



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. Sample preparation and data processing are critical in non-target/metabolomic workflows but there is currently no standardized protocol for the development of these methods. The impact of data processing parameters or the inclusion of a different matrix is not often taken into account during the selection of an extraction. Thus, this study aimed to investigate the impact of different extractions, e.g., QuEChERS, and data processing on the determination of malachite green metabolites in two different organisms, brook trout and shrimp. The results obtained confirm the need for a harmonized approach for the validation of non-target workflows, as depending on the comparison criteria, the matrix, the mode of ionization or data processing, a different extraction could be chosen. This study also identified for the first time des-methylated leucomalachite green as another metabolite in the two organisms.

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). Therapeutants are often used in aquaculture in response to stress conditions, such as high fish density and high ammonia/nitrite concentrations (EFSA, 2016). 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 des-methylated forms, e.g., 1, 2 and 3-

desmethyl LMG, and MG *N*-oxide identified as other metabolites in catfish (Doerge, Churchwell, Gehring, Pu, & Plakas, 1998) and rainbow trout (Dubreil et al., 2019). 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 (EFSA, 2016). 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 transformation products formed during food processing or cooking. The extraction and

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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-targeted analysis in food analysis. Non-targeted 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. This has been successfully applied for the screening of veterinary drug residues in seafood (e.g., fish and shrimp), including MG and LMG (Turnipseed et al., 2017). 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 analysis is based on the identification of compounds for which there is limited information, designing and validating the

Table 1
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 et al., 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 et al., 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 Target 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
	Fish, breast milk	Phahs, pharmaceuticals, pcbs, pesticides screening	Quechers (amount sorbent, ph), SPE	- Recovery of target analytes	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

extraction procedure can be challenging. Although no method will be able to offer full metabolome coverage, it should be robust, reproducible and efficient in extracting metabolites of interest (Antignac et al., 2011). Reproducibility is key for statistical analysis, like principal component or clustering analysis (Knolhoff, Kneapler, & Croley, 2019). There is currently no standardized procedure for the development and validation of extractions in non-targeted analysis/metabolomics (Rampler et al., 2021), including food metabolomics (Antignac et al., 2011). 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/suspect 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 commonly used 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).

Another critical aspect in a metabolomics workflow is the data processing step (Fisher, Croley, & Knolhoff, 2021). Incorrect data processing parameters can introduce errors during data analysis and cause inaccurate interpretation of the data (Antignac et al., 2011; Fisher et al., 2021). Similar to validation of the sample preparation step, data processing parameters should also be optimized across a metabolomics workflow (Fisher et al., 2021). For example, data processing parameters may be optimized for compound identification, in terms of false positive and false negatives (von Eyken & Bayen, 2019). However, to the best of our knowledge, there are no studies that assessed the impact of data processing parameters on the selection of an extraction method. Specifically, if the modification of one processing parameter, like peak height, would change which extraction performed best in terms of the comparison criteria, e.g., number of extracted features.

Overall, although there are studies in the literature that compared different extractions for metabolomic investigations (Table 1), the focus, especially for food metabolomics, has usually been on one sample type, and the food matrix is not often included as a comparison criterion during the sample preparation step. For MG-exposed organisms, metabolomics was found to be an appropriate strategy for the identification of other MG metabolites, e.g., des-methylated LMG in rainbow trout (Dubreil et al., 2019), but the impact of sample preparation for the purpose of metabolite identification was not studied. Furthermore, to the best of our knowledge, this metabolomic approach has yet to be used to determine MG metabolites in other species, such as shrimp, as residues have been detected in multiple species in markets in Montreal (Dinh et al., 2020). From a general non-targeted/suspect contaminant analysis approach, even though sample extraction is validated for different food matrices, is it often based on raw samples. Seafood is usually consumed following some kind of processing, and cooked samples should be included to account for the impact of thermal processing on the fate of contaminants and metabolites, as these metabolites could be used as marker of contamination. Therefore, the objectives of the present study were to: (i) compare four extraction methods (from the literature) based on various criteria, including the impact of different matrices, for the determination of MG and metabolites in two exposed

