Evaluation of different extractions for the metabolite identification of malachite green in brook trout and shrimp

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Abstract

Applications of mass spectrometry-based metabolomics in food science have developed fast in the last decade. There is currently no standard protocol for the optimization of sample extraction in metabolomics or non-target analysis in this field. In this study, different extraction methods, based on QuEChERS or solvent extraction with a freezing step for clean-up, were compared to study the fate of malachite green in two different organisms, brook trout and shrimp. Extracts were analysed using liquid chromatography coupled with high-resolution mass spectrometry. The effect on data processing parameters on extraction selection was assessed. Results showed that depending on the comparison criteria, matrix, mode of ionization or data processing, a different extraction should be chosen. These results confirm the need for a standardized procedure for the optimization of extractions in metabolomics. The selected method was applied to incurred samples and identified des-methylated leucomalachite green as another metabolite in brook trout and shrimp.

Keywords

metabolites; aquaculture; veterinary drugs; anti-fungal treatment; extraction; suspect screening

1. Introduction

Seafood has a lot of nutritional benefits as it is a good source of proteins, micronutrients such as calcium and iron, and unsaturated fats like omega-3 fatty acids, which can provide important health benefits in terms of prevention of cardiovascular diseases and aid in the development of the nervous system in children (FAO, 2016). In order to keep up with increased consumer demand, aquaculture production has greatly increased in the past years (FAO, 2016), with one consequence being the intensification of farming marked by high density and an increase in use of therapeutants (e.g. antibiotics, antifungals, pesticides) (Sapkota et al., 2008). Unfortunately, regulations and enforcement differ between countries, and some banned compounds are still detected in seafood (Dinh et al., 2020). One such therapeutant is malachite green (MG), used as an anti-fungal treatment, which despite its ban in food producing animals continues to be detected in aquaculture products, on account of its high efficacy, low cost and widespread availability (EFSA, 2016). Furthermore, it continues to be used as an industrial dye, hence its presence in seafood could be due to uptake by the fish following release of wastewater from industrial activities (EFSA, 2016). Once absorbed, MG is rapidly metabolized in fish species such as catfish to the more lipophilic and persistent leucomalachite green (LMG), with demethylated forms of LMG proposed as other metabolites (Doerge, Churchwell, Gehring, Pu, & Plakas, 1998). From a regulatory perspective, current action levels are set at 0.5 and 2 ng/g in Canada and Europe, respectively (Health Canada, 2017). With the compound still detected in seafood, a range of analytical methods have been reported in the literature for a variety of matrices, e.g., trout, shrimp and carp, that achieve the low detection limits required by regulatory levels to identify non-compliant products. These approaches involve an extraction step using a mixture of buffer and organic solvents (e.g., acetonitrile), followed by liquid-liquid partitioning

with dichloromethane to extract the less polar LMG and clean-up steps using solid-phase extraction. Quantification is often achieved using liquid chromatography coupled to mass spectrometry (LC-MS) with electrospray or atmospheric pressure chemical ionization (Doerge et al., 1998). Extractions based on QuEChERS (quick, easy, cheap, effective, rugged and safe) or multi-residue screening methods have also been applied (Turnipseed et al., 2017; Villar-Pulido, Gilbert-Lopez, Garcia-Reyes, Martos, & Molina-Diaz, 2011). However, the focus of these methods is mostly on the parent compound MG and its main metabolite LMG, thereby disregarding other compounds of interest such as other contaminants, metabolites or possible degradation products formed during food processing or cooking. The extraction and identification of any of these compounds would be useful in better evaluating the risks to human health associated with consumption of contaminated seafood. Thorough sample treatment steps could remove some of these compounds of interest, thus simpler, more generic methods are preferred; methods which cover a wide range of compound classes and are applicable to different types of food matrices (Mol et al., 2008). When coupled to high-resolution mass spectrometry (HRMS), these extractions present more advantages as they can be used for suspect and non-target analysis in food analysis. Non target analysis allows for the identification of compounds not yet described and for which no previous information is available (Knolhoff & Croley, 2016). Suspect analysis or screening can be performed based on some existing information, such as mass and formula (e.g. known list of contaminants) and in both cases, HRMS can provide the information needed to identify compounds (accurate mass, isotope abundance) with structural information obtained from MS/MS fragmentation (Knolhoff & Croley, 2016). MS/MS information can also be obtained through All Ions MS/MS or data independent acquisition, in which both precursor and fragment ions are obtained. Data independent acquisition has been

successfully applied for the screening of veterinary drug residues in honey (von Eyken et al., 2019) and in seafood (e.g. fish and shrimp), including MG and LMG (Turnipseed et al., 2017). For statistical analysis, like principal component analysis, these compounds should be detected with a good reproducibility (Knolhoff, Kneapler, & Croley, 2019). Non-targeted mass spectrometric methods have emerged as key methods in metabolomic studies for molecular fingerprinting (Arbulu, Sampedro, Gomez-Caballero, Goicolea, & Barrio, 2015; Perez-Miguez, Sanchez-Lopez, Plaza, Castro-Puyana, & Marina, 2018) and in food analysis for identification of contaminants (Kunzelmann, Winter, Aberg, Hellenas, & Rosen, 2018).

As non-targeted screening is based on the identification of compounds for which there is limited information, designing and optimizing the extraction procedure can be challenging. There is currently no standardized procedure for the comparison of extractions, and different approaches were presented in the literature, depending on the application/goal of the study (Table 1). In metabolomics, the number of features and repeatability, often expressed as the number of features with a coefficient of variation (CV) below 20 or 30%, are two parameters used for comparison of extractions. The use of representative quality control (QC) samples has been proposed as a strategy in metabolomic studies to correct for changes in metabolite responses over time and ensure the data is robust and reproducible (Dunn et al., 2011). Pooled mixes of sample extracts or standard mixtures of compounds have been proposed as QCs (Dunn et al., 2011; Knolhoff et al., 2019; Perez-Miguez et al., 2018).

For contaminant screening/non target analysis, extraction protocols are often assessed in terms of recovery and precision for specific targeted compounds. The number of detected features and their CV are less common criteria in this case. Indeed, as most of the features extracted may be endogenous matrix components (e.g., amino acids, sugars, lipids), evaluating

repeatability based on the percent features with a CV < 20% may not reflect the applicability of the method for trace contaminants. For screening approaches, optimization of extraction protocols will seek to improve recovery and precision of a target list of analytes from different compound classes (pesticides, antibiotics, etc.) which will then be applied to other samples to screen for the target analytes along with other contaminants present (Jia et al., 2017).

