EURL-SRM - Analytical Observations Report

Concerning the following…

- **Compound(s)**: Ethylene oxide (EO), 2-Chloroethanol (2CE)
- **Commodities**: Sesame seeds
- **Extraction Method(s)**: QuOil, QuECHERS
- **Instrumental analysis**: GC-MS/MS

Analysis of Ethylene Oxide and its Metabolite 2-Chloroethanol by the QuOil or the QuEChERS Method and GC-MS/MS

Version 1.1 (December 2020)

Background information / Initial Observations:

In late August 2020, Belgium initiated a RASFF notification concerning residues of the unauthorized substance ethylene oxide (EO) in various lots of sesame seeds from India at levels up to 186 mg/kg. On 9 September 2020, a notification this concerning was published in the RASFF portal (2020.3678). The affected products were delivered to several member states and were used for the production of various processed foodstuffs. Until 20 November 2020, roughly 140 notifications concerning EO in sesame from India were notified within the RASFF portal with two of them being border rejections. These notifications originated from 17 different EU-Member states and 2 EFTA countries[1]. The EO-levels encountered in the sesame samples mostly ranged between 0.1 and 10 mg/kg, all exceeding the EU-MRL (maximum residue limit) of 0.05 mg/kg.

For how long EO-fumigation has been in use or increasingly applied to sesame seeds in India will need to be investigated. In a review paper from 2004 concerning fumigation of oily seeds with focus on India[2], methyl bromide and phosphine are reported as the main fumigants used in India for oilseeds. The alternatives discussed in this review do not include EO. Given the strong antibacterial properties of EO (reportedly 10-fold more effective than methyl bromide[3]), it is conceivable that EO-fumigations may have been initiated in India as a counter-measure for reducing the incidences of sesame seed contaminations with salmonella and other fecal bacteria. These contaminations have led to numerous border rejections of sesame seeds from India by EU Member States in the past two decades. Looking at the RASFF portal,[4] the first three notifications on salmonella in sesame seeds were launched by

[1] Austria, Belgium, Croatia, Czech Republic, Finland, France, Italy, Germany, Latvia, Luxemburg, Poland, Slovenia, Spain, Sweden, The Netherlands, Norway Switzerland.
[2] Somiahnadar Rajendran, Chayadevi HS; Oilseeds -Storage and Insect Pest Control, JFST (2004); 41(4):359-367
[3] L.T. Richardson and H. A. U. Monro; Fumigation of jute bags with ethylene oxide and methyl bromide to eradicate potato ring rot bacteria. Appl. Microbiol.l0: 448451 (1962)
[4] https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1

Germany in 2001. The first border rejections were reported by Greece in 2007. By 20 November 2020 most RASFF-notifications concerning salmonella and other bacteria in sesame seeds were notified by Greece (60) followed by Italy and Poland (45 each), Germany (34) and The Netherlands (23).
On 1 October 2014, a modification of Regulation (EC) No 669/2009 came into force requiring an increased level of import controls on sesame seeds from India with 20% of lots being required to be checked for salmonella. Thereafter, the number of notifications peaked but started falling again from 2016 onwards. In an FVO-inspection report from mid-2015, concerning a visit to India in 2014[5], the auditors did not report about EO-fumigations, but reported that, according to the local authorities, fumigations with phosphine (alumina phosphide) were taking place, where pests were observed. In February 2017, a new EU-Regulation was implemented[6] requiring that a health certificate, as well as a laboratory report verifying the absence of Salmonella spp. shall accompany each consignment of sesame seeds. In 2018, a follow-up FVO-mission to India was undertaken, but fumigations are not mentioned in the respective mission report[7].
Problems with salmonella in sesame are not just limited to India. Over the years, salmonella was also encountered in sesame seeds from various African countries. This resulted in the implementation of EU regulations requiring increased import controls of sesame originating from Uganda (Dec. 2016), Nigeria and Sudan (Jun. 2017) and Ethiopia (Jan. 2019). The frequency of controls was initially set at 50% and was subsequently dropped to 20% in the case of Sudan and Uganda (May 2020 onwards).
In 2016-17, a salmonellosis outbreak with a novel salmonella subspecies was reported by five EU countries (Greece, Germany, Czech Republic, Luxembourg and the United Kingdom) that could be traced back to the consumption of contaminated sesame from Nigeria and Sudan[8].

Around 70% of the world's sesame production comes from Asia (mainly India, China and Myanmar) and 26% from Africa (mainly Sierra Leone, Sudan, Nigeria and Uganda)[9]. Europe's own sesame production is limited to less than one thousand tons, mainly from Greece and Italy. In 2019, the sesame import into the European Union was 132 thousand tones[10]. More than half of the import volume (ca. 70,000 tons annually) originate from India. Thereof 80-90% are hulled. It is estimated that ca. 1.5 Mio people are involved in sesame production within India. India also imports large quantities of sesame seeds from Africa, which are processed and partly re-exported. According to the Indian plant quarantine (Regulation of Import into India)[11], sesame imported to India from African countries such as Somalia, Sudan, Senegal as well as from Pakistan, Bangladesh and Mexico needs to undergo fumigation with methyl bromide or an equivalent treatment prior to entering India.

[5] DG(SANTE) 2014-7170 – MR: Final report of an audit carried out in India from 09 December 2014 to 17 December 2014 in order to assess the control systems in place to control microbiological contamination in seeds for human consumption intended for export to the European Union
[6] Commission Implementing Regulation (EU) 2017/186, amending Regulation (EC) No 669/2009
[7] Final report of an audit carried out in India from 23 October 2017 to 27 October 2017 in order to evaluate the control systems in place to control microbiological contamination in seeds for human consumption intended for export to the European Union
[8] A. Meinen et al.; Salmonellosis outbreak with novel Salmonella enterica subspecies enterica serotype (11:z41:e,n,z15) attributable to sesame products in five European countries, 2016 to 2017, Euro Surveill. 2019 Sep 5; 24(36): 1800543. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6737830/
[9] H.A. Abou-Gharbia, A.A.Y. Shehata and F. Shahidi; Effect of processing on oxidative stability and lipid classes of sesame oil; Food Res. Int., 33: 331-340 (2000).
[10] https://www.cbi.eu/market-information/grains-pulses-oilseeds/sesame-seeds/market-potential
[11] https://plantquarantineindia.nic.in/PQISPub/pdffiles/pqorder2015.pdf

Ethylene oxide uses beyond fumigation:

EO is one of the most widely produced chemicals worldwide. It is mainly used as a chemical intermediate in the manufacture of numerous important chemicals such as mono-, di-, tri- and polyethylene glycols as well as various ethanol-amines and glycol-ethers. These chemicals are either used directly[12], or as intermediates for the fabrication of other important products, such as polymers (e.g. PET) and surfactants (e.g. ethanol-amines and ethanol-amides). Other important products derived from EO are modified polysaccharides (e.g. hydroxyethyl cellulose[13]) and TCEP[14].

Ethylene oxide uses as a fumigant:

Only 0.05% of the global EO production is used for fumigation purposes including the sterilization of medical equipment[15] and the control of insects and microorganisms (fungi and bacteria) in dry food products, such as herbs, spices, nuts and oily seeds. The use of EO for controlling diseases in honeycombs has also been reported[16]. Empty storage facilities, wood, wool and furs are also fumigated with EO. In the EU, the use of EO for the disinfection of foodstuffs, e.g. in storage areas, is not permitted (ECHA, 2020).

EO inactivates bacterial spores and viruses by reacting with DNA and proteins such as enzymes. Due to its small size, EO shows a high diffusivity and strong penetrating properties and is thus very effective in the disinfestation or disinfection of dry food commodities. Owing to its high flammability, EO is usually mixed 1:9 with carbon dioxide. For sterilization, higher EO concentrations and prolonged treatment times are required compared to conditions for insect control. In an FAO manual from 2013[17], the recommended doses for EO-fumigation are as follows:

- Nuts / nuts with shell (560 / 640 g/m³ for min. 3 h at 20°C);
- Dates and raisins (640 g/m³ for min. 3 h at 20°C);
- Spices (640 g/m³ for min. 3 h at 20°C);
- Milk powder (720 g/m³ for min. 3 h at 20°C);
- Milled cereals and feed (800 g/m³ for min. 6 h at 25°C).

No recommendation is given for oily seeds.

Residues following fumigation:

Evaporation and reactions with matrix constituents are the main dissipation pathways of EO in food. Following EO-fumigation, the EO levels in fumigated goods decrease rapidly if products are aerated. Typically, EO levels fall below 1 ppm within 2 weeks following fumigation[18]. With no aeration, or if the samples are sealed, the EO levels decrease more slowly. The removal of EO from food products is sometimes assisted by applying vacuum or higher temperatures. The latter sometimes negatively affects the sensory properties of the food products.

[12] e.g. ethylene glycol is used as an antifreeze agent and various glycol-ethers are used as additives to cosmetics and water-based paints
[13] Hydroxyethyl cellulose is used in cosmetics, drug capsules, toys and construction materials
[14] TCEP stands for tris-(2-chlorethyl)-phosphate. It is used as a plasticizer (e.g. in PVC and polyurethanes) and as a flame retardant in various plastics and fabrics, It is a chemical of high toxicological concern as regards indoor air contamination (https://www.umweltbundesamt.de/sites/default/files/medien/pdfs/Tris-chlorethylphosphat.pdf)
[15] E.g. catheters, blood conserve bags, dialysis membranes, surgical implants; endoscopes, dentist equipment
[16] G W Bruns, R A Currie; Determination of 2-chloroethanol in honey, beeswax, and pollen; J Assoc. Off. Anal. Chem. 1983 May;66(3):659-62
[17] http://www.fao.org/3/x5042e/x5042E00.htm
[18] http://www.inchem.org/documents/jmpr/jmpmono/v071pr15.htm

Once in contact with the food EO undergoes various reactions within the matrix. Reaction products include ethylene glycol, 2-chloroethanol (2-CE)[19] and 2-bromoethanol[20]. The latter two are formed in presence of chloride and bromide ions, respectively. Diethylene glycol and dioxane are also formed to some extent. Where fumigation with methyl bromide preceded the fumigation with EO, leaving high residues of bromide, the levels of 2-bromoethanol formation can be very high[21]. EO furthermore directly reacts with matrix components, such as amino acids, purines and fatty acids forming hydroxyethyl adducts. 2-CE also undergoes reactions with fatty acids forming 2-CE esters. 2-CE and the various reaction products of EO and 2-CE are only removed at a limited extend during aeration and many of them can serve as markers for EO-fumigations.

Legal situation in the EU

The use of EO for food fumigation has been phased out in many countries worldwide, due to toxicological concerns. In Germany, fumigation of food with EO was prohibited already in 1981 due to concerns as regards the toxicological properties of the residues remaining in food. In 1985, China restricted the use of EO only to empty warehouses. In 1986, the EEC prohibited marketing and use of EO as plant protection product with certain small-scale temporary exemptions still applying at national level[22]. In 1991, the EEC fully prohibited all plant protection products containing EO as active ingredient due to health concerns as it was classified as a Category 2 carcinogen and mutagen[23].