organisms: brook trout (*Salvelinus fontinalis*) and shrimp (*Litopenaeus vannamei*), (ii) evaluate the impact of data processing parameters on the choice of the extraction method and (iii) apply a metabolomics workflow to identify other MG metabolites in the two organisms. The novel aspects of this study are the identification of other MG metabolites in brook trout and white shrimp, a comparison of extractions for this purpose, along with the inclusion of different matrices as part of comparison criteria, and the assessment of the impact of data processing parameters on the choice of the extraction method.

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 and 5 °C and pH 7.6. Trout were exposed to 2 mg/L MG for 90 min, after which they were sacrificed. Water was sampled before MG was added to the tank and at the end of exposure. 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 min 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 h. This level is in the range of those reported in the literature (0.2 mg/L for 2 h) 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 . Water was sampled before MG was added to the tank and at the end of exposure.

2.4. Sample preparation

2.4.1. Comparison of extractions

Trout muscle was homogenized using a blender, while shrimp muscle was homogenized using a mortar and pestle. For each extraction, 10 replicates were prepared along with 5 procedural blanks. Blanks were prepared following the same protocols as described above, but with no sample added. For LC-MS analysis, 100 μL of each sample was diluted with water (1/10) and 50 μL of a 0.4 $\mu\text{g}/\text{mL}$ solution of the labelled internal standards was added.

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

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

Extraction 3 was adapted from Nacher-Mestre, Ibanez, Serrano, Perez-Sanchez, and Hernandez (2013). In short, 5.0 g of trout muscle or 2.0 g of shrimp muscle was weighed into 50 mL centrifuge tubes. For trout extraction, 10 mL (80:20 v/v) acetonitrile/water with 0.1% formic acid was added, while for shrimp extraction 4 mL of the same solvent mixture was added. Samples were vortexed and centrifuged at 4400 rpm ($3000\times g$, 25°C) for 10 min. Supernatant (2 mL) was transferred to new tubes, covered in aluminum foil and stored at -20°C for 2 h. Extracts were centrifuged again for 10 min, 4400 rpm, filtered using 0.22 μm PTFE filter and stored in amber vials at -20°C .

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

Filtered water samples collected from the exposure experiments were diluted 1/10 in water and spiked with 50 μL of the internal standards (0.4 $\mu\text{g}/\text{mL}$) solution.

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

2.5. QC

Five QC injection samples were prepared by pooling 10 μL of all

extracts and blanks from all four extractions. Extraction QCs ($n = 5$) were prepared by pooling 20 μL of all five blanks and ten replicates for each extraction. For LC-MS analysis, 100 μL of each sample was diluted with water (1/10) and 50 μL of a 0.4 $\mu\text{g}/\text{mL}$ solution of the labelled internal standards was added. Extraction QCs were diluted as such to obtain 0.01 g of matrix in the vials for direct comparison between extractions. Labelled standards were not added for quantification purposes, but rather to monitor the instrumental variability.

2.6. 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 min, to ensure it was completely cooked. Shrimp was boiled for only 10 min, as a longer boiling time led to high water loss and too much disintegration of the muscle.

2.7. Instrumental analysis

Samples (muscle and water) 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 (Phenyl-Hexyl, 3.0×100 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.

2.8. Data treatment

2.8.1. Quantification target analytes

Concentrations of MG and LMG, 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.

2.8.2. Feature extraction

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 for further data filtering.

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; von Eyken & Bayen, 2019). Post-processing peak absolute height is the minimum height at which a compound is considered to be found.

In previous studies, features retained for further 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 to assess repeatability. 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 to assess repeatability.

2.8.3. Statistical analysis

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% and (iii) number of features present in all extraction QC samples at a ratio sample/blank

> 2 or not present at all in blanks.