Recently, metabolomics was found to be an appropriate strategy for the identification of other MG metabolites in rainbow trout (Dubreil et al., 2019) but to the best of our knowledge, this approach has yet to be used to determine MG metabolites in other species, including brook trout or shrimp, as residues have been detected in multiple species in markets in Montreal (Dinh et al., 2020). The objectives of the present study were to: (i) compare four different extraction methods, based on commonly used criteria in non-target analysis, to study the fate of MG in two different organisms, brook trout (*Salvelinus fontinalis*) and shrimp (*Litopenaeus vannamei*) either raw or cooked; (ii) evaluate the effect of data processing parameters on the selection of the method, and (iii) apply a metabolomics workflow to identify metabolites in muscle tissue. Extraction methods included QuEChERS, which has been successfully applied to non-target screening in seafood (Jia et al., 2017) along with simple solvent extraction coupled with freezing in order to compare different sample clean-up strategies. The impact of instrument parameters, e.g., resolving power has been assessed for their effect on features extracted (Knolhoff et.al., 2019) while the impact of data processing parameters has been assessed in non-target analysis in terms of compound identification, e.g., false positives (Kunzelmann et al., 2018; Tian, Lin, & Bayen, 2019; von Eyken & Bayen, 2019). However, to the best of our knowledge, the effect of data processing parameters like peak height thresholds, on feature extraction and repeatability have not been studied. Therefore, the novelties of this study are the identification of other MG

metabolites in brook trout and white shrimp, a comparison of extraction for this purpose, and the assessment of the impact of data processing parameters on the selection of the optimal extraction.

2. Materials and methods

2.1 Chemicals

MG chloride (>96.0%) and LMG (>98.0%) analytical standards were obtained from Sigma Aldrich (St Louis, MO, USA). MG oxalate technical grade standards used for trout exposure was obtained from Fisher Scientific (Waltham, MA, USA) and for shrimp exposure, Acros Organics (Geel, Belgium). Labelled internal standards, d_3-diphenhydramine and d_3-6-acetylmorphine, were purchased from Cerilliant (Round Rock, TX, USA). HPLC grade acetonitrile, methanol, water, LC-MS grade formic acid, acetic acid and ammonium acetate were obtained from Fisher Chemical (Pittsburgh, PA, USA). Anhydrous magnesium sulfate and sodium acetate were purchased from Sigma Aldrich (St Louis, MO, USA). Primary secondary amine (PSA) sorbent was purchased from Agilent (Santa Clara, CA, USA). All glassware used was baked in an oven at 320°C for four hours and rinsed with methanol before use. Labelled internal standard solution of 0.4 µg/mL was prepared in methanol and stored at -20°C in amber vials. MG and LMG standards of 1 mg/mL and working standards of 20 µg/mL were prepared in methanol and stored at -20°C in amber vials. All standards were prepared fresh every 6 months. Five calibration standards, from 3 to 20 ng/mL, were prepared in water (0.1% formic acid).

2.2 Trout exposure

For MG exposure, two tanks of 250 L each (one control and one for exposure) were used with ten trout (1:1 male/female) in each tank. Trout (mean length 44.6±4.5 cm) weighed between 0.6 and 2.1 kg (mean weight 1.3±0.4 kg). Water temperature was between 4-5°C and pH 7.6.

Trout were exposed to 2 mg/L MG for 90 minutes, after which they were sacrificed. Exposure time, procedure, and euthanasia followed the normalized procedures accepted by the UQAR Animal protection committee. Briefly, trout were anesthetized using MS222 (tricaine methanesulfonate) and sacrificed by severing of the spine. Exposure time was established based on earlier studies. Mean MG and LMG levels in rainbow trout exposed to 1.5 mg/L MG for one hour were 528 and 2823 ng/g respectively one day after treatment (Bajc, Jenčič, & Šinigoj Gačnik, 2011). Comparable levels, 590 ng/g for MG and 1030 ng/g for LMG, were obtained for catfish exposed to 1 mg/L MG for one hour (Doerge et.al 1998). To account for discrepancies between fish weights amongst the different exposure studies and ensure detectable levels of MG and LMG, an experimental condition of 2 mg/L for 90 minutes was used in this study. Fish were filleted using stainless steel knives, wrapped individually in aluminum foil and polyethylene bags and stored at -80°C.

2.3 Shrimp exposure

Pacific white shrimp were obtained from Planet Shrimp facilities (Aylmer, ON, Canada) Two tanks of 60 L (one control and one for exposed) each filled with distilled water were used, with 60 shrimp per tank. Artificial seawater (16 g/L) was prepared with sea salt (Instant Ocean, Blacksburg, VA, USA) based on recommendations from Planet Shrimp facilities. Water pH was 8, temperature of 29°C and dissolved oxygen 5 mg/L. Shrimp were exposed to 0.4 mg/L MG for 2 hours. This level is in the range of those reported in the literature (0.2 mg/L for 2 hours) reported to lead to muscle concentrations of 20 and 79 ng/g for MG and LMG respectively (EFSA 2016). At the end of exposure, shrimp were sacrificed by placing them on ice. Shrimp were individually wrapped in aluminum foil and polyethylene bags and stored at -80°C.

182 *2.3 Sample extraction*

183 Trout muscle was homogenized using a blender, while shrimp muscle was homogenized
184 using a mortar and pestle.

185 Extraction 1 was adapted from Dasenaki & Thomaidis (2015). Briefly, 1.0 g of sample
186 was weighed into a 50 ml centrifuge tube. Water, 2 mL (0.1% formic acid v/v) was added and
187 the samples were vortexed for 1 minute. Acetonitrile (2 mL) followed by methanol (2 mL) were
188 added, with samples vortexed for 1 minute between each solvent addition. Samples were
189 centrifuged (Eppendorf, Hamburg, Germany) for 4400 rpm (3000 × *g*, 25°C) for 10 minutes.
190 Supernatant was collected in new tubes and transferred to -20°C for 14.5 h for lipid precipitation.
191 Samples were then centrifuged again for 10 minutes at 4400 rpm, filtered using a 0.22 μm PTFE
192 filter (Canadian Life Science, Peterborough, ON, Canada) and stored in amber vials covered with
193 aluminum foil at -20°C.

194 Extraction 2 (QuEChERS) was adapted from Jia et al. (2017). Briefly, 1.0 g of sample
195 was weighed into centrifuge tubes, after which 5 mL (84:16 v/v) acetonitrile/water with 1%
196 acetic acid was added and vortexed for 1 minute. To each sample, 1.0 g of $MgSO_4$ and 0.30 g
197 sodium acetate were added, vortexed for 1 minute followed by centrifugation at 4400 rpm (3000
198 × *g*, 25°C) for 5 minutes. Supernatant (2 mL) was transferred to new tubes containing 0.24 g
199 $MgSO_4$ and 25 mg PSA, vortexed for 1 minute and centrifuged for 5 minutes at 4400 rpm.
200 Extracts were filtered using a 0.22μm PTFE filter and stored in amber vials at -20°C.