In 1994, EO was included in a list of high priority compounds for which EC-harmonized MRLs were needed[24]. Thereafter, separate MRLs were proposed for EO and its primary metabolite 2-CE at 0.05* and 0.01* mg/kg, respectively. These MRL-levels were applying to all commodities. In 2008,[25] it was decided to introduce a joint residue definition for the two components: "*Sum of ethylene oxide and 2-chloroethanol expressed as ethylene oxide*". This residue definition is still valid today The MRLs for EO (sum) were set at 0.2* mg/kg for oilseeds, teas, cocoa and spices; at 0.1* mg/kg for fruits, vegetables, sugar plants, fungi and oil fruits; and at 0.02* mg/kg for cereals and food of animal origin. In 2015[26] the EU-MRLs for teas, cocoa and spices were lowered to 0.1* mg/kg; the MRLs for nuts, oil fruits and oilseeds were lowered to 0.05* mg/kg and for fruits, vegetables, sugar plants, fungi and pulses they were lowered to 0.02* mg/kg. The MRLs for cereals and products of animal origin were kept at 0.02* mg/kg. For apicultural products, the MRLs were newly set at 0.05* mg/kg. These MRL-values are still valid today.

(*=MRL set at LOQ)

Legal situation elsewhere

Internationally, many countries (e.g. Australia[27], Japan[28], Korea, and Thailand) do not have explicit MRLs for EO implemented. In **Canada**, EO has long been classified as food additive for whole or ground spices. The permitted concentration of EO during fumigation was set at 500 mg/L and the residues of 2-CE were limited at 1500 mg/kg. In 2017, it was decided to change the status of EO from

[19] Also known as ethylene chlorohydrin (ECH)
[20] Also known as ethylene bromohydrin (EBH)
[21] http://www.inchem.org/documents/jmpr/jmpmono/v071pr15.htm
[22] Directive 86/355/EEC
[23] Currently classified as Category 1B (Reg. 1223/2009/EC)
[24] http://aei.pitt.edu/46684/1/COM_(94)_482_final.pdf
[25] Regulation 149/2008/EC
[26] Regulation 868/2015/EU
[27] https://apvma.gov.au/sites/default/files/gazette_17112020.pdf
[28] https://db.ffcr.or.jp/front/

additive to pesticide[29]. Nowadays, **USA and Canada** have common MRLs for EO and 2-CE in spices, dried herbs, dried vegetables and oily seeds (including sesame seeds) at 7 and **940** ppm, respectively[30]. In USA EO residues in walnuts are additionally regulated with a tolerance at 50 ppm[31]. In **Australia and New Zealand** the Food Standards Australia New Zealand (FSANZ), following an industry request to extend the MRLs of 20 mg/kg applying for EO till 2001, thoroughly evaluated the EO residues checking toxicological and microbiological risks as well as the actual residue situation in treated products. Finally, it was decided to phase-out EO-treatments, and only to grant a limited extension for EO-treatments to give industry time to develop alternative techniques for removing human pathogens from herbs and spices[32]. This temporary provision only applied to Australia and treated products were only allowed to be sold or imported 21 days after treatment with EO.

Other legal aspects

Apart from food products, EU-limits for EO also exist for certain **food additives** involving the use of EO in their production. Regulation 231/2012/EU, laying down the specifications of food additives, sets a maximum limit of 0.2 mg/kg for polyoxyethylene stearate (E 431); a number of polyoxyethylene sorbitan fatty acids (E 432, E 433, E 434, E 435, E 436); polyvinyl alcohol PEG copolymer (E 1209); and PEG (E 1521). For the byproduct dioxane, the respective limit in E 431-436 was set at 5 mg/kg and for E 1209 and E 1521 at 10 mg/kg. This regulation additionally states that EO may not be used for sterilizing food additives.

In the non-food area, EO is included in a list of prohibited substances within the **cosmetics** regulation[33]. In the Plastics Food Contact Regulation[34], EO is listed in Annex I (Authorized substances) with restrictions as regards the maximum amount allowed to be contained in the plastic (1 mg/kg) and as regards the Specific Migration Limit (SML) which was set at 0.01 mg/kg.

On the ECHA website[35], EO is categorized as a **biocide** falling under product type category PT2 ("Disinfectants and algaecides not intended for direct application to humans or animals")[36]. The initial application for approval is still in progress.

Toxicity, Exposure and Metabolism of EO and its reaction products:

Compared to other fumigants, EO shows a relatively moderate acute toxicity to humans. Short-term effects of EO in humans consist mainly of depression of the central nervous system and irritation of mucous membranes such as the conjunctiva of the eyes. Chronic exposure to EO in humans is reported to cause neurological disorders through damage to brain and the nervous system, even at low exposure doses.

The ability of EO to react with and damage DNA explains its sterilizing properties but it also accounts for its genotoxic, mutagenic and carcinogenic potential. Evidence in humans indicates that exposure to EO increases the risk of lymphoid and breast cancer. There is also some evidence linking EO

[29] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/8-other-accepted-uses.html
[30] Health Canada - MRL Database (choose: Ethylene Oxide & Sesame seeds); http://oasdmz01.hc-sc.gc.ca/mrl-lrm/index-eng.php, extracted on 25.11.2020
and https://www.ecfr.gov/cgi-bin/text-idx?SID=f944ee5791ae8ffd2915f089996c105b&mc=true&node=se40.26.180_1151&rgn=div8
[31] https://www.law.cornell.edu/cfr/text/40/180.151
[32] https://foodregulation.gov.au/internet/fr/publishing.nsf/Content/forum-communique-2001-September
[33] Regulation 1223/2009/EC
[34] Regulation 10/2011/EU
[35] ECHA: https://echa.europa.eu/de/substance-information/-/substanceinfo/100.000.773
[36] Annex V to Reg. 528/2012/EU (Biocide Product Regulation - BPR)

exposure to reprotoxic effects. ECHA has classified ethylene oxide in category 1B as regards carcinogenicity, mutagenicity and reproductive toxicity respectively, and in category 3 as regards the acute toxicity[37]. The US National Institute of Health (NIH)[38] classified EO as "known to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in humans, including epidemiological studies and studies on mechanisms of carcinogenesis." The US Environmental Protection Agency (EPA)[39] has concluded that EO is carcinogenic to humans by the inhalation route of exposure. Being considered a genotoxic carcinogen and as there is no acceptable threshold for exposure, no ADI was established for EO.

The pathway of EO exposure can influence its metabolism within the body. EO is readily absorbed via inhalation or dermal contact and distributes within the body via the blood stream. Available data indicate that parts of the EO taken up through inhalation are exhaled (ca. 20-25%), parts are hydrolyzed to ethylene glycol (ca. 50%) and parts are conjugated to glutathione (ca. 20%). Other parts of EO bind to various reaction partners within the body, such as amino acids (and proteins), purine bases (and DNA / RNA), fatty acids and other.

EO quickly disappears in food through evaporation or reactions, so the **exposure of consumers to EO-related residues through food consumption will mainly concern EO reaction products**, the most prominent of which being 2-CE. 2-CE is also legally relevant as it is included in the current MRL residue definition. Ingested 2-CE will be oxidized by alcohol dehydrogenase to 2-chloroacetaaldehyde, and finally to 2-chloroacetic acid. Both 2-chloroacetaaldehyde, and 2-chloroacetic acid are detoxified through glutahion conjugation[40]. 2-chloroacetaldehyde is toxicologically more critical than 2-CE[41,42,43] and is classified as Category 2 carcinogen by ECHA[44]. 2-CE and 2-bromoethanol are considered weakly genotoxic and potentially carcinogenic[45]. A thorough overview on the toxicology of 2-chloroethanol is presented by Hartwig et al. (2019)[46]. The German Risk Assessment Authority (BfR) recently evaluated the toxicity of 2-CE. Given the inconclusive toxicological picture of 2-CE, it was decided to follow the precaution approach and consider 2-CE equally toxic to EO[47].
Any intact EO remaining in food and thus orally ingested is expected to partly transform to 2-CE within the stomach, where acidic conditions and high concentrations of chloride occur.

Consumer exposure to EO-related residues in sesame (mainly 2-CE), will strongly depend on the **sesame consumption**, which can be very different from country to country. According to FAOSTAT in the decade 2004-2013, the average annual consumption of sesame seeds in the EU countries was

[37] https://echa.europa.eu/de/regulations/clp/classificationhttps://echa.europa.eu/en/regulations/clp/classification
[38] https://ntp.niehs.nih.gov/ntp/roc/content/profiles/ethyleneoxide.pdf
[39] https://www.epa.gov/sites/production/files/2016-09/documents/ethylene-oxide.pdf
[40] W. Grunow, H.J. Altmann Toxicokinetics of chloroethanol in the rat after single oral administration. *Arch Toxicol* **49**: 275–284 (1982)
[41] https://www.foodstandards.gov.au/code/applications/documents/A445_FARs37.pdf
[42] J. McCann,V. Simmon,D. Treitwieser, and B.N. Ames; Mutagenicity of chloroacetaldehyde,a possible metabolic product of 1,2-dichloroethane (ethylene dichloride), chloroethanol (ethylene chlorohydrin), vinylchloride, and cyclophosphamide; Proc. Nat. Acad. Scd. USA Vol. 72, No.8, pp.3190-3193 (1975) Genetics
[43] J Fowles, J Mitchell, H McGrath; Assessment of cancer risk from ethylene oxide residues in spices imported into New Zealand.; Food and chemical toxicology 39 (2001) 1055-1062
[44] https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/53634
[45] https://www.foodstandards.gov.au/code/applications/documents/A445_FAR_091002.pdf
[46] A. Hartwig, MAK Commission; 2-Chlorethanol [MAK Value Documentation in German language, 2019]; Volume 4; Issue (2019); https://onlinelibrary.wiley.com/doi/full/10.1002/3527600418.mb10707d0067
[47] BfR-Stellungnahme zur Toxizität von Ethylenoxid und 2-Chlorethanol, hier: Funde in Sesamsamen und in hieraus hergestellten Erzeugnissen vom 20.11.2020, Az. 30-0202-01-11345065

0.07 kg per person[48]. Looking at individual countries, the highest sesame consumption in the EU is observed in Cyprus (1.3 kg per person annually) followed by Greece (1.1 kg), Malta and Austria (0.3 kg each). In most EU countries, annual sesame consumption is below 0.1 kg per person. Considerably higher consumption figures are encountered in the Middle East (e.g. Israel 4.7 kg; Lebanon 2.5 kg). These consumption differences between EU-countries will need to be taken into account in dietary intake and risk assessment calculations as well as in MRL-setting. Individual population groups with high consumption of sesame products (e.g. immigrants from the Middle East) may also need to be considered. Beyond the average consumption figures, short-term consumptions are also of interest as high-dose exposures (especially if repetitive) increase the risk of a mutation incidence within the body and therefore the risk of developing follow-up effects such as cancer.

Alternative sources of human exposure to EO and its reaction products:

It could be shown that EO can be formed naturally through enzymatically catalyzed oxidation of ethylene; involving CYP-450 oxidases. This was demonstrated for both bacteria[49] and plants[50]. **Endogenous oxidation of ethylene to EO in the human body** through bacterial activity within the intestinal tract was reported by Ehrenberg et al. (1977)[51] and many other authors. Tornqvist (1996)[52] indicated that the transformation of ethylene to EO explains the background levels of EO-adducts with DNA and proteins, such as hemoglobin. They have also demonstrated that EO-adduct formation is linked to the metabolism of unsaturated fatty acids via ethylene by the intestinal flora. An additional ethylene precursor is the amino acid methionine, which is also metabolized to ethylene[53]. Another source of EO formation and uptake is the inhalation of **combustion gases including cigarette smoke and car exhaust gasses**[54]. Kirman (2017)[55] discusses the risks originating from endogenous and exogenous (occupational and cigarette smoke) exposure to EO.