2.8.4. Metabolite identification

Control and MG exposed raw trout and shrimp samples ($n = 10$) were extracted, along with five procedural blanks, using the optimal method chosen based on the parameters discussed below in section 3. QC samples ($n = 5$) were prepared by pooling 20 μL of each replicate and procedural blank. For LC-MS analysis, 100 μL of extract was diluted with water, followed by addition of 40 μL of labelled internal standard mixture and analyzed using the method described in section 2.7. Data alignment and feature extraction was done 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 between control and exposed samples that could be considered as other metabolites of MG. Volcano plots have been applied in non-targeted metabolomics to identify statistically significant metabolites between two groups (Li et al., 2017).

3. Results and discussion

3.1. Extraction target analytes

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 ng/g set in Canada (Health Canada,

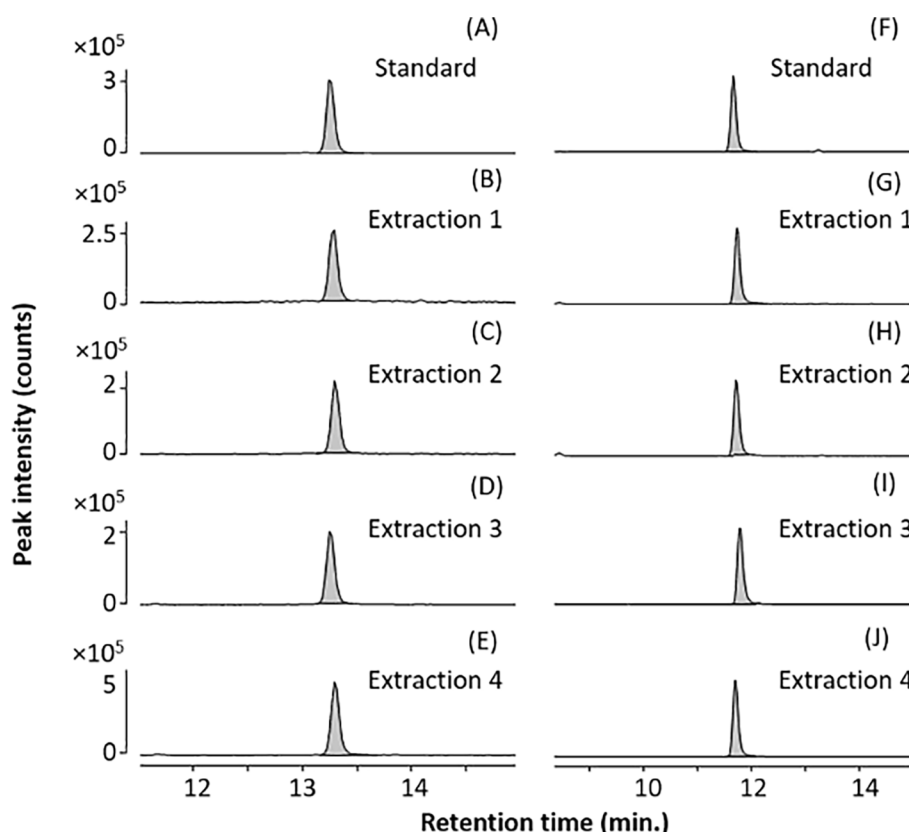


Fig. 1. Extracted ion chromatogram for MG (m/z 329.2012; Fig. A-E) and LMG (m/z 331.2168; Fig. F-J) in extracted shrimp and pure solvent.

2017).

3.1.1. MG

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, e.g., matrix effects of 89 and 82% for raw trout, was observed in Extractions 3 and 4, which can be expected as they are the simplest extractions with very little clean-up. Extraction 2 (QuEChERS) showed little matrix effect, around 100%, for MG in trout and shrimp, both raw and cooked samples. Mean values observed of 109 and 110% for raw and cooked shrimp, respectively, are similar to those reported previously by Hurtaud-Pessel et al., (2011) for MG.