201 Extraction 3 was adapted from Nacher-Mestre, Ibanez, Serrano, Perez-Sanchez, &
202 Hernandez (2013). In short, 5.0 g of trout muscle or 2.0 g of shrimp muscle was weighed into 50
203 mL centrifuge tubes. For trout extraction, 10 mL (80:20 v/v) acetonitrile/water with 0.1% formic
204 acid was added, while for shrimp extraction 4 mL of the same solvent mixture was added.

205 Samples were vortexed and centrifuged at 4400 rpm (3000 × *g*, 25°C) for 10 minutes.
206 Supernatant (2 mL) was transferred to new tubes, covered in aluminum foil and stored at -20°C
207 for 2 hours. Extracts were centrifuged again for 10 minutes, 4400 rpm, filtered using 0.22μm
208 PTFE filter and stored in amber vials at -20°C.

209 Extraction 4 followed the same protocol as extraction 3, except for no formic acid was
210 added.

211 For each extraction, 10 replicates were prepared along with 5 procedural blanks. Blanks
212 were prepared following the same protocols as described above, but with no sample added. Five
213 QC injection samples were prepared by pooling 10 μL of all extracts and blanks from all four
214 extractions. Extraction QCs (n=5) were prepared by pooling 20 μL of all five blanks and ten
215 replicates for each extraction. For LC-MS analysis, 100 μL of each sample was diluted with
216 water (1/10) and 50 μL of a 0.4 μg/mL solution of the labelled internal standards was added.
217 Extraction QCs were diluted as such to obtain 0.01 g of matrix in the vials for direct comparison
218 between extractions. Labelled standards were not added for quantification purposes, but rather to
219 monitor the instrumental variability.

220 Recovery experiments (n=6) were completed for both raw and cooked tissues of trout and
221 shrimp. Samples were spiked with MG/LMG to achieve a target concentration of 400 ng/g and
222 300 ng/g in trout and shrimp muscle respectively, and were allowed to equilibrate for 10 minutes
223 before extraction. Extracts were prepared the same way as exposed samples for LC-MS analysis.
224 Matrix effect and absolute recoveries were calculated according to the protocols set out by
225 Matuszewski, Constanzer, & Chavez-Eng (2003).

226 *2.4. Thermal treatment*

To obtain cooked samples, homogenized shrimp and trout muscle were transferred to 40 mL amber vials, capped and placed in a water bath at 100°C. Trout muscle was boiled for 30 minutes, to ensure it was completely cooked. Shrimp was boiled for only 10 minutes, as a longer boiling time led to high water loss and too much disintegration of the muscle.

2.5 Instrumental analysis

Samples were analyzed using an Agilent UHPLC 1290 coupled with an Agilent 6545 QTOF-ESI-MS, in both positive and negative ionization modes. In positive mode, mobile phases were (A) H_2O with 0.1 % formic acid and (B) acetonitrile and in negative mode, mobile phases used were (A) 0.05 M ammonium acetate and (B) acetonitrile. For both positive and negative modes, the same gradient elution was used, starting from 1 min 5% B, from 1 to 15 min gradient to 100% B, from 15 to 20 min 100% B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. An InfinityLab Poroshell 120 (Pheny-Hexyl, 3.0 x 100 mm, 2.7 µm, Agilent Technologies) with a Poroshell (4.6 mm) Phenyl Hexyl pre-column was used. Flow rate was 0.2 mL/min, injection volume was 2 µL and column temperature was 20°C. The MS parameters were as follows: sheath gas temperature 275°C, drying gas temperature 325 °C, drying gas flow 5 L/min, sheath gas flow 12 L/min, nebulizer pressure 20 psi, capillary voltage 4000, nozzle voltage 2000 V, fragmentor voltage 175 V, skimmer voltage 65 V. All Ions MS/MS mode at collision energies of 0, 10, 20 and 40 V was used. Data was collected between 100 and 1700 *m/z* at a rate of 3 spectra/s.

Each sample type was considered an individual batch and was run at the same time, i.e. all four extractions for trout raw were run together (all replicates, blanks, extraction QCs and injection QCs). Samples were kept at 4°C in the multi sampler compartment.

2.6 Data treatment

SPSS Statistics software (v.26) (IBM, NY, USA) was used for statistical analysis to compare the four extractions. A three-way ANOVA was performed with the type of extraction, sample type (shrimp and trout) and process (raw and cooked) as the independent variables to evaluate differences between recovery and matrix effect values. To evaluate the impact of data processing parameters, a four-way ANOVA was performed with type of extraction, mass window, peak filter height and post-processing peak absolute height as the independent variables. Dependent variables considered were: (i) percentage of features with RSD<20%, (ii) percentage of features with RSD<30%, (iii) number of features present in all extraction QC samples at a ratio sample/blank>2 or not present at all in blanks.

Concentrations, based on external calibration, were computed using Agilent Mass Hunter Quantitative Analysis B.07.0. Method detection limit (MDL) and limit of quantification (LOQ) were calculated as 3σ and 10σ, respectively, of the procedural blanks integrated at the retention time of the target compounds. Data alignment and feature extraction were completed using Agilent Mass Hunter Profinder software B.08.0. A feature can be defined as an entity for which a neutral mass, retention time and abundance can be assigned. Ion species and isotopes are included. To evaluate each extraction individually, molecular feature extraction (MFE) was performed for each (10 replicates, 5 procedural blanks and 5 extraction QC samples), using the following default parameters: peak filter height 200 counts, retention time window ±0.30 min, mass window ±10.00 ppm, post-processing peak absolute height 1000 counts, MFE score 80. Features were exported in Excel with only features present in all replicates at a ratio of sample/blank>2 used to calculate repeatability, expressed as CV or relative standard deviation.

To assess the impact of data filtering parameters, each parameter was changed one a time, while keeping the remaining parameters as default. The following values were assessed for each

parameter: peak filter height 500 and 1000 counts, mass window ±5 and 50.00 ppm, post-processing peak absolute height 200 and 5000 counts. Peak filter height will set a threshold for chemical and background noise, which can be set at 100-300 counts (Du et al., 2017; Mezcua, Malato, García-Reyes, Molina-Díaz, & Fernández-Alba, 2009; von Eyken & Bayen, 2019). Post-processing peak absolute height is the minimum height at which a compound is considered to be found. For metabolite identification, control and exposed trout and shrimp samples were extracted using Profinder default parameters and exported to .cef files. Files were imported into Mass Profiler Professional (v 14.8, Agilent Technologies) for statistical analysis (volcano plot, $p<0.05$, fold change>2) to identify statistically significant compounds that could be considered as other metabolites of MG.

