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Food and beverage

Determination of chlorate in agar samples using ion chromatography (IC)

Authors

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Keywords

Dionex Aquion RFIC System, Dionex IonPac AS19 column, RFIC, suppressed conductivity

Goal

To develop an IC-based product quality control process for agar-containing products and evaluate the chlorate concentration in the final products

Introduction

The European Food Safety Authority (EFSA) reported chlorate as a risk to human health, with possible adverse effects on iodine metabolism in the case of chronic exposure. The formation of methemoglobin was identified as a critical acute effect of chlorate. Methemoglobin cannot bind oxygen and thus cannot carry oxygen to tissues. This can lead to methemoglobinemia, which can cause dizziness, headache, and cyanosis (a bluish discoloration of the skin and mucous membranes due to a lack of oxygen in the blood). When ingested, chlorate can irritate the digestive system and cause nausea, vomiting, diarrhea, and abdominal pain. In severe cases, ingestion of large amounts of chlorate can lead to the formation of ulcers in the mouth and throat, difficulty swallowing, and even death.

The source of the chlorate contamination can be water used for cleaning purposes (food and work surfaces) during the food processing and production process and water used for irrigation of plants during the growing season. Chlorate, a byproduct, is formed during water treatment with chlorine, chlorine dioxide, or hypochlorite.[1]

Algae are sustainable sources of proteins, minerals, and dietary fiber, boasting an impressive array of essential amino acids, pigments, and fatty acids. These natural constituents make algae an incredibly nutritious food source. The algae species consumed and commercialized in Europe are covered under the EU novel food legislation.[2,3]

In June 2020, the European Commission set chlorate limits for various foods, including algae. This limit is 0.05 mg/kg for algae and other foods. Food manufacturers must ensure that their products comply with EU food regulations; and therefore, must collect data and find and evaluate chlorate sources in their production processes. To lower chlorate levels and reduce exposure, EU member states have agreed on a multidisciplinary action plan that includes simultaneously executed measures. These include efforts in drinking water, drinking water hygiene, and provisional maximum residue limits (MRLs) for food and feed. The provisional MRLs will be reviewed regularly, considering developments in drinking water hygiene, drinking water, and progress made by the food industry in reducing chlorate levels.[4]

Agar is produced from red algae, e.g., *Gracilaria* and *Gelidium*, which are types of seaweed rich in agarose. It is a jelly-like substance consisting of cell wall polysaccharides. It is extracted by soaking the seaweed in hot water, and the extract is processed further to remove impurities. In its natural form, its main constituents are agarose, a linear polysaccharide made of repeating units of agarobiose and agaropectin. Agaropectin is a heterogeneous mixture of smaller molecules. It consists of alternating units of D- and L-galactose modified with acidic side groups, such as sulfate and pyruvate. The processing of food-grade agar removes the agaropectin, and the commercial product is pure agarose. Agarose consists of repeating units of D-galactose and 3,6-anhydro-L-galactose, linked by glycosidic bonds. Agarose has a linear structure with few branches, allowing it to form gels when dissolved in water. Industry applies agar as a laxative, as an appetite suppressant, a vegan substitute for gelatin, a thickener for soups, ice cream, and other desserts, in fruit preserves, as a clarifying agent in brewing, for sizing paper and fabrics, and in microbiological work as a solid substrate to contain culture media.[5]

The extraction process is laborious due to the complex composition of the natural product and the presence of impurities. The seaweed extract is washed and bleached with chlorine-containing additives. Consequently, the final product needs to be controlled for the presence and concentration of chlorate. The commonly recommended method to determine chlorate in food of plant origin is based on a multi-residue method for polar pesticides. The extracted and prepared liquid sample is analyzed by liquid chromatography-tandem mass spectrometry (HPLC/MS-MS) or IC-MS/MS.[6,7]

LC-MS/MS or IC-MS/MS are the preferred analytical methods for chlorate, other disinfection byproducts, and anionic pesticide determinations. However, in product development or the methodical verification of the food production process, different aspects play a role in selecting the analytical tools. These include operation simplicity, purchase, operating costs, and the footprint of the analytical instrument. We selected a Reagent-Free IC (RFIC™) instrument with suppressed conductivity detection. Other criteria were: Electrolytic eluent generation and AutoSuppression, hydroxide gradient elution, high detection sensitivity, and selectivity, and the upgrade option to IC-MS/MS.