Possible sources of background levels in food

It is always of interest to check whether food may be contaminated with regulated pesticides and metabolites (i.e. EO and 2-CE) through pathways going beyond direct pesticide use on the product.

From a purely theoretical perspective EO and 2-CE may end up in food when ethylene, which is endogenously produced within the plants, is enzymatically oxidized to EO and further transformed to 2-CE within the plant. Ethylene could theoretically also react with hypochlorite (contained in chlorinated water) to form 2-CE. This could take place during sesame processing (soaking, washing) or in the field during (irrigation). A minor contamination with EO and 2-CE could take place when combustion gasses containing EO contaminate the samples during the drying or roasting process. Less likely, but theoretically conceivable as sources of 2-CE, would be vinyl chloride (chloroethene) and dichloroethane (ethylene chloride), that are both well-known 2-CE precursors. See also discussion under “Residues in real samples”.

[48] http://www.fao.org/faostat/en/#home

[49] De Bont JAM. 1975. Oxidation of ethylene by bacteria. Ann. Appl. Biol. 81:119–121.

[50] Dodds JH, Musa SK, Jerie PH, Hall MA. 1979. Metabolism of ethylene to ethylene oxide by cell-free preparations from Vicia faba L. Plant Sci. Letts. 17:109–114.

[51] Enrenberg L, Osterman-Golkar S, Segerback D, Svensson K, Calleman CG. 1977. Evaluation of genetic risks of alkylating agents. III Alkylation of hemoglobin after metabolic conversion of ethene to ethylene oxide in vivo. Mutation Res. 45:175–184.

[52] Tornqvist M.: Ethylene oxide as a biological reactive intermediate of endogenous origin; Adv Exp Med Biol (1996) 387: 275-283.

[53] Lieberman, M. and Mapson, L.W. Genesis and biogenesis of ethylene Nature, 204, 343-345 (1964)

[54] Törnqvist, M., Osterman-Golkar, S., Kautiainen, A., Jensen, S., Farmer, P.B., Ehrenberg,L., 1986. Tissue doses of ethylene oxide in cigarette smokers determined from adductlevels in hemoglobin. Carcinogenesis 7 (9), 1519–1521.

[55] C.R. Kirman, S.M. Hays; Derivation of endogenous equivalent values to support risk assessment and risk management decisions for an endogenous carcinogen: Ethylene oxide; Regulatory Toxicology and Pharmacology; Volume 91, December 2017, Pages 165-172

Analytical procedures

Various methods for the analysis of EO, or the sum of EO and 2-CE have been presented in literature. In 1988, K.G. Jensen[56] published a method involving conversion of 2CE to EO under alkaline conditions. The produced EO (consisting of any EO originally present in the sample and the EO formed from 2-CE) is purged using a nitrogen flow into an aqueous reservoir containing sodium iodide and sulfuric acid. There, EO is converted to iodoethanol, which is subsequently partitioned into ethyl acetate and measured via GC-ECD. The method was adapted in a modified form and extensively validated in an inter-laboratory study by Gilsbach et al.[57] ultimately becoming an official standard in Germany[58]. The method also foresees the analysis of intact EO, originally present in the sample. This is achieved by purging EO from the sample without any alkaline treatment at the initial step. The 2-CE levels can then be determined through difference calculation between intact EO and EO (sum).
In 1988, Aitkenhead et al.[59] presented a method for the analysis of 2-CE (and 2-bromoethanol) in spices. The analytes are extracted from the sample using a mixture of acetonitrile and methanol and then determined by GC-FID. In 1995, Woodrow et al.[60] published a method involving direct headspace sampling for the analysis of EO in spices. In 2002, Ayoub et al.[61] published a method involving headspace sampling or direct immersion SPME to determine EO from medical devices. In 2006, Tadeo and Bononi[62] published a method for the analysis of 2-CE in pepper. The method involved conversion of any residual EO into 2-CE, using sulfuric acid and sodium chloride, followed by an extraction of the total 2-CE with ethyl acetate, an evaporation step for enrichment and GC-MS analysis.

Analyte properties and analytical strategies

The choice of the analytical approach depends on both the analytes and the matrix to be analyzed. The available equipment and the methods already routinely employed in a lab also need to be considered. When following a single-residue analysis approach, the procedure can be optimized towards better analytical performance (sensitivity, recovery rates etc.) by taking advantage of the specific physicochemical properties of the analytes (e.g. polarity, volatility). Some methods focusing only on EO and/or 2-CE were described above. When trying to integrate those two analytes into an existing multiresidue procedure, there are more restrictions when it comes to optimization possibilities, but at the same time, this approach it is more efficient for the labs.
Tables 1 and 2 give an overview of the physicochemical properties of 2-CE and EO. The logKow values of EO (-0.3) and 2-CE (0.24) suggest a decent partitioning of both analytes from an aqueous phase into an organic solvent if not too non-polar. Satisfactory recovery rates by the QuEChERS approach are thus expected. The volatility and reactivity of EO raise concerns as regards the handling of standard solutions and the possibility of losses due to evaporation or interactions with the matrix. The chromatographic separation of EO and its isomer acetaldehyde is an additional important aspect

[56] K.G. Jensen; Determination of ethylene oxide residues in processed food products by gas-liquid chromatography after derivatization. Z. Lebensm Unters Forsch 187, 535–540 (1988)
[57] W. Gilsbach, R.D. Weeren; Ringuntersuchungen zur Validierung einer gaschromatographischen Methode zur Bestimmung von Rückständen an Ethylenoxid und 2-Chloroethanol in Gewürzen aus Paprika und Chili, Dtsch. Lebensm. Rundsch. 95, 83–90 (1999).
[58] Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB, L53.00-1 (formerly ASU §35 LMBG L53.00-1)
[59] P. Aitkenhead and A. Vidnes;. Simple and accurate method for determination of ethylene chlorohydrin in dried spices and condiments. J. Assoc. Off. Anal. Chem. 71, 729–731 (1988).
[60] J. E. Woodrow, M. M. McChesney, and J. N. Seiber; Determination of Ethylene Oxide in Spices Using Headspace Gas Chromatography; J. Agric. Food Chem., 43, 8, 2126–2129 (1995)
[61] K. Ayoub, L. Harris, B. Thompson; Determination of low-level residual ethylene oxide by using solid-phase microextraction and gas chromatography. J. AOAC Int. 85 (6), 1205–1209 (2002)
[62] F. Tateo, M. Bononi: Determination of ethylene chlorohydrin as marker of spices fumigation with ethylene oxide; Journal of Food Composition and Analysis 19, 83–87 (2006)

to consider in GC analysis[60;63]. 2-CE, the main analyte expected to be present as residue in samples, shows a remarkably low volatility for its size, which is attributed to strong intramolecular hydrogen bonds[64]. It also appears to be quite stable in the matrix and during GC analysis. The non-amenability of 2-CE and EO to standard LC-ESI-MS/MS measurement[65] and the amenability of both compounds to GC-MS(/MS) makes the latter approach the first choice to focus on. In the case of GC-analysis, the removal of matrix co-extractives of low volatility is needed. Fortunately, lipid removal in QuEChERS or QuOil is easily achieved by C_{18} sorbents or by a freeze-out step.

Table 1: Comparison of physicochemical properties of EO and 2-CE compared to common solvents

Compound	LogKow	Molecular weight	Vapour pressure at 20 °C	Boiling point
Ethylene oxide	-0.3	44.05	1,45 bar	10.4 °C
Acetonitrile	-0.34	41.05	0.1 bar	80.2 °C
Ethanol	-0.3	46.07	0.06 bar	78.5 °C
Water	---	18.02	0.02 bar	100 °C
2-Chloroethanol	0.14	80.52	0.007 bar	129 °C

Commodities relevant for residues of EO/2-CE are primarily spices, oily seeds and nuts. Nuts and oily seeds (including some oily spices, such as cumin seeds) contain lipids in the range between 20 and 70%. The lipid content of sesame (currently in the focus), ranges between 42 and 56% according to Girmay (2018)[66] and between 53 and 67%, with an average at 60%, according to Hanine et al. (2019)[67]. Processed products derived from sesame, such as oils, tahini, halva and bakery goods are also of relevance. When it comes to such commodities (high lipid content, low water content), many labs nowadays employ the QuOil method. This method involves extraction with acetonitrile (in the case of oils) or acetonitrile containing 5% of water (in the case of oily seeds and nuts). Co-extracted lipids and fatty acids are removed by dSPE with C_{18} and PSA sorbent respectively. $MgSO_4$ can reduce the water content in the acetonitrile extract of QuEChERS and QuOil extracts from 7-8% and 5% respectively, down to 2-3%.

Direct GC-analysis of the QuEChERS or QuOil extracts, can be problematic due to the large expansion volume of acetonitrile and the negative impact of acetonitrile and the residual water contained in extracts to the column and the filaments. A way out is split-injection and in case of PTV-injectors, the evaporation of the solvent in the in the liner by a gentle nitrogen stream. In the latter case, evaporation losses of EO during purging would be expected to a certain extent.

Based on all above considerations, it was decided to target EO and 2-CE by QuOil, in the case of dry high oil content commodities, and by QuEChERS when dealing with other commodities and to analyze the extract directly by GC-MS/MS following the removal of the lipids. Both QuEChERS and QuOil are routinely employed in many labs for multiresidue analysis.

[63] https://www.agilent.com/cs/library/applications/A01291.pdf

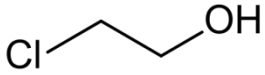
[64] Microwave Spectra and Intramolecular Hydrogen Bonding in the 2-Haloethanols: Molecular Structure and Quadrupole Coupling Constants for 2-Chloroethanol and 2-Bromoethanol J. Chem. Phys. **52**, 5299 (1970)

[65] According to pre-experiments of the EURL-SRM

[66] A. B. Girmay; Sesame Production, Challenges and Opportunitiesin Ethiopia; Agri Res & Tech: Open Access J 15(5): ARTOAJ.MS.ID.555972 (2018); https://juniperpublishers.com/artoaj/pdf/ARTOAJ.MS.ID.555972.pdf

[67] M. El Harfi, A. Nabloussi, H. Rizki, S. Ennahli and H. Hanine; Proximate Composition and Fatty Acid Composition, Phytochemical Content of Sesame (*Sesamum indicum L.*) Seeds Landrace from Morocco; Adv Crop Sci Tech 2019, 7:3

