



Performance evaluation of the LC-MS/MS method for determination of chlorates in plant-origin foodstuffs

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ARTICLE INFO

Keywords:

Chlorate
LC-MS/MS
Validation
SANTE
Hypercarb™

ABSTRACT

Chlorate (ClO_3^-) is a monovalent inorganic anion obtained by deprotonation of chloric acid. Its widespread historic use (as an herbicide) and numerous present applications result in notable findings of chlorate residues in food commodities. The aim of this paper is to present validation data confirming the fitness for purpose of the modified “QuPpe” method for LC-MS/MS determination of chlorate in plant-origin foodstuffs with high water content. The validation study was conducted according to SANTE 11312/2021 in the form of a full initial validation on three matrices representative of the leafy vegetable group.

The results of the key validation parameters demonstrate the fitness for purpose of the proposed method. Recovery values (96.5–105.3%), repeatability (4.1–16.2%), intermediate precision (3.9–16.4%) and expanded measurement uncertainty (12.6–32.1%) are in compliance with the criteria laid down in the SANTE document. Use of a graphitized carbon column for the separation of chlorate introduces the necessary step of frequent preconditioning with planar compounds e.g., chlorophyll. However, this process does not result in significant delays or extended run times if carried out regularly.

1. Introduction

Chlorate (ClO_3^-) is a monovalent inorganic anion obtained by deprotonation of chloric acid. Due to its strong oxidizing properties, it was used as a herbicide for many decades, mainly in the form of its sodium salt, NaClO_3 (EFSA, 2015). In 2008, it was banned as a pesticide in the EU according to Commission Decision No 2008/865/EC (European Commission, 2008).

However, usage of this anion goes beyond the scope of plant protection products since chlorate is a common disinfectant of drinking water and

of water that is used for production and washing of plant-origin foodstuffs. Chlorate is also extensively used in other applications, e.g., production of chlorine-based biocides (active chlorine, chlorine dioxide, sodium hypochlorite, calcium hypochlorite) and the bleaching of paper. It should be noted that use of chlorate in herbicide formulations outside the EU is still relevant.

As a result of this cumulative process, findings of chlorate residues in food are common, and since the ban of plant protection products containing chlorate was carried out without setting a maximum

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Paper received September 9th 2025. Paper accepted September 15th 2025.

The paper was presented at the 63rd International Meat Industry Conference “Food for Thought: Innovations in Food and Nutrition” – Zlatibor, October 05th–08th 2025.

Published by Institute of Meat Hygiene and Technology – Belgrade, Serbia.

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residue level (MRL) in foodstuffs under Regulation (EC) No 396/2005 (European Commission, 2005), the default MRL of 0.01 mg/kg is applied. The World Health Organization (WHO) has established a guideline level of 0.7 mg/L for chlorate in drinking water (EFSA, 2015), and in the EU, a regulatory level of 0.25 mg/L in drinking water is set by Directive (EU) 2020/2184 (European Commission, 2020b). Having in mind the MRL of 0.01 mg/kg in food, the majority of tested foodstuffs would be deemed non-compliant with the legal limits. Positive samples were reported in significant numbers since 2014, according to the EFSA Scientific Opinion, and multiple studies were conducted in order to establish an MRL for chlorate residues in foodstuffs. Therefore, the EU has adopted Commission Regulation 2020/749 (European Commission, 2020a) amending Annex III to Regulation (EC) No 396/2005 (European Commission, 2005), which set the MRL for chlorate in or on some products.

Besides acute toxicity, potential chronic health risks associated with the presence of chlorate anion in food and drinking water are related to the thyroid gland and (in the case of chronic exposure) can cause bone marrow hyperplasia (EFSA, 2015). Chlorate anion is chemically similar to perchlorate anion, exhibiting thyrotoxic activity due to its strong oxidizing properties (EFSA, 2015, CVUAS, 2014). The presence of this substance in the body can also cause damage to red blood cells (methemoglobinemia and haemolysis) (Steffen and Wetzel, 1993).

Having in mind the ubiquitous and often inevitable presence of chlorates in the food chain, as well as their harmful effects, it is necessary to develop reliable and accurate analytical methods for their quantitative determination in various food commodities. Analytical methodology for measurement of chlorates is historically based on spectrophotometric/colorimetric techniques. Together with ion chromatography with conductometric detection, these methods are suitable for “clean” samples (e.g., water), while for complex matrices, such as plant-origin food, liquid chromatography coupled with mass spectrometric detection (LC-MS/MS) is a technique of choice for sensitive and accurate determination of chlorate anion (EFSA, 2015).