3. Results and discussion

3.1 MG and LMG extraction

All four extraction methods extracted both MG and LMG from the raw tissues of exposed brook trout and shrimp with the average concentrations listed in Table S1. Fig.1 shows the chromatograms for MG and LMG in standard solutions and incurred shrimp extracts. For all four extractions, the extracted ion chromatograms showed clear peaks with little background signals. In general, somehow better LOQs (Table S2) were achieved for LMG compared to MG; for example, LOQ of 1.6 ng/g for LMG was determined for *Extraction 1*, compared to 3.0 ng/g for MG. LMG MDLs for *Extraction 2* for trout and shrimp were below the set interim limit of quantification of 0.5 ppb set in Canada (Health Canada, 2017).

Matrix effects for MG ranged between 82 and 106% in raw trout (Table S3). Values below 100% indicate signal suppression, while values above 100% indicate signal enhancement (Matuszewski et al., 2003). Slight ion suppression, with matrix effects for raw trout of 89 and

296 82%, was observed in *Extractions 3* and *4*, which can be expected as they are the simplest
297 extractions with very little clean-up. In cooked samples, further suppression was observed for
298 *Extraction 4* e.g., matrix effects of 63%. *Extraction 2* (QuEChERS) showed little matrix effect,
299 around 100%, for MG in both raw and cooked samples. Hurtaud-Pessel, Couedor, & Verdon
300 (2011) reported a matrix effect for MG of 88% in raw rainbow trout based on a similar extraction
301 with acetonitrile/magnesium sulfate, but no clean-up with sorbents. For shrimp, little matrix
302 effect was observed for MG for any of the extractions, with values ranging between 91 and
303 110%. For *Extraction 2,* mean values observed of 109 and 110% for raw and cooked shrimp,
304 respectively, are similar to those reported previously by Hurtaud-Pessel et al. (2011) for MG
305 (i.e., 103.7 and 105% in raw and boiled shrimp, respectively).
306 In terms of MG recovery, the lowest values were found for *Extraction 1* (<50%), with
307 *Extraction 2* providing the best recoveries for raw and cooked trout and shrimp (67-105%) and
308 best precision (RSD<30%). Recoveries of MG between 48 and 78% (depending on the spiking
309 level) have been reported in shrimp with a QuEChERS-like extraction based on
310 acetonitrile/water and magnesium sulfate/sodium acetate, but with the sorbent clean-up step
311 omitted (López-Gutiérrez, Romero-González, Plaza-Bolaños, Martínez-Vidal, & Garrido-
312 Frenich, 2012). *Extractions 3* and *4* showed lower MG recoveries for shrimp (41-67%) and trout
313 (37-69%). Statistical analysis (Table S4) showed no significant interaction, in terms of MG
314 matrix effect and recovery between the three variables, type of extraction, sample type (trout or
315 shrimp) and process (raw or cooked) ($p>0.05$). However, based on between-variables
316 comparison, the type of extraction has a significant effect on MG matrix effect while a
317 significant difference was found for MG recovery between raw and cooked samples.

For LMG, *Extraction 2* again appeared to provide the best results in terms of recovery and precision. For raw and cooked trout and shrimp, matrix effect ranging from 54 to 96% with recoveries between 71 and 97 % were observed, similar to other studies where recoveries between 62-112% and 101-104.8% were found in shrimp and rainbow trout (Hurtaud-Pessel et al., 2011; López-Gutiérrez, Romero-González, Martínez Vidal, & Frenich, 2013).

For *Extractions 3* and *4*, the 2-hour freezing time appeared to be insufficient for removal of lipids and proteins, as precipitate formation was observed in the filtered extracts during storage at -20°C, even after a few days, which could have an effect on the matrix effect and impact quantification. Indeed, for raw trout, a matrix effect of 13% was measured for LMG (Table S3), indicating almost complete suppression. Another issue arising from the presence of precipitates and insufficient removal of proteins from samples is column clogging and poor performance associated with protein interactions (Sitnikov et al., 2016). Large variability in the response for recovery samples was also observed for *Extractions 3* and *4*, leading to a poor precision in terms of both matrix effect and recovery. Statistical analysis (Table S4) found a significant interaction between the three variables for LMG recovery, with the type of extraction and process type (raw and cooked) having a statistically significant effect. Although the use of an internal standard could correct for the poor precision for LMG observed in *Extractions 3* and *4*, due to the precipitate formation throughout storage *Extraction 2* was considered to provide the best results for MG and LMG, in terms of recovery and reduced matrix effects.

3.2 Number of extracted features

The number of features is a common parameter used for comparison of extractions in metabolomic studies (Table 1), as it may reflect the metabolome coverage.

The inspection of individual features revealed a large number present only in a single sample and many others present in procedural blanks. Features retained for statistical analysis are often filtered based on their occurrence in all or in a minimum of replicates (e.g., two out of three) (Arbulu et al., 2015; Knolhoff et al., 2019; Sitnikov et al., 2016; Theodoridis et al., 2012). Completely removing features that are present in blanks may remove key molecular features, so features present in blanks or resulting from the chemical noise are often filtered based on a specific intensity ratio comparing samples and blanks (Knolhoff et al., 2019). In this study, for trout samples, features only present in all five replicates of each of the two fish samples and extraction QCs, and absent in blanks or present at a sample/blank ratio (based on peak height) above 2, were retained. For shrimp matrices, features only present in extraction QCs and absent in blanks or present at a sample/blank ratio (based on peak height) above 2 were retained. In general, the highest number of features for both raw and cooked trout and shrimp were observed in *Extraction 3* and *4* (Fig. 2, Tables S7 to S12). As these extractions are the most generic extractions of the four, they may also extract other matrix components as showed by the higher number of features and confirmed by the more pronounced matrix effects observed for the two extractions, especially in the case of LMG in trout.

Modification of the MFE parameters, especially peak height and post-process peak absolute height significantly decreased the number of features extracted from the matrices (Tables S5, S7-S12). This can be expected as setting higher thresholds for peak height will eliminate smaller peaks (e.g., chemical noise). For example, for *Extraction 2* in cooked trout negative mode, increasing the peak height from 200 to 1000 counts decreased by more than half the number of detected molecular features. Still, with the above later threshold, *Extraction 2* performed the best amongst all four extractions. In some cases, such as shrimp positive mode

(Table S11), a slightly higher number of extracted features were obtained for *Extraction 2* (868) compared to *Extraction 4* (804), when setting 5000 counts as the absolute peak height, whereas *Extraction 4* had the highest extracted features when the default parameters are used. This indicates that features detected through *Extraction 4* had relatively lower intensities compared to *Extraction 2* and were not detectable with increasing absolute peak height. In the case of cooked trout positive mode, the highest number of features (1576) were obtained through *Extraction 2*, when using the default absolute peak height of 1000 counts. When the absolute peak height was increased to 5000 counts, it was *Extraction 4* that resulted in in a slightly higher number of features (875), compared to *Extraction 2*, through which only 760 features were extracted. Hence, for cooked trout samples, features detected through *Extraction 2* had lower intensities compared to *Extraction 4,* which is the opposite of what was observed in shrimp samples.