Table 2: Compound details at a glance

Ethylene oxide (CAS: 75-21-8), Other names: Oxiran, Epoxyethane, Oxacyclopropane; Abbreviations: EO, EtO, ETO			
Parameter	Value	Notes	
Molecular Mass	44.052 g/mol		
Formula	C_2H_4O		
Boiling point	10.4°C		
pKa	No dissociation in water		
LogD	-0.05[68]	pH independent; LogKow -0.3 (WHO); computed by Chemicalize.com	
Water solubility	infinite	pH independent	
Stability	The main route of losses is evaporation. In neutral water at 25 °C, ethylene oxide is hydrolyzed to glycol with a half-life of 14 days (at 0 °C, half-life is 309 d). In presence of halide ions (chloride, bromide, iodide), 2-haloethanol is formed next to glycol and half-life is shortened (Conway et al., 1983). When heated, EO isomerizes to acetaldehyde through surface catalyzed rearrangement[69]		
Residue definition EU	Ethylene oxide (sum of ethylene oxide and 2-chloro-ethanol expressed as ethylene oxide) (F) (Note by EURL-SRM: the (F) attribute is questionable given the LogD value and the lack of bioaccumulation properties)		
Approved in…	No authorization in place within the EU		
Properties of concern	Being an electrophilic agent, EO reacts with nucleophilic groups (e.g. amino, thiol, hydroxyl). 2-hydroxyethyl adducts are formed with amino acids within proteins (e.g. with cysteine, histidine and N-terminal valine) and bases within DNA and RNA (e.g. adenine, guanine). Shown to be mutagenic, carcinogenic and reprotoxic in animals. Endocrine Disruption properties are being assessed (ECHA). IARC[70] classified EO as carcinogenic to humans (Group 1). Certain plastics are permeable by ethylene oxide, so not all cloves offer full protection.		
Other sources	Naturally developed at very low levels in plants and animals as a metabolite of the growth hormone ethylene. Formed during combustion of organic material (e.g. 2-7 µg per cigarette) Diethylene glycol and polyurethanes produced thereof may contain residues of EO Polyethylene glycol-based detergents (used in cosmetics) may contain residues (Legal limits established).		
2-Chloroethanol (CAS: 107-07-3) Other names: 2-Chloroethan-1-ol; Ethylene chlorhydrin; Glycol chlorohydrin; Abbreviations: 2-CE; 2CE; CE; ECH			
Parameter	Value	Notes	
Molecular Mass	80.51 g/mol		
Formula	C_2H_5ClO		
Boiling point	129°C (due to intramolecular hydrogen bonding, the boiling point is comparably high and volatility low)[71]		
pKa	Strongest acidic pKa: 14.86		
LogD	0.15	pH independent	
Water solubility	miscible	pH independent	
Stability	Hydrolyzed to ethylene glycol, Oxidized via chloroacetaldehyde to chloroacetic acid At high temperatures acetaldehyde is formed though HCl elimination[72]		
Residue definition EU	Ethylene oxide (sum of ethylene oxide and 2-chloroethanol expressed as ethylene oxide)		
Properties of concern	Forms phosgene upon combustion, Reported to have a moderate mutagenic potential		
Acute toxicity	Fatal if swallowed, in contact with skin and if inhaled (ECHA) Classified as an extremely hazardous substance in the United States		
Other sources	Metabolite in the degradation of 1,2-dichloroethane (widely used in the production of vinyl chloride) and also metabolite of vinyl chloride. Furthermore formed when ethylene interacts with hypochlorite		

[68] Computed by Chemicalize.com
[69] M.L. Neufeld and A.T. Blades; The kinetics of the thermal reactions of ethylene oxide; Canadian Journal of Chemistry; Volume 41, Number 12, December 1963 (https://cdnsciencepub.com/doi/pdf/10.1139/v63-434)
[70] IARC: International Agency for Research on Cancer (2008 report)
[71] Microwave Spectra and Intramolecular Hydrogen Bonding in the 2-Haloethanols: Molecular Structure and Quadrupole Coupling Constants for 2-Chloroethanol and 2-Bromoethanol J. Chem. Phys. 52, 5299 (1970)
[72] The thermal decomposition of 2-chloroethanol; DC Skingle and VR Stimson Australian Journal of Chemistry 29(3) 609 – 615(1976)

Chemicals and Materials

The materials needed can be found under EN-15662 (QuEChERS) or CEN/TS 17062:2019 (QuOil) with the exception of the grinding balls that are needed to improve residue accessibility and reduce extraction time (e.g. SPEX 2155 (9.5 mm)).

Analytical Standards:

Table 3 gives an overview of the analytical standards used for this study.

Table 3: Analytical standards[73]

Compounds[74]	Details on standards used	Provider	Other providers, notes
Ethylene oxide	50 mg/mL in methanol (code: CRM48838) or 50 mg/mL in dichloromethane (code: CRM48891)	Both by Merck	Temperate the purchased 1 mL ampule containing EO solution in the refrigerator. Open it while still cold and quickly transfer its content into a sealable container (e.g. a GC-Vial) and store at -18°C.
Ethylene oxide D4	≥98 atom % D, ≥99% (CP) (code: 457833)	Merck	In lecture bottle. Difficult to handle
2-Chloroethanol	Pure substance >99% (code: 23000-50ML) or 2000 µg/mL solution in MeOH (code: CRM48085)	Both by Merck	LGC (Dr. Ehrenstorfer); HPC Standards GmbH
2-Chloroethanol D4	98 atom% (code: DLM-1928-PK)	Cambridge Isotopes	HPC Standards GmbH; TRC Merck (delays) LGC: soon available

Stock and working solutions of analytes and ILISs

IMPORTANT SAFETY REMARKS: Ethylene oxide and, to a lesser extent, 2-chloroethanol are considered dangerous to health (see introductory text). The use of a fume hood and wearing cloves is recommended to minimize exposure (Note: some clove materials can be penetrated by EO over time and should be avoided[75]).

2-chloroethanol (density 1.21 g/cm^3): A stock solution at **5** mg/mL may be prepared as follows: Pipette 83 µL of 2-chloroethanol (tempered to room temperature) into a reservoir of 20 mL acetonitrile[76]. This solution is diluted with acetonitrile to prepare various working solutions as required by each experiment. Alternative ready to use solutions may be purchased.

2-chloroethanol-D$_4$: A stock solution at **5** mg/mL is prepared by pipetting 41 µL of 2-chloroethanol (tempered to room temperature) into a reservoir of 10 mL acetonitrile. This solution is diluted with acetonitrile to prepare working solutions (at e.g. 40 µg/mL for use during sample preparation and at e.g. 4 µg/mL for calibration solutions[77].

[73] **Disclaimer**: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

[74] Under IATA regulations, EO and 2-CE are not allowed to be transported by air.

[75] https://www.lyondellbasell.com/globalassets/documents/chemicals-technical-literature/ethylene-oxide-manual.pdf (page 78 ff)

[76] 82.64 µL 2-CE and 19,92 mL ACN would be more precise figures, assuming 100% purity of 2-CE

[77] Spiking 100 µL of this standard to a 2 g sample portion will correspond to 2 mg/kg

Ethylene oxide: Commercial certified ethylene oxide solutions, as those listed above, can be considered as stock solutions. Prepare the necessary working solutions by dilution with acetonitrile. Overall, it is preferable to handle EO-solutions at low temperatures to reduce evaporation losses. Open cold vessels only for a short time to reduce water-condensation. As the density of methanol, dichloromethane and acetonitrile shifts similarly when moving from room- (~ 22°C) to refrigerator- (~ 4°C) and freezer- (~ -18°C) temperatures[78], volumetric errors become negligible, if the standard solutions to be diluted and the solvents used for dilution have similar temperatures. You may also pipette the acetonitrile reservoir at room temperature and place it in the fridge or freezer afterwards, but in this case, the volume contraction needs to be considered[79].
For the preparation of further diluted working solution, follow the same principles. If these solutions are used in cold condition for spiking, volume contraction must be considered[80].

Ethylene oxide D_4: As soon as commercial EO-D_4 solutions become available (as in the case of native EO), working solutions may be prepared in the same way as described above for EO[81].
For the purpose of the experiments shown in the following, the EURL-SRM has prepared EO-D_4 solutions in two different ways:
a) by carefully trapping gaseous EO-D_4 into a cold methanol reservoir. The concentration of the solution prepared in this way was estimated at ~20 mg/mL[82]. This solution was stepwise diluted with acetonitrile to obtain a working solution at ~10 µg/mL, which was for spiking the analytical portions prior to extraction. A 10-fold dilution thereof in acetonitrile, was prepared freshly before each experiment day and used for the preparation of calibration standards.
b) by chemically transforming 2-CE-D_4 to EO-D_4 under alkaline conditions and partitioning the latter into acetonitrile[83]. The final EO-D_4 concentration in the prepared solution was expected to be around 120 µg /mL and this was confirmed by a measurement against a native standard. Residual levels of 2-CE-D_4 in the final solution were checked and found being negligible. This solution was further diluted with acetonitrile to a working solution containing ~5 µg/mL, which was used for spiking analytical portions during analysis. For calibration purposes, a 10-fold diluted working solution of this standard was freshly prepared on each experiment day.

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[78] Density figures and density ratios at 22°C, 4°C and -18°C, respectively
- **ACN**: Density: 782.2, 801.8, 825.2 (mg/mL);
- **MeOH**: Density: 790.5, 806.7, 825.7 (mg/mL); density ratio MeOH/ACN: abs.: 1.010; 1.006; 1.001; normalized: 1.000; 0.995; 0,990
- **DCM**: Density: 1324.4, 1356.1, 1393.8 (mg/mL); density ratio DCM/ACN: abs. 1.693; 1.691; 1.689; normalized: 1.000; 0.999; 0,998
The density figures for DCM were calculated by http://www.ethermo.us using the "Antoine" approach and for ACN and MeOH they were retrieved from Dortmund Database (http://ddbonline.ddbst.com/DIPPR105DensityCalculation/DIPPR105CalculationCGI.exe).
The small of error <1% can be considered acceptable considering that working at RT increases the risk of larger errors through evaporation.
[79] Pipette 2.5% ACN more at room temperature if the EO standard you would like to dilute is tempered in the fridge (~4°C) and 5.5% more ACN if the EO-solution is tempered in the freezer (~-18°C). Examples: 10,19 mL ACN at room temperature corresponds to 9.9 mL at 4°C and 10.45 mL ACN at room temperature corresponds to 9.9 mL at -18°C.
[80] When working at refrigerator temperature increase the volume of acetonitrile-based solutions by 2.5% (e.g. 102.5 µL corresponds to 100 µL at room temperature) and when working at freezer temperature by 5.5% (e.g. 105.5 µL corresponds to 100 µL at room temperature)
[81] The concentrations of ILIS solutions do not have to be exactly known (unlike native EO). It is, however, important that the amount of ILIS added to the analytical portion and the amount of ILIS added to the calibration solutions is at an exactly known proportion (e.g. 10:1). This means that only the last dilution step will need to be conducted in a way ensuring exactness.
[82] Approximate concentration was estimated by comparing the GC-MS signals against those of native EO solutions
[83] Procedure followed: Add 2 mL 5N NaOH + 3 mL ACN + 1 mL 2CE-D_4 (1000 µg/mL ACN) into a 50 mL PP falcon tube. Close the trube and let it react for 2 h at room temperature, occasional shaking. Cool down in the freezer. Neutralize with 2 mL 5N H_2SO_4, add partitioning salt (1.5 g $MgSO_4$), + Buffer mix (200 mg NaAc + 20µL HAc) centrifuge shortly, cool down in the freezer and transfer 3,5 mL of the ACN-Phase into a 15 mL PP centrifuge tube containing 300 mg $MgSO_4$. Shake, centrifuge shortly, and cool down. Transfer twice 1.3 mL into two GC-Vials with screw caps. Assuming full transformation and partitioning of ca. 80% of EO-D_4 into the ACN phase the expected EO-D_4 concentration was ~120 µg/mL. A signal comparison against native EO confirmed this.

Remarks on stability of EO / EO-D_4 solutions: To minimize evaporation losses the EO-solution should be preferably added to the acetonitrile reservoir rather than into an empty vessel. Screw-caped bottles are preferable to bottles with fitting glass stoppers. Stock and working solutions of EO in screw-cap glass bottles with Teflon seals can be stored in the refrigerator for at least a month and for longer periods in the freezer. Chemical degradation does not seem to contribute significantly to ethylene oxide losses, but evaporation losses can be critical and should be minimized (i.e. avoid large head-space, repeated tempering of working standards to room temperature and frequent opening of vessels). Diluted EO and EO-D_4 working solutions used for the preparation of calibration solution are recommended to be prepared freshly on the day of the experiment.