This work describes a straightforward method to determine chlorate in seaweed-derived agar products. A sample preparation adapted to the matrix circumvents unwanted gelation, thus avoiding clogging capillaries and columns. Our approach is suitable for analyzing chlorate in process water or raw materials for products where the final agar content does not exceed 0.5–1.0%. We simplified and automated the computation and reporting of the analytical figures of merit with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) templates of the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS). Calculation and report generation within the CDS eliminates data transfer to external spreadsheet programs and transfer errors.

Experimental

Equipment*

- Thermo Scientific™ Dionex™ Aquion™ RFIC system with Chromeleon CDS software, version SE, with Degas (P/N 22176-60128)
- Thermo Scientific™ Dionex™ Aquion™ Column Heater (P/N 070063)

Consumables

- Thermo Scientific™ Dionex™ EGC III KOH Eluent Generator Cartridge (P/N 074532)
- Thermo Scientific™ Dionex™ CR-ATC Continuously Regenerated Anion Trap Column (P/N 060477)
- Thermo Scientific™ Dionex™ ADRS 600 Anion Dynamically Regenerated Suppressor (4 mm, P/N 088666CMD)

Software

Thermo Scientific™ Chromeleon™ Chromatography Data System software version 7.2

*This application can be run on any Thermo Scientific™ Dionex™ IC system equipped with Thermo Scientific™ Dionex™ RFIC™ technology. For higher sample throughput, an autosampler can be added.

Reagents and standards

- Deionized (DI) water, resistivity >18 MΩ·cm at 25 °C, TOC <10 µg/L (for minimum requirements, see ASTM type I standard specifications for reagent water)[8]
- Thermo Scientific™ Dionex™ Chlorate Standard, 1,000 mg/L, 125 mL (P/N 303170)
- Thermo Scientific™ Dionex™ Nine Anion Standard (P/N 303173)
- Sodium hydroxide solution (50% w/w/Certified) (Fisher Chemical™; P/N SS254-500)
- Cellulose nitrate filter 25 mm, 5.0 µm pore size (Sartorius; P/N 11342-25-N)
- Disposable syringe filter CHROMAFIL™ Xtra RC, 25 mm, 0.2 µm (Macherey-Nagel; P/N 729230)

Diluent and standards

Diluent

A dilute sodium hydroxide solution (*w*(NaOH) = 0.02) was prepared by adding 0.4 g of the concentrated stock solution (*w*(NaOH) = 50) to 999.6 g DI water.

Standards

Calibration standards were prepared by diluting the 1,000 mg/L chlorate stock solution with DI water to the final concentrations of 0.2, 0.5, 1, 5, 10, and 20 mg/L.

Samples

The agar samples analyzed in this study were of different origins, such as dried red algae and product mixtures.

Sample preparation

Agar is a neutral polysaccharide with broad molecular weight distribution and gelation properties. High molecular weight samples (>150.000–200.000 g/mol), such as those from drying red algae, must be depolymerized with NaOH before the analytical determination.[9] In contrast, products with low gel strength (or low molar mass <100.000 g/mol) can be dissolved directly in water.

For the general procedure, 1 g of the sample was weighed into a 50 mL beaker; 15 mL of DI water and 5 mL of the diluent were added. The solution was stirred at 35 °C for about 30 min. The solution was filtered (first 5.0 µm, then 0.2 µm), collecting at least 2–3 mL of sample. In the case of samples, which clog the 0.2 µm filter, an additional 0.45 µm filter can be used before the 0.2 µm filter. Depending on the expected chlorate concentration, the weighed amount of product was adjusted to 0.5 to 2 g.