The European Reference Laboratory for Single Pesticide Residue Methods (EURL-SRM), have developed and proposed the modification of their “QuPpe” method (Quick method for the Analysis of Residues of Highly Polar Pesticides), extending it to analysis of (among other substances) chlorate

(Anastassiades *et al.*, 2021). For the less complex matrices, the method is based on extraction and dilution steps only (in acidic methanol-water mixture). Another specific issue in chromatographic analysis of chlorate is that low mass anions cannot be separated on traditional reverse-phase phenyl LC columns used for the majority of other pesticides. In the case of chlorate, the method recommends porous graphitized carbon columns or hydrophilic interaction liquid chromatography (HILIC).

The aim of this paper is to present the validation data confirming the fitness for purpose of the modified QuPpe method for LC-MS/MS determination of chlorate in plant-origin foodstuffs with high water content. This type of foodstuff was selected because chlorate findings are frequent in prepacked and pre-washed lettuces, endives and mixed salads, since water used for washing can be previously treated with hypochlorite. Validation was performed according to the Sante protocol “Analytical quality control and method validation procedures for pesticide residues analysis in food and feed” (SANTE 11312/2021, 2021).

2. Materials and methods

Standard solution (1000 mg/L) of chlorate was purchased from Sigma (St. Louis, USA). Water and methanol (HPLC grade) were purchased from Honeywell (Charlotte, USA). Formic acid ($\geq 98.0\%$) was supplied by Merck (Darmstadt, Germany). The LC-MS/MS system consisted of Shimadzu (Kyoto, Japan) components: two LC-40D xs UHPLC pumps connected in binary gradient mode, DGU-405 degassing unit, SIL-40C xs autosampler, CTO-40C column oven, SCL-40 system controller and LCMS 8045 triple-quadrupole mass spectrometer. A LC column Thermo Scientific (Waltham, USA) Hypercarb™, 100×2.1 mm, $5\mu\text{m}$ particle size was used for separation of chlorates.

The QuPpe method (Anastassiades *et al.*, 2021) provides detailed description of sample preparation. An aliquot of 10 g of a previously homogenized sample (lettuce, endive, mix salad) was extracted with 1% formic acid in methanol in 50 mL polypropylene centrifuge tube. After vigorous shaking, tubes were centrifuged for five minutes at 3500 g. This extract was filtered through $0.2\mu\text{m}$ PTFE syringe filter directly into the HPLC vial, and $5\mu\text{L}$ of extract was injected into LC-MS/MS system.

Mobile phases consisted of water containing 1% acetic acid and 5% methanol (Solvent A) and

1% acetic acid in methanol (Solvent B). Flow rate was 0.35 mL/min, and the gradient elution program was as follows: 0.0 min: 10 % A; 0.0–2.5 min, linear gradient up to 100 % A, hold for 3 min and back to initial conditions. Total run time was 8 minutes. The mass spectrometer was set to negative electrospray ionisation and operated in MRM mode. Transitions for chlorate were 83>67 and 85>69 with collision energies of -25 and -28 V respectively.

Validation was performed on three matrices (lettuce, endive and a mix of lettuce, rucola and baby spinach). According to the *SANTE 11312/2021* (2021) protocol, the following sample scheme was used per matrix: calibration standards (corresponding to chlorate concentrations of 0.025, 0.050, 0.5 and 1 mg/kg); reagent blank; blank sample; five spiked samples at 0.05 mg/kg; five spiked samples at 0.5 mg/kg; calibration standards. The chosen concentrations are below the MRL for chlorate in Commission Regulation (EU) 2020/749 (*European Commission*, 2020a) which is set to 0.7 mg/kg for the whole group (leaf vegetables, herbs and edible flowers).