Sample Preparation

Homogenization: For oily seeds, it is recommended to pre-cool the knife mill[84] by grinding a small portion of dry ice. Reopen the mill; add the material to be homogenized (oily seeds, nuts or spices) and if no dry ice is left in the mill add a small amount of dry ice (sample to dry ice ratio ca. 5:1) and grind for a few seconds at high speed to obtain fine free-flowing grit[85].

When dealing with very small oily seeds, such as sesame seeds (ca. 3 mg per seed) or poppy seeds (ca. 0.3 mg per seed), intact seeds may also be employed for analysis, but the whole sample should be mixed thoroughly before withdrawing the analytical portion. In this case, the use of extraction aids, such as stainless steel balls, will be needed to ensure good residue accessibility.´

Tahini should be thoroughly mixed to ensure a homogeneous distribution of the solids. Experiments by the EURL-SRM have shown that no significant losses of 2-CE occur when the sample is milled[86].

QuOil-Method (CEN/TS 17062:2019 modified)[87]

Weigh 2 g ± 0.02 g[88] of sample homogenates (oil, nuts, or oily seeds[89]) and add 10 mL of acetonitrile containing 5% v/v of water. Optionally add ILIS-solution(s)[90], add extraction aids (e.g. 3-4 9.5 mm stainless steel balls), close the vessel and extract by a mechanical shaker for 5 minutes in the case of oils, 15 minutes in the case of finely comminuted material (e.g. tahini) and for 15-30 min in the case of intact seeds or laboratory-homogenized material[91]. Centrifuge at >3000 g[92] and then subject the

[84] The mill should have a hole to allow the evaporated dry ice to escape. Mill at high speed for a short time. If dry ice is left in the material after milling, mill further until the dry ice is evaporated.

[85] Homogenizations of high oil content commodities for extended periods or without cooling often lead to pasty material sometimes still containing larger insufficiently comminuted pieces.

[86] The impact of milling on EO concentrations needs to be checked on samples containing incurred EO-levels. Such samples were not available to the EURL.

[87] **QuOil** is a multiresidue method for pesticides in **commodities of high lipid and low water content** such as oils, oily seeds and nuts. In such matrices, the method achieves higher recoveries for lipophilic pesticides compared to QuEChERS. The current version of CEN/TS 17062:2019 only includes the **module for pure oils in which pure acetonitrile is used as extraction solvent**. The module for **oily seeds and nuts** is currently in the implementation process and foresees **acetonitrile containing 5% v/v of water as extraction solvent**. For labs already employing the QuOil method routinely, the inclusion of EO and 2-CE in the analyte scope would be a convenient and efficient step.

[88] 2 g are used within the multiresidue approach to keep the overall lipid amount small and reduce losses of lipophilic compounds. **Where this method will only target EO and 2CE**, which are polar enough not to partition into the lipid phase, the **sample weight may be increased from 2g to 5 g**. This improves method sensitivity without negatively influencing recoveries.

[89] Seed spices can be also regarded as oily seeds, e.g. nutmeg (25-40%, mace 20-30%); cumin seeds (22%) and coriander seeds (18%)

[90] e.g. 50 or 100 µL of appropriately concentrated ILIS solutions 2-CE-D_4 and EO-D_4. Conduct pre-experiments to determine the appropriate amount of 2-CE. If EO-D_4 is used as ILIS, it should be spiked to the vessel after adding the acetonitrile to minimize evaporation losses.

[91] **Intact seeds can only be extracted properly when adding grinding aids** (e.g. metal balls) during extraction. Adding extraction aids, such as stainless steel balls to the extraction vessels, improve residue accessibility and extractability, thus allowing for shorter extraction times. EURL-SRM experiments have shown that without extraction aids extraction times of 15 min (typical with QuOil) are barely sufficient for very finely milled material (e.g. tahini) but insufficient for laboratory milled material (here, 45-60 min extraction times are recommended).

[92] Cold centrifugation at -10°C for 10 minutes will also remove certain amounts of lipids and fatty acids

extract to dispersive SPE cleanup with C_{18}/PSA/$MgSO_4$ (25/25/150 mg/mL extract) to remove lipids and fatty acids[93]. The extract is filled into GC-vials for GC-MS/MS measurement.

QuEChERS-Method (EN 15662)[94]

For **cereals, spices and other dry commodities of low lipid content** the procedure described in EN-15662 is followed but extraction time is prolonged to 15 min in case extraction aids are used and to 45 min if they are not used.

Nuts and oily seeds (such as sesame) are actually not included in the scope of EN 15662. Still, this procedure is suitable for the analysis of EO and 2-CE as these compounds are polar and show only little tendency to partition into a separated lipid phase. Weigh 2 g ± 0.02 g[95] of sample homogenates (nuts, or oily seeds[89]) and add 10 mL of acetonitrile. Optionally add ILIS-solution(s)[90], add extraction aids (e.g. 3-4 9.5 mm stainless steel balls), close the vessel and extract by a mechanical shaker for 5 minutes in the case of oils, 15 minutes in the case of finely comminuted material (e.g. tahini) and for 15-30 min in the case of intact seeds or laboratory-homogenized material[91]. Add the QuEChERS buffer-partitioning salts mixture and shake for 1-2 minutes. Centrifuge at >3000 g[92] and then subject the extract to dispersive SPE cleanup with C_{18}/PSA/$MgSO_4$ (25/25/150 mg/mL extract) to remove lipids and fatty acids. The extract is filled into GC-vials for GC-MS/MS measurement.

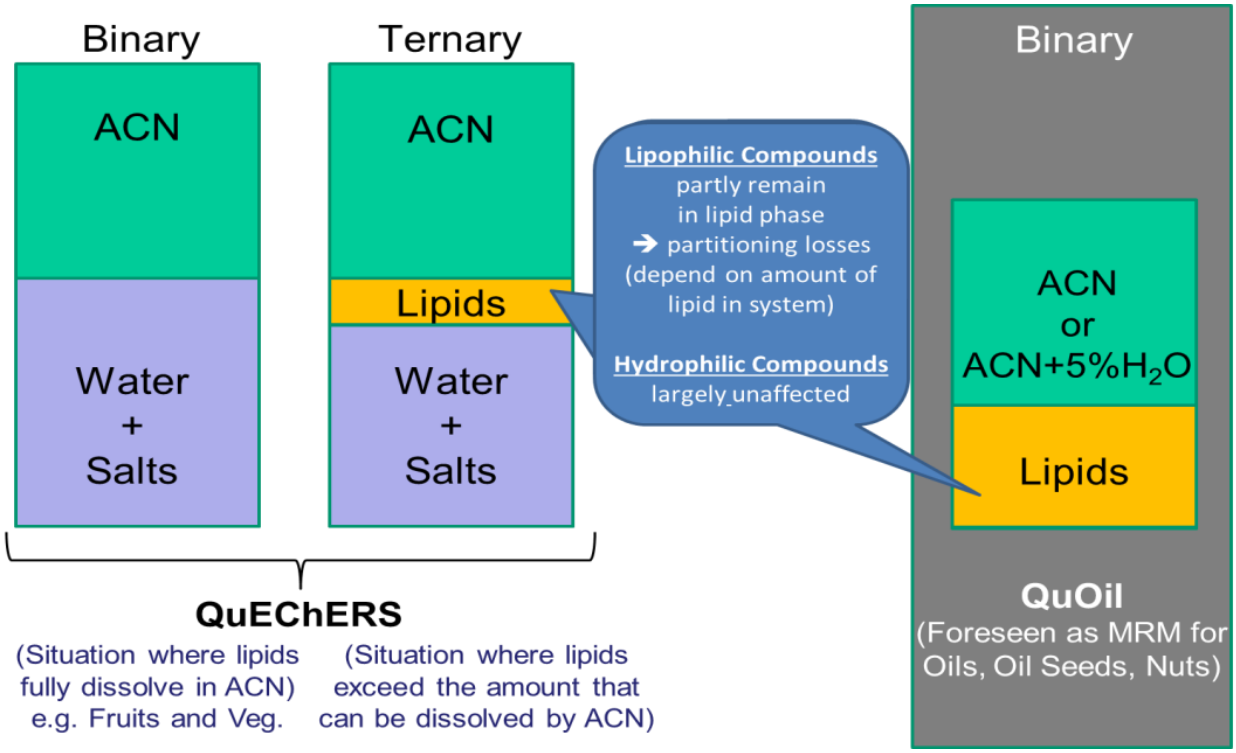

*Figure 1: Overview of QuEChERS and QuOil as regards the role of lipids and the partitioning of pesticides between the phases depending on their polarity (see also comments under Note **94**)*

[93] This mixture corresponds to the standard mixture used in the QuEChERS procedure (EN-15662)

[94] **QuEChERS** is a multiresidue method for the analysis of pesticides in commodities of **low lipid content**. At high lipid content, the lipids not dissolven in CAN will form a separate phase into which lipophilic compounds will partly partition. When targeting lipophilic compounds the amount of the lipid phase should be kept low to minimize the partitioning losses of lipophilic pesticides. Typically the amount of lipids within the analytical portion is kept < 0.35 g (i.e. < 7% lipids in sample if 5 g analytical portions are employed (e.g. cereals) and < 18% lipids in samples where 2 g portions are employed (e.g. spices).

[95] Increasing the **sample weight from 2g to 5 g** improves method sensitivity without negatively influencing recoveries.

Measurement:

The cleaned-up extract is directly measured by GC-MS/MS. Exemplary measurement conditions are given in Table 4 and Table 5.

Table 4: Instrumentation details

GC	Thermo Trace 1310 with TriPlusRSH Autosampler
Injector	Gerstel KAS 6
MS/MS	Thermo TSQ 8000
Column	J&W HP-VOC GC Column, 30 m, 0.20 mm, 1.10 µm, Part Number:19091R-303
Pre-column	Fused Silica Tubing, Deactivated, Diameter: 0.25 mm, Agilent J&W, Length: 3 m
Mobile Phase / Flow	Helium, 1 mL constant flow
Injection mode	Split 1:4
Injection volume	2 µL
Injection temp. program	Start at 90°C and hold for 0.8 min, ramp with 12°C/s to 250°C, hold 10 min,
Oven temp. program	Start: 45 °C, hold 2 min, ramp to 150°C reached at 4.1 min; ramp to 280°C reached at 5.1 min, hold till 16 min
MS Parameters	Mass axis tune mode: LowMass Ionisation mode: EI Transfer line temperature: 250°C; Ion source temperature: 270°C

Table 5: MRM Details of EO, 2-CE and their respective ILISs

Name of Compound	Retention time min	Parent (m/z)	Daughter (m/z)	CE
Ethylene oxide	2.57	44 44 44	14 28 29	20 5 5
Ethylene oxide D_4	2.57	48 48	16 30	20 5
2-Chloroethanol	4.61	80 80 82	31 43 31	5 5 5
2-Chloroethanol D_4	4.61	84 86	33 33	5 5

Calibration and Matrix Effects

Both EO and 2-CE show moderate matrix effects (ME) in GC. Unlike the general trend in GC, in which analyte signals increase in presence of matrix, in the case of EI and 2-CE signals are slightly larger in absence of matrix. Matrix components, however, improve the peak shape of 2-CE which shows slightly

better peak shapes in QuEChERS compared to QuPPe extracts. For improving the peak-shape of 2-CE[96] **analyte protectants (APs)**[97] may be added to sample extracts and calibration solutions alike. All available options for **dealing with ME** may be used including external matrix-matched calibration, standard addition to extract aliquots, standard addition to sample portions, and procedural calibration. The later two options will also correct for recovery, although recovery rates are generally satisfactory with both QuPPe and QuEChERS. **The use of ILISs** (2-CE-D_4 and EO-D_4) can be combined with any of the above approaches and it will correct for recovery if added at the beginning of the procedure. In the case of matrix-matched and external calibration, make sure to select a blank matrix showing in-significant background levels. This is of high importance when dealing with low levels.