Method

Parameter	Value
Column	Thermo Scientific™ Dionex™ IonPac™ AG19 Guard, 4 × 50 mm (P/N 062887) Thermo Scientific™ Dionex™ IonPac™ AS19 Analytical, 4 × 250 mm (P/N 062885)
Eluent	Dionex EGC III KOH Eluent Generator Cartridge with Dionex CR-ATC
Gradient	Time (min) KOH (mM) 0.0 10.0 10.0 10.0 40.0 62.0 40.1 10.0 45.0 10.0 45.0 Stop run
Flow rate	1 mL/min
Injection volume	25 µL
Temperature	30 °C
System backpressure	<2,200 psi (13.8 MPa)
Detection	Suppressed conductivity, Dionex ADRS 600 Suppressor 4 mm, AutoSuppression, regeneration current 154 mA
Background conductivity	<0.7 µS/cm
Cycle time	45 min

Results and discussion

Method and calibration

A Thermo Scientific Dionex IonPac AS19 column is a high-capacity column explicitly designed for use with hydroxide eluents and developed with an optimized selectivity for determining trace bromate, chlorite, and chlorate in complex matrices.[10] These properties recommend the Dionex IonPac AS19 column for determining chlorate in process water and agar samples. Figure 1 shows a representative separation of common anions and oxyhalides separated on this column, applying a hydroxide eluent gradient.

Column:	Dionex IonPac AS19, 4 × 250 mm		
Eluent:	10 mM KOH from 0 to10 min,		
	10 to 45 mM from 10 to 25 min		
Eluent source:	Dionex EGC-KOH cartridge with Dionex CR-ATC		
Flow rate:	1.0 mL/min		
Temperature:	30 °C		
Suppressor:	Dionex ADRS 600 4 mm		
Injection vol.:	25 µL		
Peaks:	1.	Fluoride	1mg/L (ppm)
	2.	Chlorite	10
	3.	Bromate	20
	4.	Chloride	6
	5.	Nitrite	15
	6.	Chlorate	25
	7.	Bromide	25
	8.	Nitrate	25
	9.	Carbonate	–
	10.	Sulfate	25
	11.	Phosphate	30

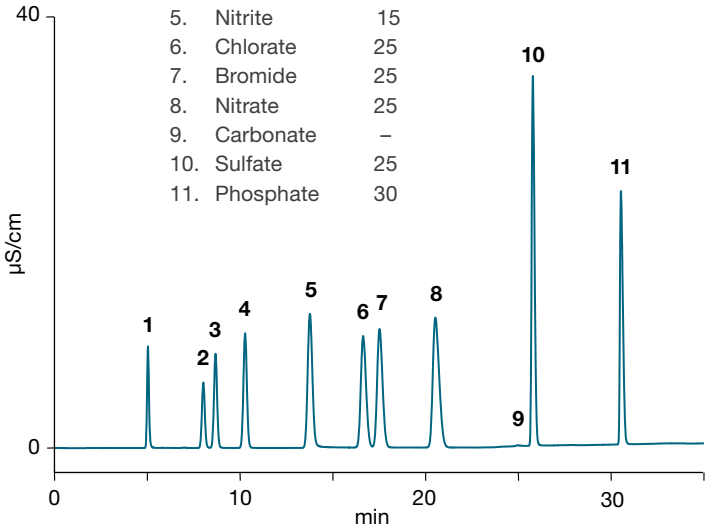


Figure 1. Separation of common anions and oxyhalides on a Dionex IonPac AS19 column

An electrolytically generated potassium hydroxide eluent eliminates the need to prepare eluents manually, thus simplifying the instrument operation, improving the method's precision and transferability, and saving the operator's time. Additional advantages are a wider linear working range for anions of strong acids such as chlorate, lower background conductivity, and improved sensitivity compared to conventional IC applications that use carbonate eluents. In this work, the KOH eluent was electrolytically generated by a Dionex EGC III cartridge and converted to water after the separation using a Dionex ADRS 600 dynamically regenerated suppressor before conductivity detection. To determine chlorate in an agar matrix, the chromatographic conditions were adapted (see Method), accommodating the complex composition of some sample types (vide infra).

The total run time was 45 min, with chlorate eluting at about 15 min as a highly efficient chromatographic peak (Figure 2).