3.3 Repeatability

Repeatability, often expressed as the number of features present in all replicates with coefficient of variation (CV) or relative standard deviation (RSD) below 20 (Knolhoff et al., 2019) or 30% (Sitnikov et al., 2016) is another parameter used to compare extractions in non-target analysis. Particular attention should be paid to this parameter. If replicates are performed per more than one sample (i.e., one replicate per one individual fish), as opposed to all replicates performed per one sample (i.e., ten replicates per one individual fish), then high variability between features will not necessarily be due to poor method precision, but it could be due to variability among organisms (i.e., different metabolism, fat content). Sources of variability also include experimental preparation (i.e., extraction of replicates on different days). In this study, trout replicates were performed on two individuals (5 replicates/trout), while shrimp replicates were performed on individual shrimps, as their weights were too low to perform all replicates on

one individual. In trout, for the same extraction, different repeatability was observed between the two individuals. For example, in trout raw positive mode, for *Extraction 4,* 66.9% of features have an RSD<20% in fish 1. However, for fish 2, *Extraction 4* only has 38.8% of features with an RSD<20%. Similarly, for cooked trout samples analysed in negative mode, *Extraction 2* has 55.6% of features <20% for fish 1, but only 11.1% of features<20% for fish 2. Therefore, comparison of extractions based on the repeatability criterion was done based on CV of features detected in extraction QCs. Trout samples, both raw and cooked in positive mode, had around 50% of features with RSD<20% across all four extractions (Fig. 3). On the other hand, in negative mode, *Extraction 2* had the lowest percent features with a CV<20% between the four extractions. This trend was different in shrimp samples; *Extraction 2* had the second highest % features (62.6%) in negative mode, but the lowest in positive mode.

The modification of peak heights parameters had a statistically significant impact on feature repeatability (Table S5). Increasing the noise threshold (peak height) should eliminate these smaller peaks and could theoretically improve the repeatability of features. Indeed, in some cases, for example in shrimp analyzed in positive mode, increasing the peak height from 200 counts to 500 and 1000 counts, increased the percent features with CV<20% in *Extraction 1* from 51.7% to 61.8 and 63.5% respectively. But, when comparing all CV values between the four extractions at the same parameter, e.g., 500 counts, *Extraction 1* still performed the best, an identical conclusion as with default parameters. However, extractions that have good precision for extracted features may not always have the best precision for some target compounds, which was the case in this study.

409 *3.4 Overall extraction comparison*

410 The above results confirm that each comparison criteria varies with the matrix, the
411 extraction method, the instrumental analysis conditions but also with the data processing
412 approach. Overall, not one single extraction performed the best based on all comparison criteria
413 investigated (Table 2, Fig. 4) and depending on which criterion takes precedence a different
414 extraction would be considered optimal. Consequently, a standardization of the approaches for
415 the validation of metabolomics workflows, that can offer some guidance on the choice of sample
416 extraction method, is critically needed. Due to the high throughput of metabolomic studies,
417 repeatability remains a key parameter in sample preparation (Bekele et al., 2014). For example,
418 Sarafian et al. (2014) used a point-based system for the optimization of extraction procedures in
419 lipidomics, where the highest marked criteria (5 marks each) were given to repeatability and
420 lipid recovery compared to 2 marks for lipid coverage (i.e. different lipid groups). In cases where
421 the extraction that provided the highest number of features did not have the best repeatability, the
422 latter was preferred over number of features when choosing the optimal extraction (Arbulu et
423 al.2015). However, despite the importance of this evaluation parameter, there are currently
424 different ways of evaluating repeatability or reproducibility, with this being an aspect of sample
425 preparation that would benefit from a systematic approach. For instance, Xu et al. (2019) used
426 six biological replicates for their study on cell metabolomics with reproducibility evaluated
427 based on the grouping of replicates in a PCA model and CV calculated for a targeted list of
428 metabolites. In other cases, CV of features was also determined based on features extracted
429 across three replicates of the same sample (Knolhoff et al., 2019). As this study has shown,
430 different CVs for the extracted features were observed between two individual fish, therefore
431 more representative samples are needed when assessing this parameter. For this purpose, there

are several options proposed for determining feature repeatability. (i) based on pooled QC samples from each replicate after extraction or (ii) based on a pooled sample from each individual which is then extracted by all methods. For example, Theodoridis et al. (2012) prepared replicates for their solvent optimization study on grapes from a homogenous 1 kg sample. QC standard mixtures, composed of compounds with different chemical properties and present at high and low concentrations (Knolhoff et.al, 2019) spiked before extraction could also be used for assessment of repeatability, besides having other advantages. They have been used for further appraisal of data quality, e.g., mass accuracy and generation of formula for the spiked standards and could enable comparison of different data sets (Knolhoff et al., 2019). As this study has shown, data processing parameters e.g., peak height, had a statistically significant effect on the detectable molecular features and repeatability and should be taken into account as part of the sample preparation protocol for non-target analysis. The integration of the QC standard mixtures in routine non-target analysis can allow for optimization of the data processing parameters to improve compound identification and reduce false positives or false negatives. Another detail that must not be disregarded is the treatment of data obtained through negative ionization mode. The results in this study for negative ionization mode showed that while extractions were comparable in terms of molecular features, they were not in terms of feature repeatability. Although generally most compounds, including the two target compounds in this study, are ionized in positive mode, analysis in negative ionization mode could also be of benefit to identify other interesting compounds (Knolhoff et al., 2019). Therefore, the quality of data obtained through negative ionization mode and the effect of data treatment parameters must also be assessed.

Overall, despite a lower number of detected molecular features in raw positive mode observed for *Extraction 2* for trout, the generated data is still of good quality with good repeatability observed. *Extraction 2* also provided the best results amongst the four extractions in terms of precision and recovery for MG and LMG in both trout and shrimp. Although *Extraction 2* extracted a lower number of features in shrimp and had the lowest percentage of features with a CV<20% amongst all four extractions, since the same method should be applied to both matrices to identify common or unique compounds, it was also considered the best extraction method for shrimp muscle. Good results were also obtained for cooked trout for *Extraction 2* which means that this extraction could also be applicable for other objectives, such as identification of possible thermal transformation products formed during cooking. Therefore, *Extraction 2* was chosen in this study as the optimal extraction for identification of metabolites of MG in exposed brook trout and shrimp.