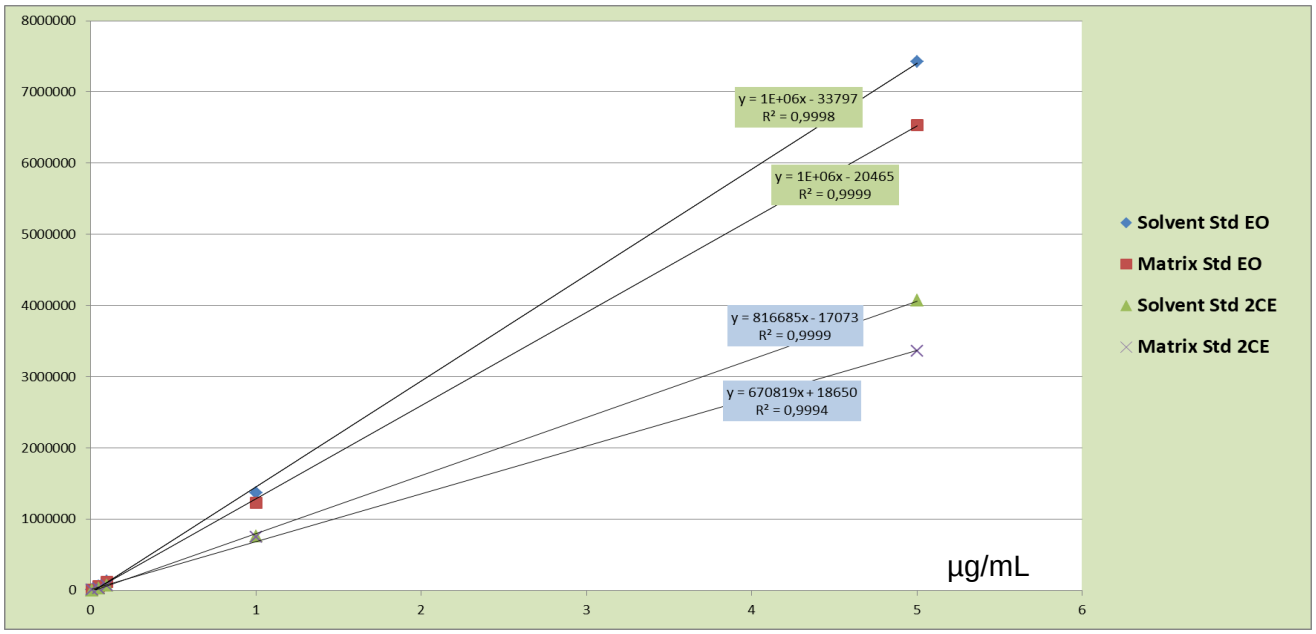

Figure 2: Exemplary calibration curves of EO and 2CE without AP. Matrix effects at the 5 µg/mL level were -12% for EO and -17% for 2CE. No use of AP

[96] In general, the 2-CE peak form when injecting QuEChERS extracts is narrower than when injecting QuOil extracts, which is attributed to the presence of polar co-extractives in QuEChERS extracts, that interact with active sites in the GC-system thus protecting the analytes.
[97] https://www.eurl-pesticides.eu/library/docs/srm/EURL_Observation-APs.pdf

Experiments and observations:

GC-Analysis:

Some exemplary chromatograms of EO in sesame extracts (at 0.2 g sesame/mL) are shown in Figure 3. Peak shape is overall satisfactory and matrix interference moderate. At the low level, the second transition is interfered.

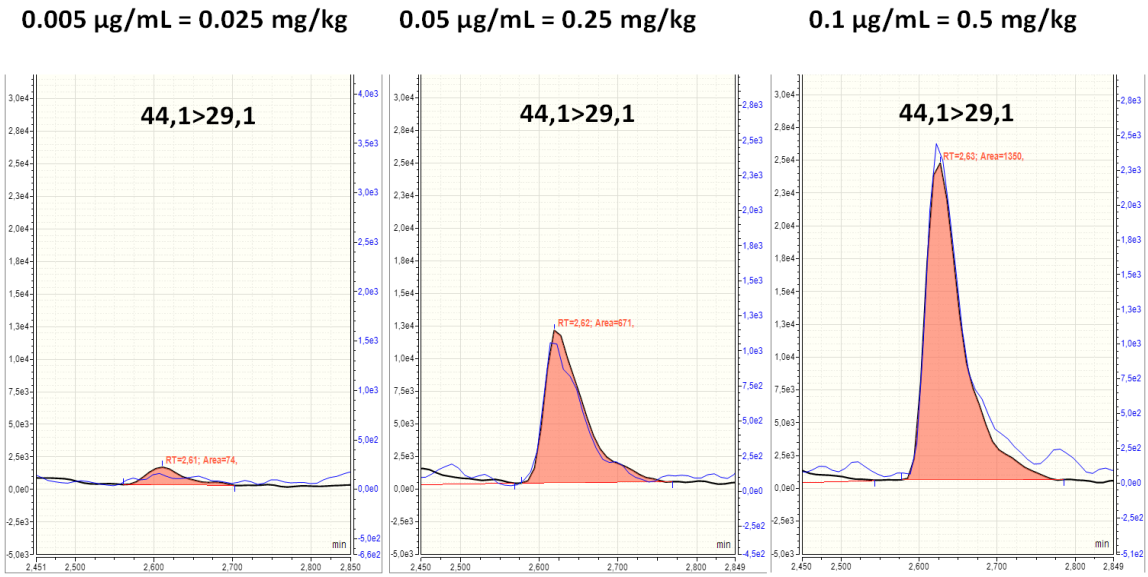

Figure 3: Exemplary chromatograms for EO spiked on QuOil extracts of blank sesame at levels corresponding to 0.025 mg/kg; 0.25 mg/kg and 0.5 mg/kg. Matrix Concentration: 0.2 g/mL.

Some exemplary chromatograms of 2-CE in sesame extracts (at 0.2 g sesame/mL) are shown in Figure 4. Peak shape is overall satisfactory and matrix interferences moderate.

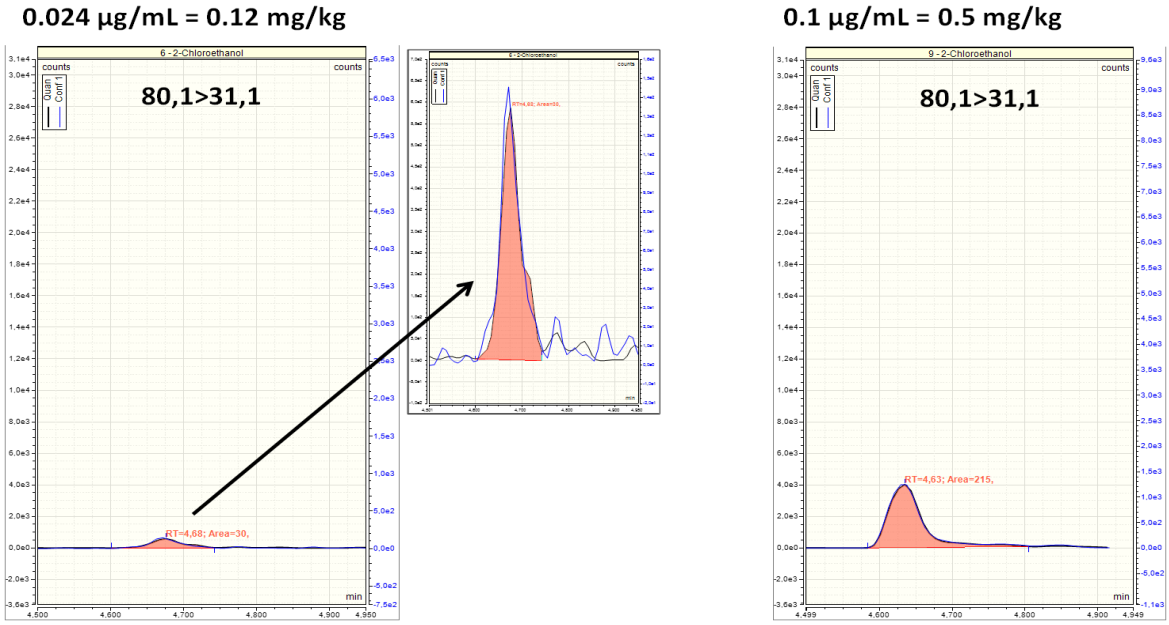

Figure 4: Exemplary chromatograms for 2-CE spiked on QuOil extracts of blank sesame at levels corresponding to 0.12 mg/kg and 0.5 mg/kg. Matrix Concentration: 0.2 g/mL

Dealing with acetaldehyde: Some sesame samples contain high levels of acetaldehyde (AA). AA can be of natural origin, but it may be also be formed at high temperatures from EO (through thermal rearrangement) or from 2-CE (through HCl elimination, see more on this below). As AA is an isomer of EO with which it shares several MRM transitions, a chromatographic separation between EO and AA is paramount. Fortunately, the two compounds are sufficiently separated under the presented chromatographic conditions. Figure 5 gives an example of a sesame extract containing AA that was overspiked with AA or EO. Experiments to check whether AA is formed at the high temperatures, to which the compounds are exposed during GC analysis, have shown that, under the GC conditions employed, the transformation of EO and 2-CE to AA in the injector is negligible.

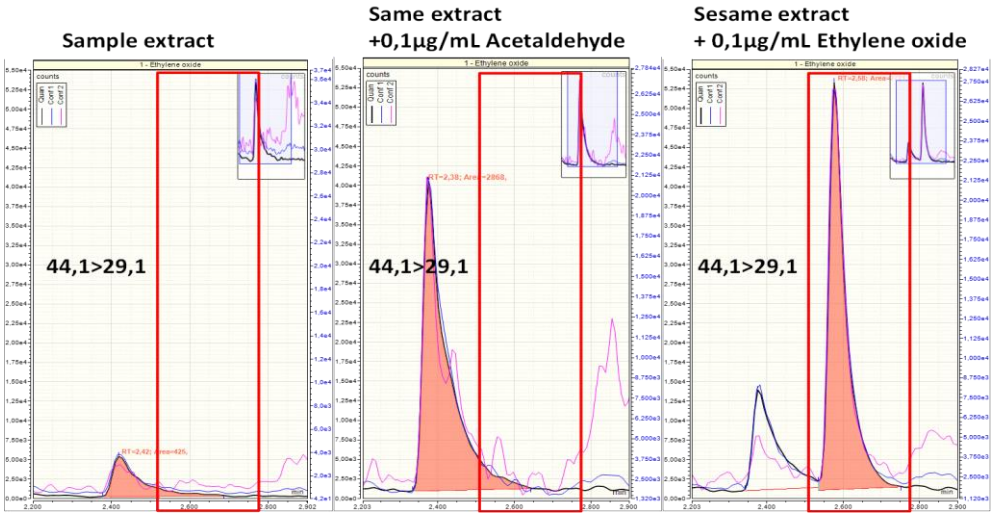

Figure 5: Chromatographic separation between EO and acetaldehyde. Extracts of sesame were overspiked with acetaldehyde (center) or ethylene oxide (right).