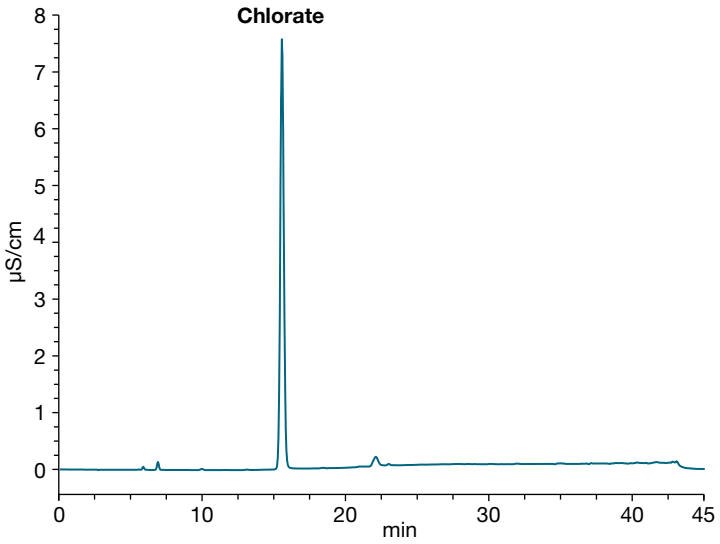


Figure 2. Gradient separation of chlorate using anion-exchange chromatography on a Dionex IonPac AS19 column. Standard concentration: 20 mg/L. Chromatographic conditions: see Method.

The calibration curve was constructed with six concentration levels ranging from 0.2 to 20 mg/L. The results yielded a linear relationship of peak area to concentration with a coefficient of determination (r^2) of >0.9999 (Figure 3 and Table 1).

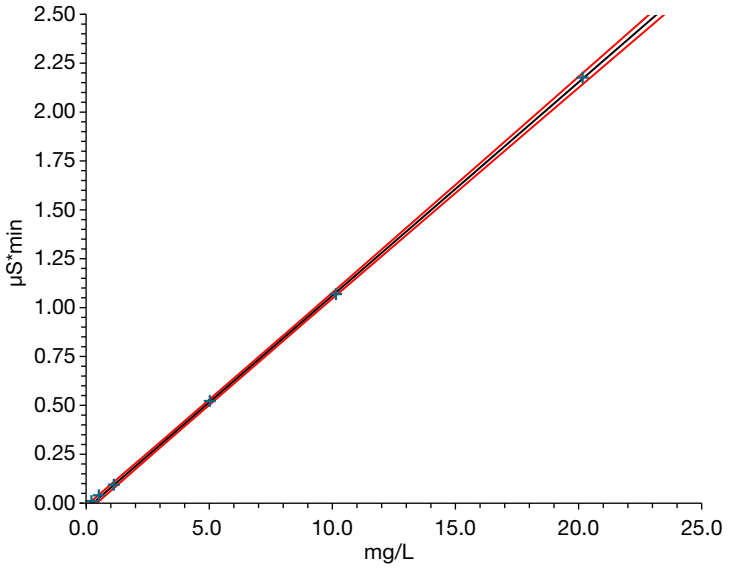


Figure 3. Calibration plot for chlorate. Calibrated range: 0.2 to 20 mg/L, evaluation: linear with offset, confidence intervals (red traces; upper and lower probability): 99.5%.

Table 1. Characteristic calibration data obtained for chlorate

Peak name	Ret. time (min)	Calibration type	Numbers of points	Coeff. of determination (r^2)	Rel. std. dev.	Offset	Slope
Chlorate	15.5	Linear with offset	6	>0.9999	1.05	0.0085	0.11

Determination of limit of detection (LOD) and limit of quantification (LOQ)

We used the ICH-provided recommendations for validating analytical procedures. Advised tests include method accuracy, detection limit, linearity, precision, quantitation limit, and robustness.[11] Chromeleon 7 CDS contains templates that allow quick and easy validation of an analytical method, for example, the evaluation of LOD and LOQ based on the calibration curve. All required items to perform the calculation are combined in an eWorkflow™: sequence templates, processing methods, report templates, and custom variables. These items become accessible following the installation instructions found in the folder "Extension Pack\ICH Templates" on your Chromeleon CDS installation CD-ROM. Open the PDF file "ICH LOD LOQ (Curve) – User Manual" and follow the described process. Calculating and reporting all data requested by ICH can be tedious and time-consuming. The ICH method validation templates provided in the Chromeleon 7 CDS Extension Pack automate these steps and simplify the creation of method validation reports.