3. Results and discussion

The results shown in Table 1 indicate that all key investigated parameters were in compliance with the requirements of *SANTE/11312/2021* (2021) (Recovery 70–120 %; $RSD_{rep} < 20\%$; $RSD_{ip} < 20\%$). The interpretation of the raw data showed that other parameters show satisfactory performance as well. The specificity of the method was proven by the absence of interferences at the retention times of interest in blank samples. The ion ratio was consistent and in all samples, was within the acceptable tolerances ($\pm 30\%$ (relative) of average of calibration standards from same sequence). The obtained results were in accordance with findings from other authors (*Huertas-Perez et al*, 2022, *Constantinou et al*, 2019).

Retention times were also consistently within specified times (± 0.1 min), providing suitable conditioning of the chromatographic columns had preceded the run. In general, porous graphitized carbon columns, such as Hypercarb™, have to be prepared (primed) in a specific way prior to analysis in order to enhance the chromatographic behaviour of the

Table 1. Validation data obtained for the investigated matrices according to the most significant criteria laid down in *SANTE 11312* (2021).

Matrix	Fortification level (g/kg)	Trueness (%)	Repeatability (RSD_{rep} %)	Intermediate precision (RSD_{ip} %)	Expanded uncertainty (%)
Lettuce	0.05	105.3	4.1	8.3	20.7
	0.5	110.0	16.2	14.9	32.1
Endive	0.05	100.6	11.6	13.8	28.6
	0.5	96.5	13.7	16.4	27.1
Mixed salad	0.05	101.6	6.1	3.9	12.6
	0.5	97.3	12.5	9.7	19.3

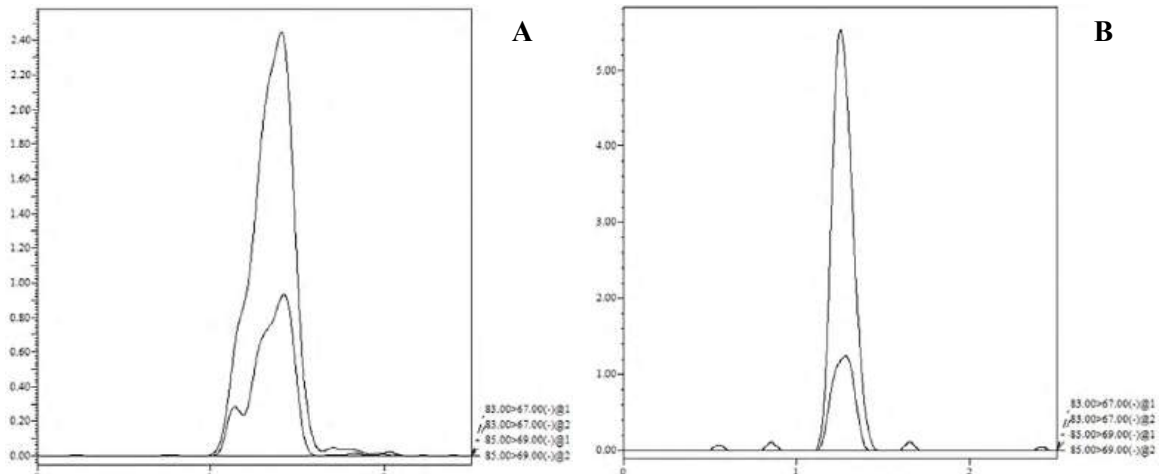


Figure 1. Peak shape before (A) and after (B) priming with 50 injections of spinach extract

column. The purpose of priming is to mask the active sites on the carbon with planar molecules, such as chlorophyll (*Anastassiades et al.*, 2017). This is accomplished by injecting 50–100 extracts of spinach blank (with no chlorate) upon receiving a new column, and least 5 injections before and after each test batch. Spinach extracts are prepared according to the QuPpe method. Without this step, Hypercarb™ columns will exhibit shifted retention times and uneven peak shapes. Figure 1 illustrates the chlorate peak before and after treatment of the column. This applies to all porous graphitized carbon columns.

Disclosure Statement: No potential conflict of interest was reported by the authors.

Funding: This study was supported by the Ministry of Science, Technological Development and Innovation, Republic of Serbia, Grant No. 451-03-136/2025-03/200050 from 04.02.2025.

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4. Conclusion

On the basis of the obtained results, it can be concluded that the QuPpe method provides an efficient means for the monitoring and control of chlorate levels in plant-origin leafy foods at the levels laid down in the relevant EU regulation. Additional steps are needed to ensure retention time consistency and peak shape when using graphitized carbon LC columns; however, this has no significant effect on overall analysis time if performed regularly.