Recovery experiments:

Results of recovery experiments using QuOil or QuEChERS are shown in Table 6. Absolute recovery rates are satisfactory, but EO shows tentatively a bit lower recoveries compared to 2-CE. Based on these results the use of ILIS to correct for recover is not paramount.

Table 6: Recovery figures obtained using QuOil and QuEChERS method

Compound	Method	Portion [g]	n	Spiking Level [mg/kg]	ILIS	Mean Rec. [%]	RSD [%]
Ethylene oxide	QuOil	2	5	0.04	Yes	90%	12.9%
					No	92%	8.1%
	QuOil	2	5	0.5	Yes	94%	3.6%
	QuEChERS	2	5	0.5	No	83%	7.8%
2-Chloroethanol	QuOil	2	5	0.12 (expr. as EO: 0.066)	Yes	91%	11.5%
					No	93%	9.8%
	QuOil	5	5	0.05 (expr. as EO: 0.0275)	Yes	95%	2,5%
	QuEChERS	2	5	0.08 (expr. as EO: 0.044)	Yes	102%	7,4%
	QuEChERS	2	5	0.5 (expr. as EO: 0.275)	Yes	102%	8.1%
					No	91%	7.1%
	QuEChERS	5	5	0.05 (expr. as EO: 0.0275)	Yes	97%	13.7%

Impact of comminution on extraction of incurred residues

Experiments on sesame samples containing incurred 2-CE residues have revealed a delay in the extraction of incurred residues from both intact and homogenized seeds.
Figure 6 shows the extraction yields from a sesame sample prepared in two different ways:
a) milled shortly at cryogenic conditions (dry ice) to a fine grit and
b) milled further for 3 minutes at room temperature to a paste.
As can be seen in the figure comminution to a fine paste accelerates extraction but still 15 min extractions (typical for QuOil) were not sufficient to reach the plateau, whereas 30 min extractions were just sufficient. It seems that the plateau is only reached somewhere between 30 and 45 min. It seems that ethylene oxide penetrates deep into the sesame corn where is converts to 2-CE which is then difficult to extract. Another important conclusion from this experiment was that 2-CE was not noticeably affected by the longer comminution at higher temperatures.

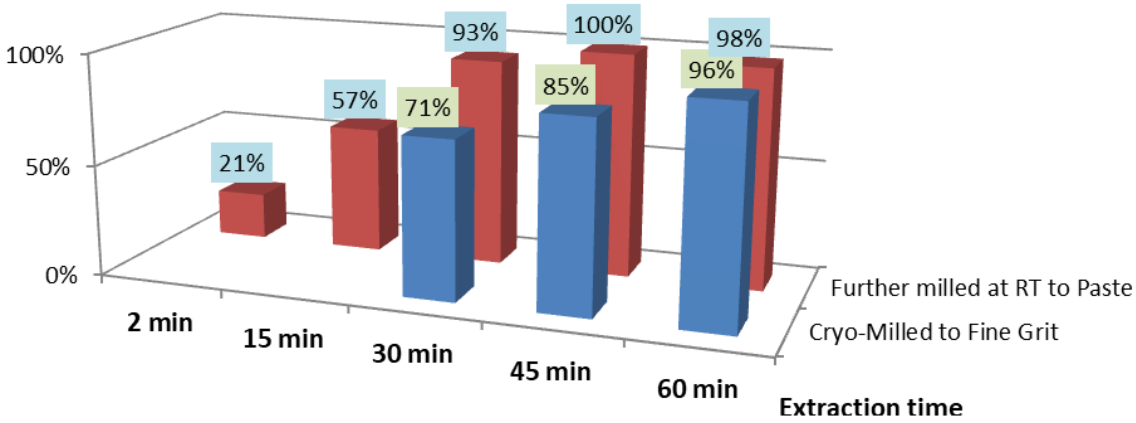

Figure 6: Impact of milling on extraction yields of incurred 2-CE from sesame at different extraction times (average of n=3). The average 2-CE yield obtained when extracting the paste for 45 min was set at 100%.

In a subsequent experiment, intact sesame seeds were extracted with and without stainless steel grinding balls. As shown in Figure 7, without these extraction aids 2-CE yields were very low even after 45 min extraction. The stainless steel balls crush the sesame seeds thus accelerating the extraction and shortening the soaking period. Using extraction aids, satisfactory 2-CE yields were already achieved at 15 min extraction times onwards. A 15 min extraction of intact seeds with balls seems to provide equivalent yields as 15 min extraction of a milled material. Furthermore, these results confirm that milling does negatively affect 2-CE residues.
Sesame seeds weigh around 3 mg on average, which means that in 2 g sample there is around 600-700 units. In theory, it should be possible to obtain a representative 2 g subsamples if the entire sample is mixed well. To check how homogeneous the residues are distributed when the sesame seeds are not milled and whether milling would improve homogeneity of the material, six replicate extractions were conducted on milled and non-milled sesame seeds. The intact sesame seeds were thoroughly mixed before withdrawing the analytical portions. Milling was conducted cryogenically with the help of dry ice. In both cases the analytical portion size was 2g and the extraction time was 15 min with balls being added to assist extraction. The RSD of the six replicate extractions was at 6.1% in the case of the non-milled material and at 3.3% for the milled material, which is in both cases within the

normal analytical variability range. It can thus be concluded, that intact sesame seeds may be extracted from the portion-to-portion variability point of view, but extraction will need to be assisted by extraction aids, such as grinding balls, for achieving satisfactory extraction yields. Even when using extraction aids the duration of the extraction should be at least 15 min, preferably 30 min.

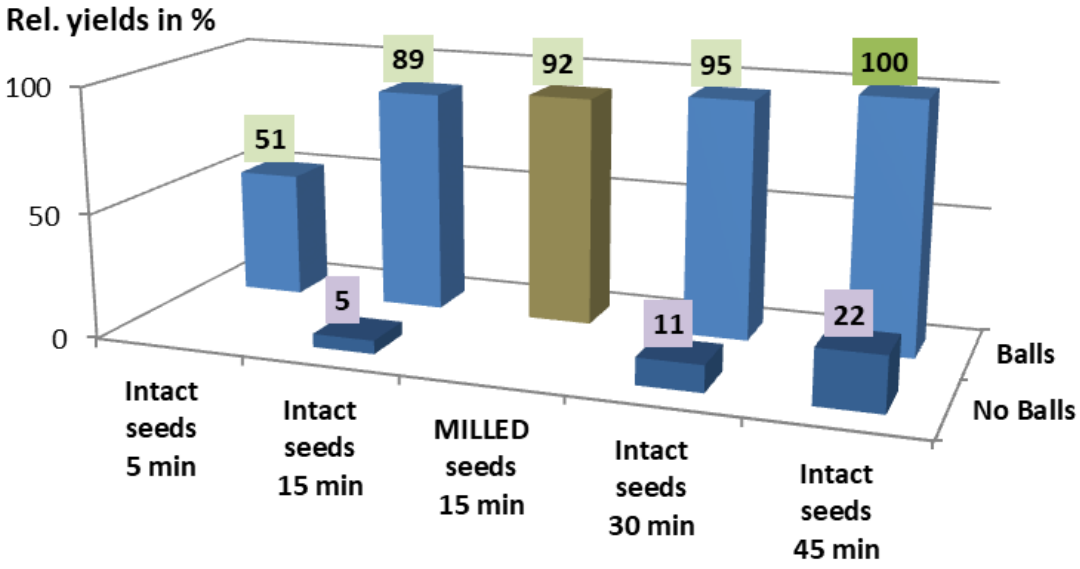

Figure 7: Impact of stainless steel balls as extraction aids to accelerate the extraction of incurred 2-CE residues from intact sesame seeds (n=3 each and in some cases n=6; see text).Yield obtained at 45 min extractions with extraction aids was set at 100%.

When extracting tahini, 2-CE extractions are less retarded. In tahini production, sesame seeds are industrially milled with very small particle sizes being obtained. This results in a larger surface area, a better accessibility of the residues and consequently a faster extraction. As can be seen in Figure 8, satisfactory yields of 2-CE were already obtained at 15 min extractions, without the addition of stainless steel balls.

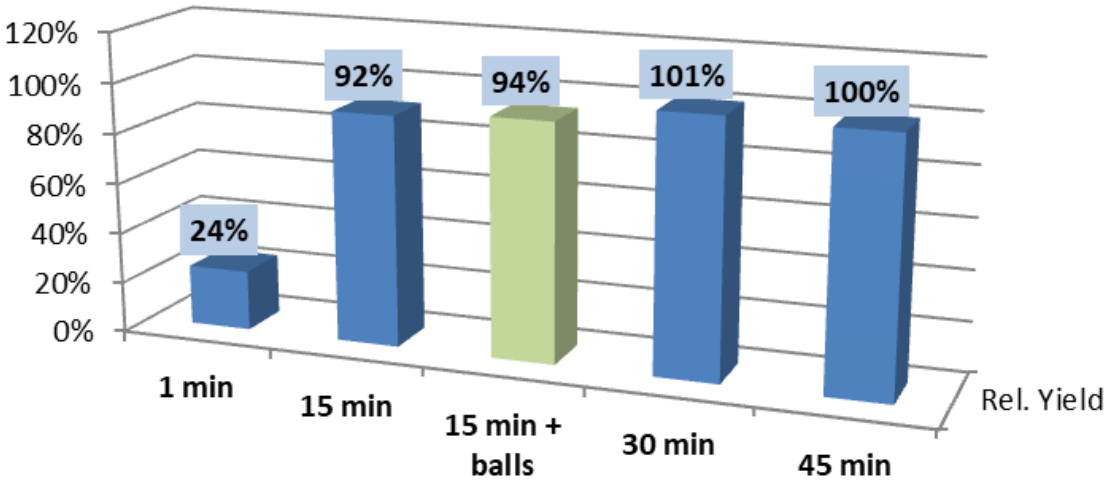

Figure 8: Extraction of incurred 2-CE residues from tahini (n=2 each).The average yield obtained at 45 min extraction was set at 100%.

In another experiment it was further observed that in the case of tahini, soaking of the sample in the extraction solvent for 20 min (without shaking), followed by a 1 min extraction, will also lead to quantitative extraction yields. This approach is interesting for labs that do not possess any mechanical shaker.

Overall extraction aids (stainless steel balls) clearly helped to reduce extraction times significantly, also enabling the extraction of intact sesame seeds. This may be of advantage when dealing with EO or other fumigants that are by far more volatile than 2-CE. Without extraction aids extraction times of 45-60 min were required to reach the plateau even for samples that were previously homogenized.

Impact of cleanup on removal of co-extractives

To minimize contamination of the GC-system a cleanup step is needed. dSPE with ODS and PSA sorbents (25 mg /mL extract each) removes considerable amounts of co-extractives.
Table 7 shows the dry residue of extracts that were cleaned-up by different procedures.
It was furthermore observed, that PSA considerably reduces the signals of acetaldehyde. This may be due to the interaction between the amine groups of the sorbent and the aldehyde group of acetaldehyde, that possibly result in the formation of covalent imine bonds.

Table 7: Dry residues obtained when applying different cleanup procedures.