3.5 Metabolite identification

Ten exposed and ten control trout and shrimp samples were extracted using the selected QuEChERS method (*Extraction 2*). Following volcano plot analysis, 6 and 102 down-regulated compounds (higher in exposed compared to control samples), including MG and LMG, were identified in trout and shrimp respectively. Between those compounds, only four were common for both matrices (Table S13). The mass and generated formula for *Compound 4* matches the mass and formula for des-methylated LMG, (ratio LMG/des-methyl LMG 6.6) which has been previously identified as a metabolite in rainbow trout (Dubreil et al., 2019) and catfish (Doerge et al., 1998). It was not found in exposure water samples but was retroactively detected in calibration standards with an average ratio LMG/des-methyl LMG of 12.7. *Compound 3* corresponds to des-methylated MG (ratio MG/des-methyl MG of 20.1) which was found in trout

and shrimp exposure water samples with an average ratio MG/des-methyl MG of 1.3, compared to an average ratio of 2.5 across the calibration standards. The des-methylated forms of the parent compounds can also occur due to natural degradation of the compounds. Based on the higher abundance of des-methylated LMG in incurred tissues and the fact it was not detected in water samples, this indicates its presence in muscle is due to possible metabolism. Based on the generated formula, *Compound 1* is a possible product following cleavage of the conjugated structure to yield a benzophenone derivative. It has been described as a photodegradation product of MG and identified as 4-(dimethylamino)-benzophenone (DMBP) (Perez-Estrada, Aguera, Hernando, Malato, & Fernandez-Alba, 2008). MS/MS analysis and database search through ChemSpider (Royal Society of Chemistry) provided a match but with a low score of 80.6% for this benzophenone derivative. It was found in calibration standards with an average peak height of 4773 (ratio MG/DMBP 111) almost 15 times lower compared to the levels observed in exposed samples (ratio MG/DMBP 3.5). On the other hand, the peak height in exposure water samples was much higher (581425) with a ratio MG/DMBP of 0.58, which could be expected from a photodegradation product. The detection of this compound in muscle tissues could be due to oxidation of MG by hydroxy radicals. Its uptake by trout or shrimp directly from water needs to be further investigated.

4. Conclusion

Four extraction methods based on simple, solvent extraction were successfully applied for the screening for MG and LMG in brook trout and shrimp. Extractions were compared based on commonly used criteria in metabolomics and contaminant screening studies. Results show that based on different approaches, different extractions could be selected. A novel aspect of this study was the evaluation of data processing parameters on the number of features and

repeatability in terms of extracted features. Peak height was found to significantly influence these two parameters, and even in some cases lead to a different extraction offering the best results. Consequently, some compromises might be required and although a "one approach fits all" is not always applicable, some standardization of the comparison criteria, for example on how to evaluate repeatability or blank subtraction (should features present in blanks not be considered at all or should there be a minimum ratio between replicate/blank) is needed. Further development on the inclusion and utilization of QC samples can allow for better assessment of the impact of data processing parameters and comparison of different data sets.

The optimal extraction method, based on QuEChERS, chosen in this study was used to extract pacific white shrimp and brook trout exposed to MG. Other metabolites beside LMG had yet to be described in these two matrices previous to this study. Based on statistical analysis, des-methylated LMG was tentatively identified and proposed as another metabolite of MG in muscle.

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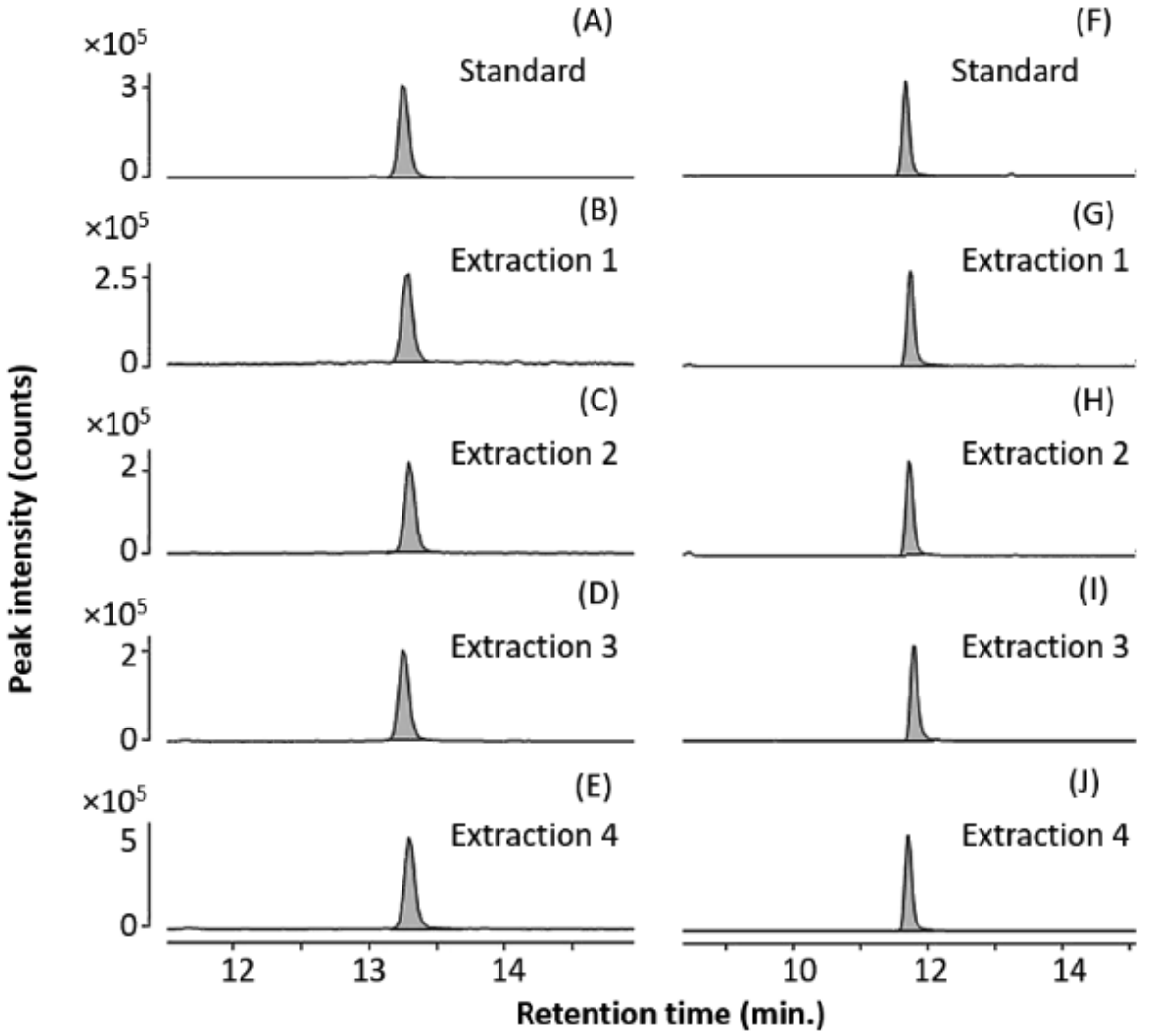
676 **Table 1:**