For the calculation of LOD and LOQ, the following formulas were used in the automatic validation report.

$$LOD = \frac{3.3 \times \sigma}{S} \qquad LOQ = \frac{10 \times \sigma}{S}$$

Where σ is the standard deviation of the calibration curve's y-intercepts, and *S* is its slope.

We used five calibration solutions near the expected LOD (chlorate) in the 0.05 to 0.3 mg/L range. The estimated LOD was 0.025 mg/L, and LOQ was 0.077 mg/L. All calculations and the report generation were done in Chromeleon CDS. No data export and import to external spreadsheet calculation programs were necessary, preventing data handling errors and ensuring data integrity and compliance.

The values provided in Figure 4 need to be recalculated based on sample preparation. For example, a typical dilution is 1 to 20 (or 1 gram of sample dissolved in 20 mL of solvent). The values of LOD and LOQ should then be multiplied by a factor of 20, thus providing the following estimated values:

LOD = 0.51 mg/kg LOQ = 1.53 mg/kg

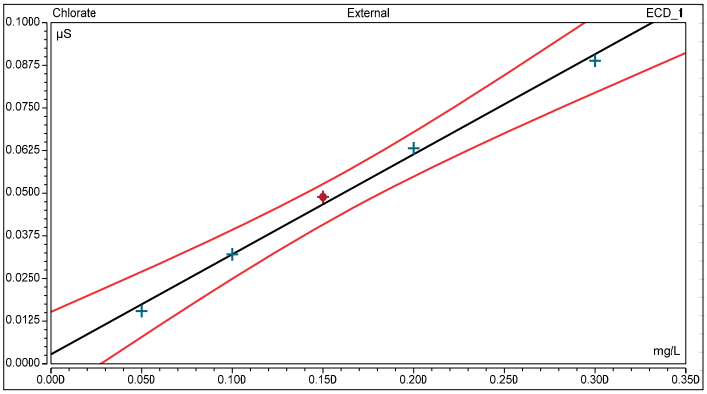
LOD/LOQ (Based on Calibration Curve)

Component Details

Component Name	Chlorate
Calibration Type	Lin, WithOffset
Evaluation Type	Height
Y-Intercept	0.0028
Slope	0.2933
Regression SD	Line
Standard Deviation of Line	0.0023
Standard Deviation of Intercept	0.0021

Calibration Details

No.	Name	X Value	Y Value
		mg/L	Height
		ECD_1	ECD_1
		Chlorate	Chlorate
1	Standard 1	0.0500	0.0154
2	Standard 2	0.1000	0.0321
3	Standard 3	0.1500	0.0489
4	Standard 4	0.2000	0.0632
5	Standard 5	0.3000	0.0888



LOD/LOQ Results

Units	mg/L
LOD	2.538E-02
LOQ	7.691E-02

Figure 4. Automated estimation of LOD and LOQ with original data in Chromeleon CDS

Sample measurement

Following are the chromatograms of agar samples of different molecular weight distributions.

Figure 5 displays a representative example of a high molecular weight sample (HMW) agar bleached with hypochlorite. This agar preparation creates an intricate sample matrix. After the initial peak, several analytes, such as chloride, sulfate, and phosphate, are detected. A large citrate peak that elutes from the column in under 30 min dominates the chromatogram. Chlorate was found in the solid agar sample at 140 mg/kg. A chlorate value of between 100 and 600 mg/kg is anticipated for agar samples bleached in this manner. Due to the Dionex IonPac AS19 column's high ion exchange capacity and exceptional chromatographic performance using straightforward RFIC gradient settings, these complex samples were simple to separate and analyze.