Extract	**Dry residue [mg/mL]**
Raw extract	4.0
Raw extract +dSPE C_{18} (25 mg/mL)	3.1
Raw extract +dSPE PSA (25 mg/mL)	2.2
Raw extract + dSPE with C_{18}/PSA (25/25 mg/mL)	1.7
Raw extract + dSPE with C_{18}/PSA (25/50 mg/mL)	1.4
Cold centrifugation (-10°C) 10 min	3.7
Cold centrifugation (-10°C) 10 min + dSPE with C_{18} (25 mg/mL)	3
Cold centrifugation (-10°C) 10 min + dSPE with C_{18}/PSA (25 mg/mL)	1.4
Freezer (-18°C) 2h	3.3
Freezer (-80°C) 30 min	2.9

Impact of Processing on 2-CE concentrations

To estimate the impact of processing on the concentration of 2-CE in sesame, intact sesame kernels containing incurred 2-CE were processed in two ways. A). **Roasting** in a pan until golden color (ca. 5 min); b) Baking, following attachment of the seeds on the surface of a flat dough and baking at 180°C for 30 min followed by a drying period of 3h at 40°C.
In **the roasting experiment** 2-CE concentration dropped dramatically to ca 7% of the original value. At the same time it was observed that the acetaldehyde peak areas increased by more than 80 fold (not quantified). This indicates that at high temperature 2-CE converts to acetaldehyde possibly following elimination of hydrochloric acid (HCl) to form vinylalkohol and a subsequent keto/enol tautomerism.

In the **baking experiment,** the dry bread was homogenized and analyzed via QuEChERS. Considering the original amount of sesame attached to the dough surface and the final weight of the dried bread the 2-CE loss following baking was calculated at 61% (39% loss). The peak area of acetaldehyde increased 8-fold.

To estimate the **processing factor when producing sesame oil** from EO-treated sesame seeds, a tahini sample with incurred 2-CE residues was analyzed both in its entirety (following a thorough mixing) and then additionally only the oil phase (separated through centrifugation). The 2-CE residue in the tahini was 5.1 mg/kg and in the oil only 0.85 mg/kg. Considering the oil content of 60%, and assuming that the oil-solids ratio roughly corresponds to the original ration in the seeds, it can be calculated that only ca. 10% of the original 2-CE residues end up in the raw sesame oil fraction. Further experiments on tahini will follow.

Analysis of real samples

20 official sesame samples (14 conventional and 6 organic) from the market were analyzed for 2-CE and EO residues. Five of these samples contained residues exceeding the MRL of 0.05 mg/kg (expressed as EO). Table 8 gives an overview of samples exceeding the MRL of 0.05 mg/kg for EO (sum) and Figure 9 shows the distribution of the residues in conventional and organic samples. Most samples with high levels originated from India or Africa. The levels below the LOQ of 0.05 mg/kg are to be considered as semi-quantitative and are only reported with one significant figure.

Table 8: Samples containing EO/2-CE residues exceeding the MRL.

Description of Sample	Origin	2-CE mg/kg	EO (sum) mg/kg	Assessment
Sesame seeds, hulled	India	19.1	10.5	>MRL
Sesame seeds, hulled	India	14.8	8.1	>MRL
Sesame seeds, hulled	Africa (unspecified)	12.4	6.8	>MRL
Sesame seeds, hulled	India	9.6	5.3	>MRL
Sesame seeds, black	India	0.23	0.13	>MRL

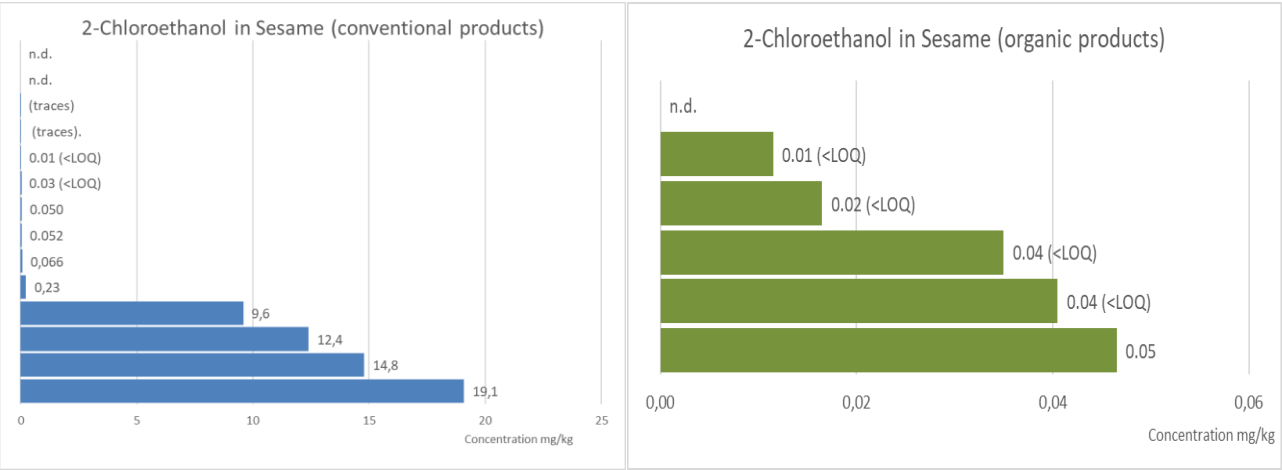

Figure 9: Findings of 2-CE residues in conventional and organic sesame samples.

Various additional sesame or sesame-containing products from local groceries originating from other countries (e.g. Turkey, Egypt, Greece, and Cyprus) were also analyzed showing either 2-CE residues at low levels between 0.01 (semi quantitative) and 0.1 mg/kg, or no detectable residues.
None of the official samples analyzed contained any measurable residues of EO. Samples received from another laboratory, containing very high 2-CE levels, showed EO residues at trace levels below the limit of quantification. These findings need to be verified. Also, none of the samples analyzed so far was found to contain any measureable levels of 2-bromoethanol or dioxin, which can be potentially generated during fumigations with EO.

Some exemplary chromatograms of extracts derived from samples from the market are shown in Figure 10.

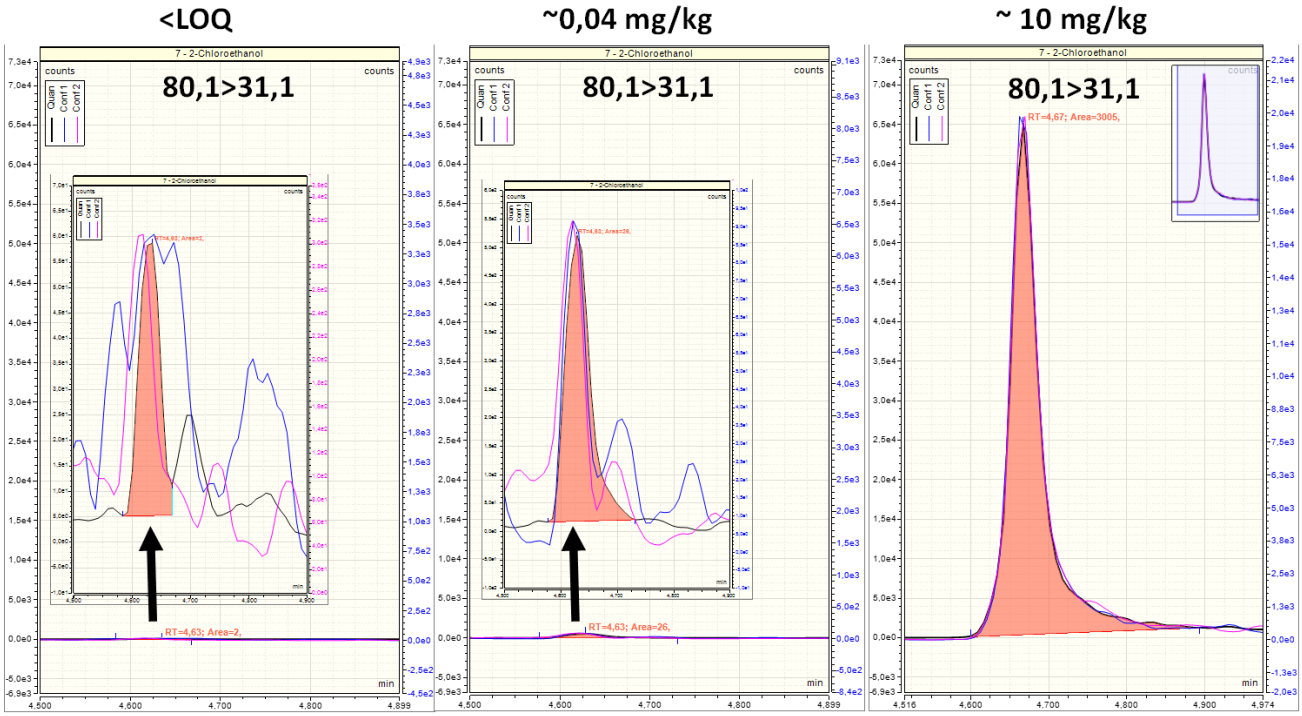

Figure 10: Peaks of 2-CE obtained from the extraction of three samples containing incurred residues.

The frequency with which 2-chloroethanol is encountered in sesame samples at low levels (< 0.1 mg/kg), raises the question whether there are alternative sources (unrelated to direct EO-fumigations) leading to 2-CE background levels. This aspect needs to be investigated further and, if verified, it would need to be considered in MRL-setting and the judgement of residue levels. The existence of samples with no detectable 2-CE levels, suggests that these low 2-CE levels, encountered in many samples, are more likely related to exogenous factors, rather than to purely natural processes within the sesame plant. The behavior of conjugated 2-CE residues, e.g. to fatty acids, during analysis will need to be studied, as these are not part of the residue definition. Also the possibility of cross-contaminations between lots, during processing, storage or transport will need to be checked.

Conclusions and Outlook:

Two methods, one based on QuEChERS and one on QuOil, are presented allowing the simultaneous analysis of ethylene oxide (EO) and its reaction product 2-chloroethanol (2-CE) in sesame. Following a standard dSPE cleanup the samples are measured by GC-MS/MS

The extraction of 2-CE from sesame, was found to be considerably delayed compared to other analytes. It is thus of high importance to use extraction aids (e.g. stainless steel balls) during extraction to disintegrate the sample and improve the accessibility of the residues.

For labs routinely applying these methods for multiresidue analysis of pesticides, the inclusion of these analytes into their scope is convenient. Still, an extra GC-run is required for measurement, due to the high volatility of the two analytes compared to typical multiresidue compounds. Expanding the method with further fumigants is planned to increase the efficiency and attractiveness of this approach. Efforts will be furthermore undertaken to increase the sensitivity of the method, which currently just sufficient for controlling the existing MRLs for EO.

An ad-hoc proficiency test on 3 sesame samples, containing incurred residues, has been initiated to enable a first comparison of results obtained by various methods.

Sesame samples from the market almost exclusively contained 2-CE. Next to samples with relatively high levels (>5 mg/kg), there were also numerous samples with findings in the lower range (<0.1 mg/kg), which are not likely to be a result of proper fumigation. Further investigations are required to study the origins of background levels including mixing of lots, cross-contaminations during processing, transport or storage as well as the possibility that low 2-CE levels end up in plants or food products through alternative pathways that go beyond fumigation.

Expanding and validating the method on other types of dry commodities, beyond sesame, is also planned as well as a random analysis of various products to localize other hotspots of ethylene oxide fumigations.

History

Action	When	Document Version
Experiments	October-December 2020	
Observation document placed online	December 2020	V1
• Title amended to include QuEChERS • Typos corrected (incl. US-MRL 970->940 ppm) • Corrected conc. of 2-CE and 2-CE-D_4 stock solutions, now 5 mg/mL		V1.1
Updated document placed online	December 2020	