In terms of MG recovery, the lowest values were found for Extraction 1 (<50%), with Extraction 2 providing the best recoveries for raw and cooked trout and shrimp (67–105%) and best precision (RSD < 30%). Recoveries of MG between 48 and 78 have been reported in shrimp with a QuEChERS-like extraction (López-Gutiérrez, Romero-González, Plaza-Bolaños, Martínez-Vidal, & Garrido-Frenich, 2012). Extractions 3 and 4 showed lower MG recoveries for shrimp (41–67%) and trout (37–69%).

Statistical analysis (Table S4) showed that the type of extraction has a significant effect on MG matrix effect with process type showing a significant difference was found for MG recovery (raw vs. cooked).

3.1.2. LMG

Extraction 2 again appeared to provide the best results for LMG 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 and 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, defined by a pronounced matrix effect, is column clogging and poor performance associated with protein interactions (Sitnikov, Monnin, & Vuckovic, 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. 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

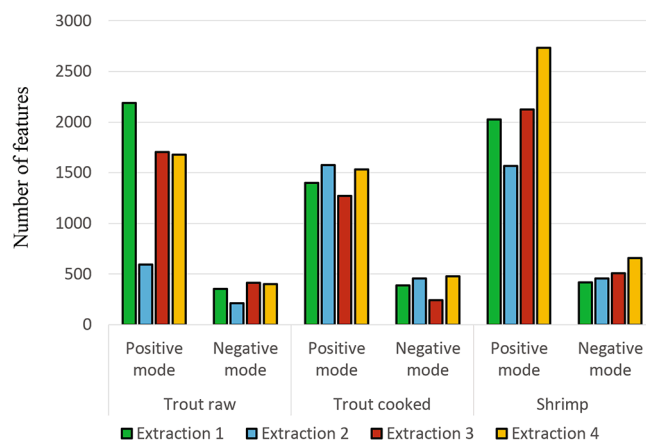


Fig. 2. Number of molecular features extracted in trout and shrimp samples in different ionization modes.

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). One of the goals of the study was to assess if the modification of one processing parameter would change which extraction would be considered best amongst the four. Indeed, in a few 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 parameter (1000 counts) is 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.

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-targeted analysis. 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. Based on the preliminary results for trout, different repeatability was observed between the two individuals (Tables S7–S12). For example, in trout raw positive mode, for Extraction 4, 66.9% of features in fish 1 and 38.8% of features in fish 2 have an RSD

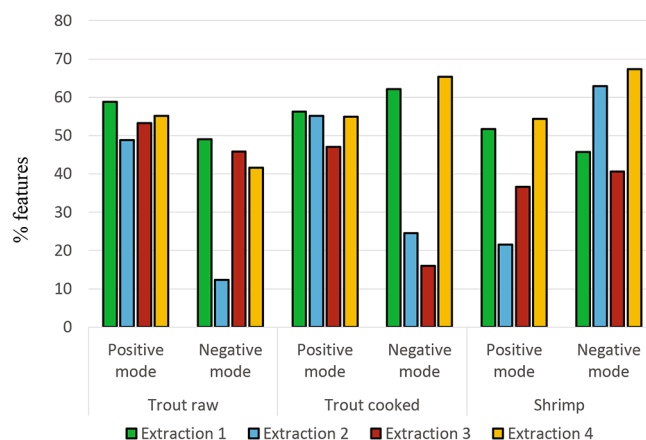


Fig. 3. Percentage of molecular features with CV < 20% in extracted trout and shrimp in different ionization modes.

< 20%. Hence, the comparison of extractions was done based on features identified in all extraction QC replicates. 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, it must be noted that 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.

3.4. Overall extraction comparison

The above results confirm that each comparison criteria varies with the matrix, the extraction method, the instrumental analysis conditions but also with the data processing approach. Overall, these results show not one single extraction performed the best based on all comparison criteria investigated (Table 2, Fig. 4) and depending on which criterion takes precedence a different extraction would be considered "optimal" for a metabolomics investigation of MG-exposed organisms. Currently there are also different approaches in the literature on data treatment that may potentially impact the choice of sample preparation, e.g., treatment of features found in procedural blanks. Consequently, a harmonization of the approaches for metabolomics workflows, further discussed below, that can offer some guidance on validation of the sample extraction method, is critically needed.