677 Criteria used for extraction comparison in non target/screening analysis

Approach	Matrix	Application	Extractions tested	Criteria	Reference
Metabolomics	wine	identification of non-volatile/semi-volatile metabolites	centrifugation filtration direct injection	• number of features • repeatability (expressed as CV of features)	Arbulu et al., 2015
	rice	metabolome profiling and geographic discrimination	different solvents	• extraction efficiency of various compound classes (lipids, sugars, lysophospholipids) • ability to discriminate between different geographic rice samples	Lim et al., 2018
	green tea	metabolome profiling	accelerated solvent extraction benchtop extraction	• extraction efficiency of catechins (expressed as concentration) • repeatability (expressed as standard deviation of extracted catechins) • metabolome profile (based on Principal Component Analysis clustering)	Kellogg, Wallace, Graf, Oberlies, & Cech, 2017
	apple	extraction polar metabolites	different solvents	• extraction efficiency of target polar metabolites (expressed as ratio between metabolite response vs. maximum response across all methods) • repeatability (expressed as relative standard deviation RSD of target metabolites) • recovery of target metabolites	Bekele, Annaratone, Hertog, Nicolai, & Geeraerd, 2014
	grapes	metabolome profiling	different solvents	• number of features • repeatability (expressed as RSD of features)	Theodoridis et al., 2012
	coffee	metabolite identification related to the roasting process	different solvents	• number of features	Perez-Miguez et al., 2018

	plasma	lipid profiling	different solvents	• protocol simplicity • lipid recovery • lipid coverage • protein removal efficiency • repeatability (expressed as CV of features)	Sarafian et al., 2014
	plasma	non lipid metabolome profiling	different solvents, SPE	• recovery of target metabolites • matrix effects • number of metabolites detected • repeatability of features (expressed as RSD)	Sitnikov, Monnin, & Vuckovic, 2016
	glioma cell lines	global metabolomics	different quenching solvents, cell disruption methods and solvent extraction	• PCA analysis • reproducibility and reliability (assessed as grouping of replicates in PCA and CV of metabolites) • metabolite coverage extraction efficiency (expressed as intensity of 68 target metabolites)	Xu et al., 2019
Contaminant screening	shrimp, fish, eel	veterinary drug screening	acetonitrile/SPE extraction (with different levels of acids)	• recovery of analytes	Turnipseed et al., 2017
	fish liver, water	organic contaminant screening	accelerated solvent extraction (different adsorbents and solvents) QuEChERS	• number of features • rate % false negatives	Du et al., 2017
	egg	antimicrobials and mycotoxins screening	QuEChERS (different solvents, pH, sample weight to solvent volume ratio)	• recovery, matrix effect and RSD of target compounds	Capriotti, Cavaliere, Piovesana, Samperi, & Lagana, 2012
	tilapia	veterinary drug screening	QuEChERS (solvent volume, pH, amount sorbent)	• recovery of target analytes	Jia et al., 2017

	wastewater	pharmaceuticals screening	SPE (different cartridges, pH)	• recovery of target analytes	Gros, Petrovic, & Barcelo, 2006
	fish, breast milk	PAHs, pharmaceuticals, PCBs, pesticides screening	QuEChERS (amount sorbent, pH) SPE	• recovery of target analysis	Baduel, Mueller, Tsai, & Gomez Ramos, 2015
General	infant rice cereal, orange juice, yogurt	general chemical coverage	dilute and shoot acetonitrile extraction QuEChERS	• number of features • repeatability (expressed as CV of features) • unique features • chemical coverage (molecular weight, chromatographic retention)	Knolhoff et al., 2019



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687 **Fig. 1**: Extracted ion chromatogram for MG (m/z 329.2012; Fig. A-E) and LMG (*m/z* 331.2168;
688 Fig. F-J) in extracted shrimp and pure solvent.
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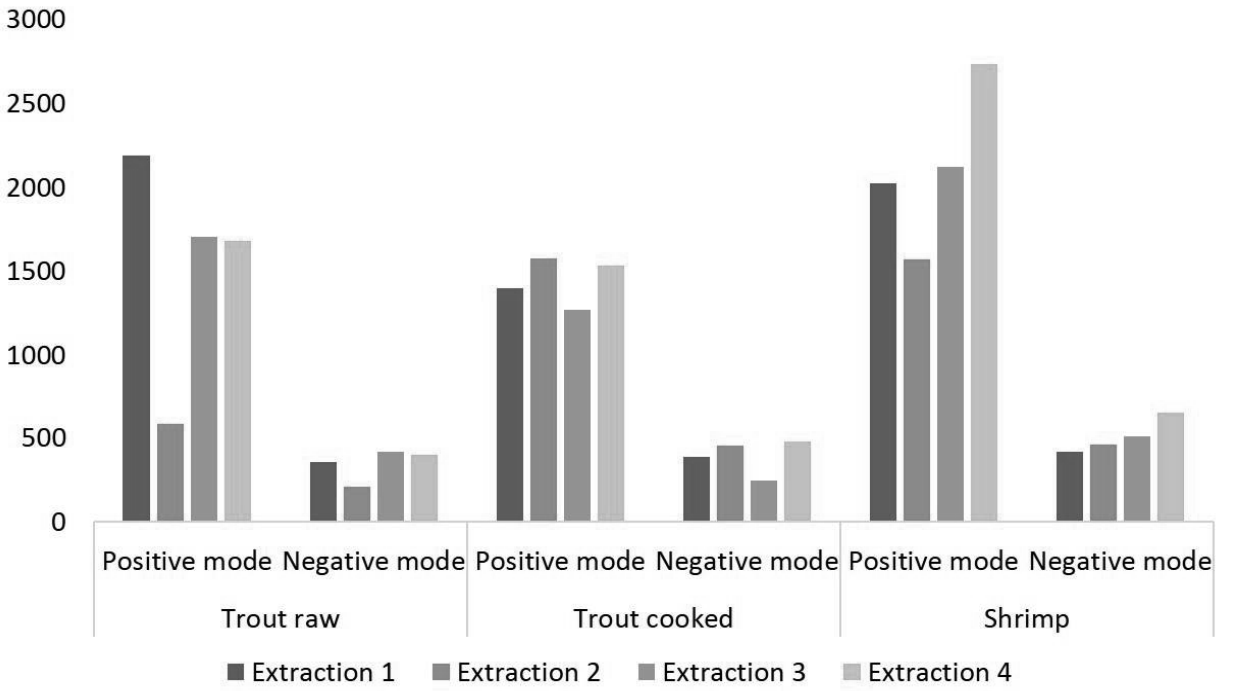


Fig.2: Number of molecular features extracted in trout and shrimp samples in different ionization modes