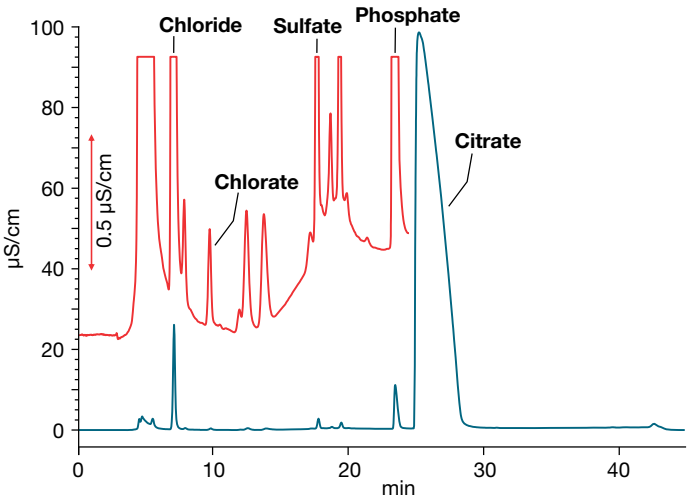


Figure 5. Chromatographic analysis of a hypochlorite bleached HMW agar sample. Chromatographic conditions: see Method. Sample preparation was as described above. The chlorate amount was determined to be 140 mg/kg. The red trace is an enlargement of the blue trace.

The exemplary chromatographic analysis of a low molecular weight (LMW) agar sample is displayed in Figure 6. The substantially reduced phosphate and citrate signals and the more clearly separated monovalent acids demonstrate that the sample composition is simplified compared to the HMW samples. The chromatogram was magnified between 9 and 17 min to show the interference-free chlorate separation. It is significant in this context because this sample underwent a novel bleaching procedure. This procedure will eventually replace hypochlorite bleaching, which should be viewed in light of the initiatives to lower the chlorate content mentioned in the introduction. With an intra-assay repeatability of 0.6% for six replicates, the measured concentration of chlorate in the LWM agar sample was 40 mg/kg.

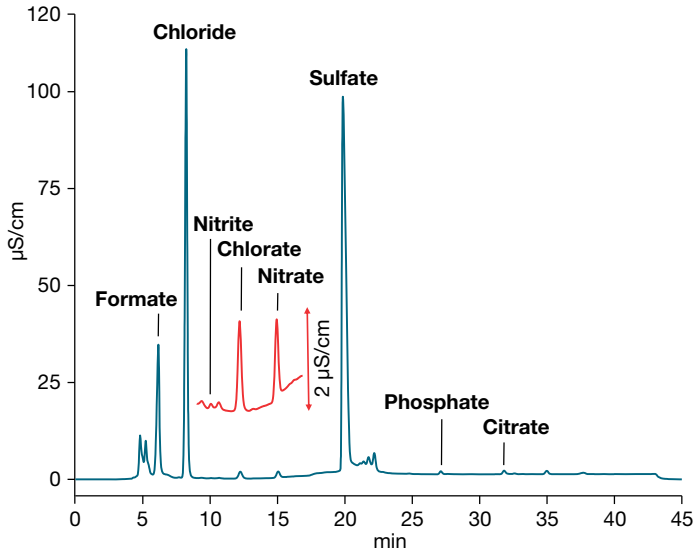


Figure 6. Chromatographic analysis of a LMW agar sample. Chromatographic conditions: see Method. Sample preparation was as described above. The chlorate amount was determined to be 40 mg/kg. The red trace is an enlargement of the blue trace.

A contracted third-party laboratory used the published EURL-SRM QuPPe method[6] to analyze the chlorate content of several agar samples. Analyte determination was carried out using LC-MS/MS. Table 2 shows that the results align with those from the easier-to-use RFIC approach described here.

