Beans, peas (dried)	beans, peas, lentil	10		9,5
Other				
	beer	90	1,0	
	cereals (grain, flour, etc.)	10		9,5
	coffee (raw)	10		9,5
	mushrooms	90	1,0	
	must (grape)	90	1,0	
	potato	80	2,0	
	tea	10		9,5
	wine	90	1,0	

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5.3 Clean-up

Apply 5 ml of the diluted centrifugate *(= V_3)* from 5.2 to a 5 ml cartridge (3.11). After 5 minutes, elute into a 50 ml round bottom flask (4.8), using 12,5 ml of dichloromethane (3.5). Repeat the elution with another 12,5 ml of dichloromethane. Reduce the combined eluates almost to dryness using the rotary evaporator (4.11). Remaining dichloromethane should be removed with a stream of nitrogen.

SIST EN 15637:2009

Add 500 µl methanol (3.6) to the round bottom flask and weigh with stopper. Carefully dissolve the residue by swirling the flask in the ultrasonic bath (4.5), but avoid losses of methanol. If losses of methanol occur (re-weighing), add methanol to obtain the previous total weight. Filter the obtained sample test solution of 0,5 ml *(= V_{end})* through a PTFE-filter (4.12) into a sample vial (4.13) for injection.

To obtain a larger amount of sample test solution for the preparation of matrix-matched standards (3.10.3) a 20 ml cartridge (3.12) can be used. In that case, 400 % of all volumes mentioned above have to be used.

NOTE The sample test solution contains the extractable components of 2,5 g sample per millilitre final extract (or 1,25 g/0,5 ml).

5.4 Determination

The sample test solutions (5.3) and calibration solutions (3.10.1, 3.10.2 or 3.10.3) are injected into the LC-MS/MS instrument in an appropriate sequence. This can involve bracketing of the sample extracts with the calibration solutions. In the injector needle of the HPLC system the sample test solution should be diluted with eluent A. The LC-MS/MS instrument shall be operated in the selected reaction monitoring (SRM) mode with transitions selective for the pesticides under investigation. For suitable experimental conditions see CEN/TR 15641 [4]. Nevertheless, individual tuning of the compounds on the instrument that is used for measurement usually provides better sensitivities.

The measurement can be performed using various instruments, instrument parameters and columns. Some instrument parameters and columns are listed in Annex A. These conditions have been shown to provide satisfactory results.

NOTE Most validation results listed in Annex B have been obtained after mixing of sample test solution with water in a LC vial and not in the injector of the HPLC system. In that case a ratio between methanolic extract and water of 1:4 (V/V) was used. Also, standard solutions were diluted with water in the volume ratio 1:4 (V/V). Most samples contained small amounts of co-extracted components that are not soluble in the resulting methanol/water mixture. As a result a turbid emulsion (or suspension) was obtained. It was recognized that recovery of some less polar pesticides was reduced, if such emulsions have formed.

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5.5 Test for interference and recovery

Prepare reagent blanks and carry out spiked recovery tests at levels appropriate to the maximum residue level. The chromatogram of the reagent blank should not show any significant peak (e. g. 10 % of relevant MRL) at the retention time of the analytes.

6 Evaluation of results

6.1 Identification and quantification

To identify analytes compare the retention times obtained from the sample test solution with those obtained from the calibration solutions. Positive findings are confirmed by comparing the peak intensity ratios of the first and second compound specific m/z transition with the peak intensity ratios found in standards. If the peak ratio of a residue peak differs more than 20 % from the expected response ratio, check the EU-quality control guidelines described in the SANCO/2007/3131 document [2]. A different LC column, another eluent or an additional m/z transition may be used, if additional measures are necessary.

Use standard solutions (3.10.1 or 3.10.2) or matrix-matched standards (3.10.3) to check linearity and to determine the calibration functions for each active substance by plotting the peak areas or heights (if ISTDs are not used) or peak ratios (if ISTDs are used) of one SRM transition against the analyte concentration [ng/ml] of the standard solution.

For a first estimate of the residue level of pesticides in the food or to show their absence, the standard solutions (3.10.1 or 3.10.2) in pure methanol can be used. They can be also used for quantification if preliminary experiments indicate that any suppression or enhancement effects experienced do not significantly affect the

SIST EN 15637:2009

results obtained. As soon as relevant residue concentrations are detected (e.g. suspected MRL violations), a more precise determination using matrix-matched standards (3.10.3) or the standard addition method should be preferred.

NOTE 1 Matrix effects influence the response of target analytes in sample extracts compared to the response of standard solutions in pure solvent.

NOTE 2 The calibration range should be appropriate to the residue concentrations to be quantified. Thus, it can be necessary to construct more than one calibration graph from the results of calibration measurements.

When using ISTDs it is important to know that any shift in the ISTD signal will directly influence the calculated concentration of the analytes. Ideally, the ISTD signal should only shift due to volume differences and thus improve the accuracy of measurement. However, there are also other, non-desirable, factors that may also affect the signals of the ISTD thus introducing errors in the analyte quantification. Losses of the ISTD during clean-up will result in an overestimation of analyte concentration. Such losses should thus be minimal. A specific suppression of the ISTD signal, potentially occurring in LC-MS applications due to co-eluting matrix components, will also result in analyte overestimations. Matrix effects will depend on whether the commodity extract contains specific components that will co-elute with the ISTD and affect its ionisation process.

In any case it is always crucial to introduce quality control measures to ensure that any error introduced by the ISTD remains insignificant. Quality control measures may include the use of backup ISTDs and quality control standards that may be added at other stages of the analytical procedure (e.g. to the final extract) and that may help to identify any non-volume related shifts of the ISTD signal. Very helpful for quality control is the observation of the signal intensity of the ISTD in every sample within a sequence. Should a significant signal shift occur, quantification should be performed using a backup ISTD or without using ISTD. In the latter case exact liquid transfers and equalisation of the volumes of the standard solutions and the sample extracts are mandatory.

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6.2 Calculation of residue concentrations without standard addition

If standard addition method is not used, the residue level w_R of a pesticide in the food sample is calculated from the obtained peak area (or height) using Equation 2:

SIST EN 15637:2009
https://standards.iteh.ai/catalog/standards/sist/0cd93c63-424f-407d-a7e4-69541e821510/sist-en-15637-2009

$$w_R = \frac{A-c}{b} \times \frac{V_{ex}}{m_a} \times \frac{V_{end} \times V_2}{(V_2 - V_1) \times V_3} \times 1000 \left(\frac{\text{mg}}{\text{kg}} \right) \quad (2)$$

where:

A is the peak area, peak height or peak ratio for one SRM transition measured, in arbitrary units (a.u) or without dimension;

c is the intercept of the corresponding calibration graph, in a.u. or without dimension;

b is the slope of the corresponding calibration graph, in a.u. × ml/ng (without ISTD) or ml/ng (with ISTD);

V_{ex} is the total volume of extraction solvents and natural water (30 ml);

m_a is the initial sample weight, in grams;

V_1 is the volume of NaCl solution (2,5 ml);

V_2 is the volume of measuring flask used in 5.2 (10 ml);

V_3 is the volume used for solid supported liquid/liquid extraction (5 ml);

V_{end} is the final volume of extract obtained after clean-up (0,5 ml);

1000 is the conversion factor.