Due to the high throughput of metabolomic studies, repeatability remains a key parameter in sample preparation (Sarafian et al., 2014, Bekele, Annaratone, Hertog, Nicolai, & Geeraerd, 2014). In cases where the extraction that provided the highest number of features did not have the best repeatability, the latter was preferred over number of features when choosing the optimal extraction (Arbulu et al., 2015). However, despite the importance of this evaluation parameter, there are currently different ways of evaluating repeatability or reproducibility, with this being an aspect of sample preparation that would benefit from a systematic approach. For instance, Xu et al., (2019) evaluated reproducibility based on the grouping of replicates in a PCA model and CV

Table 2
Optimal extraction of MG exposed trout and shrimp based on different criteria of comparison.

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

calculated for a targeted list of metabolites. In other cases, CV of features was determined based on features extracted across three replicates of the same sample (Knolhoff et al., 2019). To ensure representative samples are used when assessing this parameter, pooled QC samples, from pre- or post-extraction replicates, can be used. 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-targeted analysis. The integration of the QC standard mixtures in routine non-targeted 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. The focus of this study was to first identify metabolites, resulting for example from demethylation, which has been proposed as a mechanism in other organisms (Doerge et al., 1998). We hypothesized that those metabolites would have behave similarly to the parent compounds, where good recoveries and precision of MG and LMG would translate to good precision for the metabolites. For the identification of common or unique metabolites, the same method should be applied to both matrices. Therefore, based on the best recoveries/precision of the target analytes, satisfactory number of features extracted and repeatability across the two matrices and different ionization modes, *Extraction 2* was chosen in this study as the suitable 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, 12 compounds with a matching score >70, beside MG and LMG were identified as present in statistically significant higher abundance in exposed compared to control organisms (Table S13). This match score is often used in non-targeted analysis for confident compound identification (Du et al., 2017). Although a search of the Agilent PCDL Metlin database yielded some possible matches, based on further MS/MS analysis, the identity of the compounds was not confirmed. Between those compounds, only four were common for both matrices. *Compound 5* corresponds to des-methylated MG which was also found retroactively in trout and shrimp exposure water samples and calibration standards. *Compound 6* matches the mass and formula for des-methylated LMG, 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 detected in calibration standards. The presence of the des-methylated forms in calibration standards or water samples may be due to natural degradation of