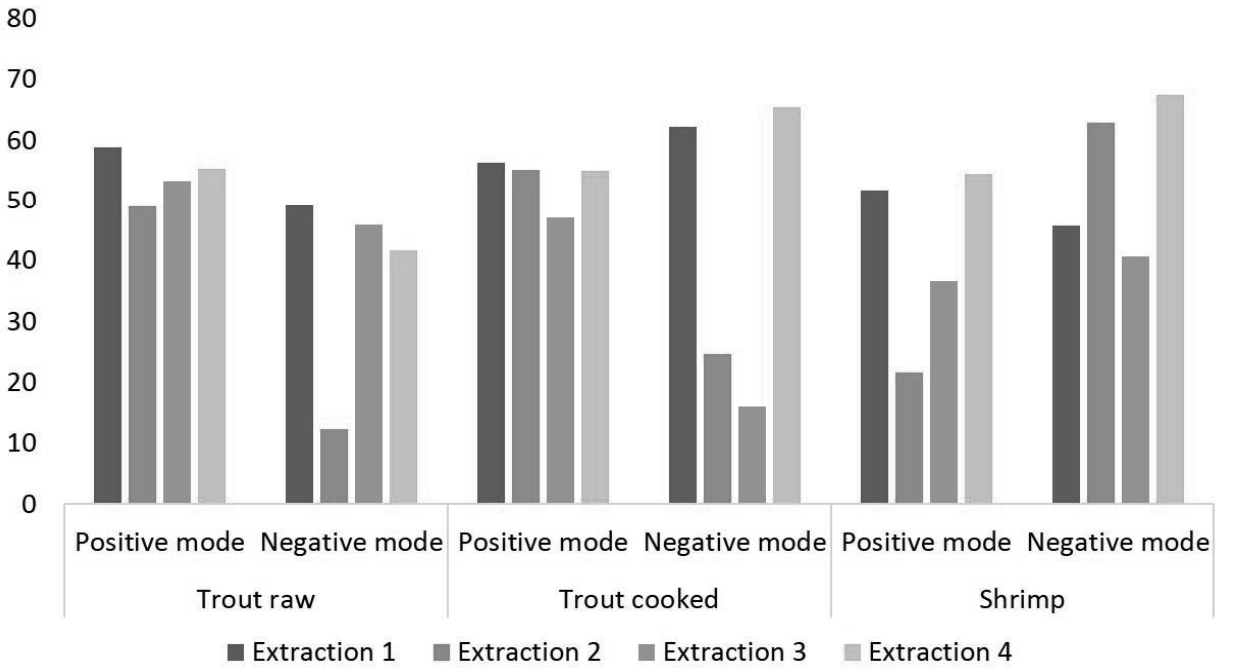
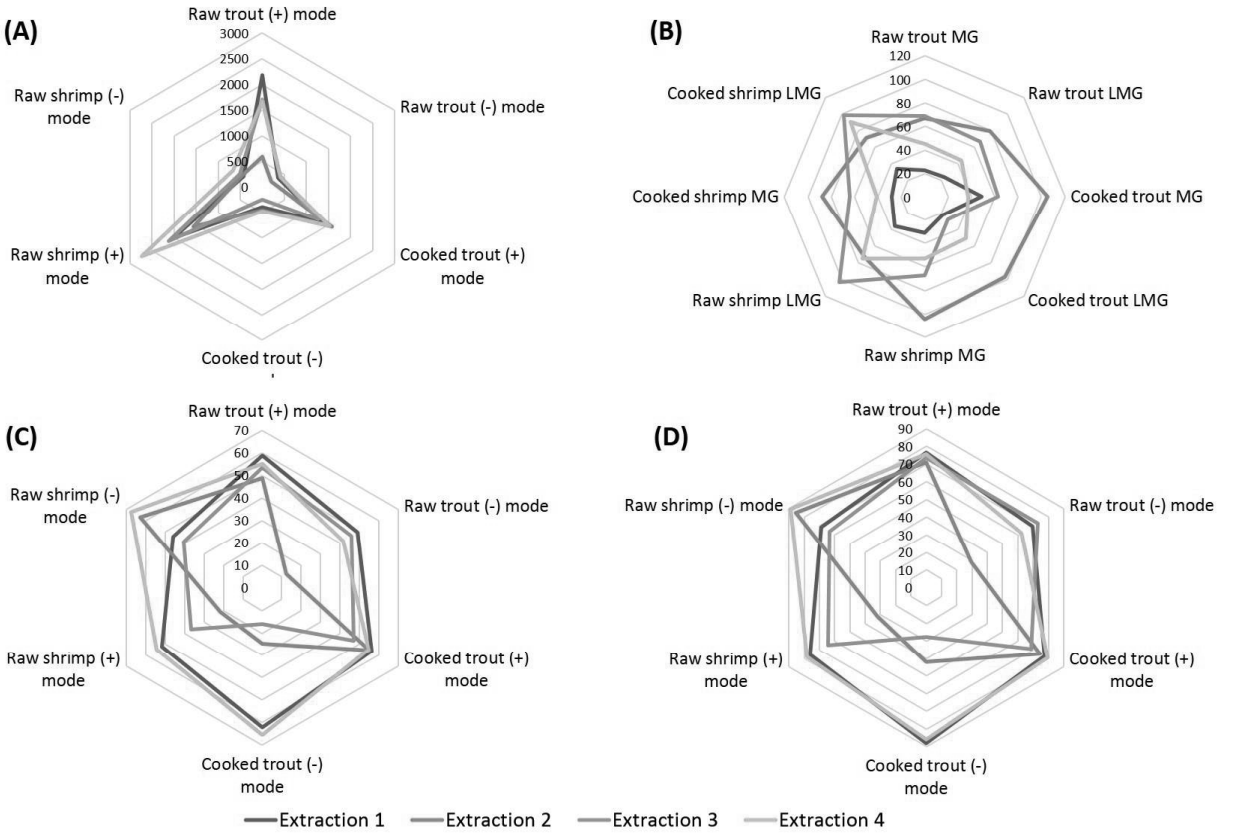


Fig.3: Percentage of molecular features with CV<20 % in extracted trout and shrimp in different ionization modes

Table 2:

Optimal extraction of MG exposed trout and shrimp based on different criteria of comparison

Criteria	Best extraction
Recovery of target analytes	• *Extraction 2* for both trout and shrimp
Precision of target analytes	• *Extraction 2* for both trout and shrimp
Number of molecular features	• Trout raw positive mode: *Extraction 1* • Trout raw negative mode: *Extraction 3* • Trout cooked positive mode: *Extraction 2* • Trout cooked negative mode: *Extraction 4* • Shrimp positive mode: *Extraction 4* • Shrimp negative mode: *Extraction 4*
Repeatability of features	• Trout raw positive mode: *Extraction 1* • Trout raw negative mode: *Extraction 1* • Trout cooked positive mode: *Extraction 1* • Trout cooked negative mode: *Extraction 4* • Shrimp positive mode: *Extraction 4* • Shrimp negative mode: *Extraction 4*



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709 **Fig. 4**: Extraction comparison based on: (A) number of features in QC ratio sample/blank>2 and absent in blanks, (B) recoveries MG

710 and LMG, (C) features with CV<20% and (D) CV<30%