Table 2. Comparison of the approach described here with the recommended LC-MS/MS practice for two HMW and two LMW agar samples each[6]

	RFIC with suppressed conductivity (mg/kg)	LC-MS/MS (mg/kg)
HMW Sample 1	493	481
HMW Sample 2	454	492
LMW Sample 3	2.6	<LOQ
LMW Sample 4	55.1	57

Conclusion

This study outlines a novel, straightforward technique for determining chlorate in agar samples. The analytical procedure comprises a simple sample pretreatment to prevent unwanted gelation of the agar sample in the IC and a streamlined chromatographic approach on a Dionex IonPac AS19 column. The KOH gradient and the regenerant of the continuously regenerated ADRS membrane suppressor were electrolytically produced. The analyst has just to supply DI water for the Dionex RFIC system to work. The chromatograms were evaluated automatically by Chromeleon CDS, also used to determine the analytical figures of merit. LOD and LOQ were in the low mg/kg for the agar samples.

The high degree of automation provided by the ICH templates integrated into the CDS further improves the workflow and analytical method validation. Application and data transfer errors are minimized. The final analytical procedure is sensitive, selective, optimized, and efficient, meeting the expectations of modern food development laboratories.

References

1. EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain). Scientific Opinion on risks for public health related to the presence of chlorate in food. *EFSA journal* **2015**, *13*(6), 4135.
2. European Commission. REGULATION (EU) 2015/2283 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 November 2015. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32015R2283 (accessed Feb 05, 2023).
3. Mendes, M. C.; Navalho, S.; Ferreira, A.; Paulino, C.; Figueiredo, D.; Silva, D.; Gao, F.; Gama, F.; Bombo, G.; Jacinto, R.; Aveiro, S. S.; Schulze, P. S. C.; Gonçalves, A. T.; Pereira, H.; Gouveia, L.; Patarra, R. F.; Abreu, M. H.; Silva, J. L.; Navalho, J.; Varela, J. C. S.; Speranza, L. G. Algae as Food in Europe: An Overview of Species Diversity and Their Application. *Foods*, June 24, **2022**, 1871–1906.
4. European Commission. Commission Regulation (EU) 2020/749, amending Annex III to Regulation (EC) No 396/2005. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020R0749&rid=1 (accessed Oct 15, 2022).
5. Wikipedia, the free encyclopedia. Agar. https://en.wikipedia.org/wiki/Agar (accessed Dec 19, 2022).
6. Anastassiades, A.; Kolberg, D. I.; Eichhorn, E.; Benkenstein, A.; Wachtler, A.-K.; Zechmann, S.; Mack, D.; Wildgrube, C.; Barth, A.; Sigalov, I.; Görlich, S.; Dörk, D.; Cerchia, G. Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement (QuPPe-Method); Version 12, 2021. EurlSrm_meth_QuPPe_PO_V12.pdf. https://www.eurl-pesticides.eu/userfiles/file/EurlSRM/EurlSrm_meth_QuPPe_PO_V12.pdf (accessed Oct 15, 2022).
7. Huang, B.; Rohrer, J. AppsLab Library, Thermo Fisher Scientific, 2021. Application Note 73981 Determination of chlorate and perchlorate in milk. https://appslab.thermofisher.com/App/4542/determination-chlorate-perchlorate-milk (accessed Oct 15, 2022).
8. ASTM International. Standard Specification for Reagent Water, 2017. ASTM D1193-99e1. https://www.astm.org/d1193-99e01.html (accessed Dec 18, 2022).
9. Aspinall, G. O.; Krishnamurthy, T. N.; Furda, I.; Khan, R. Base-catalyzed Degradations of Carbohydrates. VII. Alkaline Degradation of 3,6-Di-O-substituted Hexoses. *CAN. J. CHEM.* **1975**, *53*, 2171–2177.
10. DeBorba, B.; Rohrer, J. AppsLab Library, Thermo Fisher Scientific. Application Note 167: Determination of Trace Concentrations of Oxyhalides and Bromide in Municipal and Bottled Waters Using a Hydroxide-Selective Column. https://appslab.thermofisher.com/App/1389/determination-bromate-drinking-water (accessed Oct 15, 2022).
11. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Q 2 (R1) Validation of Analytical Procedures: Text and Methodology, 2006. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf (accessed Dec 17, 2022).

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