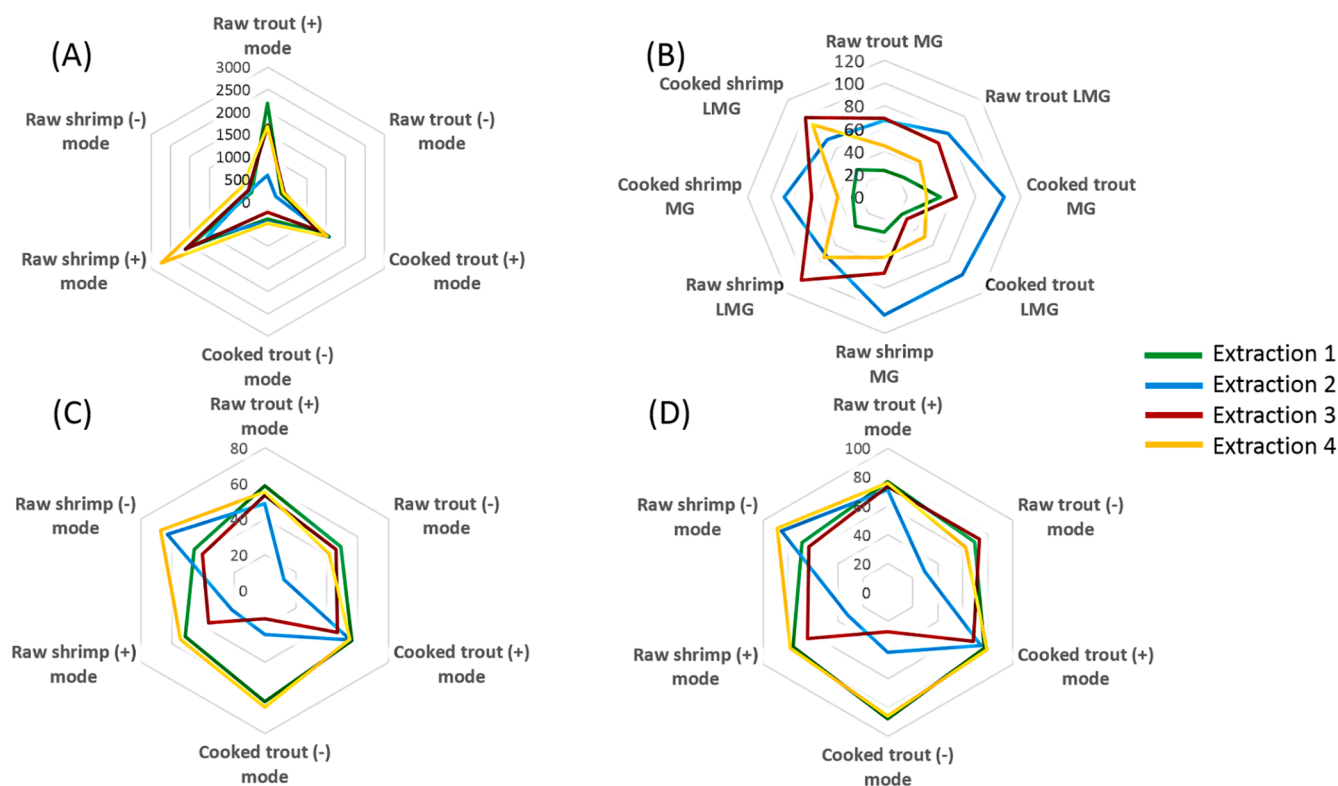


Fig. 4. Extraction comparison based on: (A) number of features in QC ratio sample/blank >2 and absent in blanks, (B) recoveries MG and LMG, (C) features with CV < 20% and (D) CV < 30%

the compound. Based on the fact that des-methylated LMG was not detected in exposure water samples, this could indicate its presence in muscle is due to possible metabolism. Currently, there are no analytical standards available for the complete identification and quantification of the des-methylated forms. 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 photo-degradation 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, 2020) provided a match but with a lower score of 80.6% for this benzophenone derivative. It was also found to increase in abundance compared to MG in water samples (ratio DMBP/MG of 1.7) while the ratio DMBP/MG in exposed samples was 0.29. 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 studied.

In order to investigate the hypothesis that the MG derived metabolites would behave similarly to the parent compounds, the precision of the tentatively identified des-methyl LMG was assessed, based on the peak height RSD across the 5 QC replicates, in the four extractions studied. For raw trout, the precision of the compound was 23.9, 6.3, 5.8 and 10.8% for Extractions 1, 2, 3 and 4 respectively. For raw shrimp, the precision of the compound was 32.5, 8.6, 42.6 and 23.0% for Extractions 1, 2, 3 and 4 respectively. It must be noted that in the case of raw shrimp, des-methyl LMG was only identified in three QC replicates for Extraction 3. These results support the suitability of Extraction 2 for the identification of MG derived metabolites in brook trout and shrimp.

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. This information is key for regulatory bodies involved in the surveillance of illegal dyes in seafood.

CRediT authorship contribution statement

Anca Baesu: Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Writing - original draft. : . **Céline Audet:** Methodology, Resources, Writing - original draft. : . **Stéphane Bayen:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing - original draft. : .

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.130567>.

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