Multiresidue GC-MS/MS pesticide analysis for evaluation of tea and herbal infusion safety

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To link to this article: https://doi.org/10.1080/03067319.2018.1518439
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ABSTRACT
A fast, simple, low-cost and high-throughput multiresidue pesticide analysis method was developed and validated for 300 pesticides in herbal and fruit infusion samples based on modified QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure combined with gas chromatography coupled with tandem mass spectrometry method (GC-MS/MS). The objectives were to develop low cost GC-MS/MS method, validate the method in accordance to SANTE/11,813/2017 guidance document and application in routine. The results obtained using different GC and MS/MS parameters were evaluated in order to develop quick, robust, accurate and effective multiresidue method. Total analysis time was 28 min with 0.6 µL injection volume. For accurate quantification, matrix-matched calibration (MMC) curves (in range of 10 µg/kg – 250 µg/kg) were applied to compensate matrix effect. The limits of quantification (LOQ) were ranged between 0.06 µg/kg and 135 µg/kg, and for the majority of the pesticides the LOQ were below the regulatory maximum residue limits. Most recoveries at 10 µg/kg and 100 µg/kg were in the range 70%–120% indicating satisfactory accuracy. The validated method was applied to commercial herbal and fruit infusion products detecting chlorpyriphos, DEET, tebuconazole, terbuthylazine, piperonyl butoxide, biphenyl, pendimethalin, pirimiphos-methyl and p,p′-DDE in more than 100 samples from 1,466 so risk assessment on human health was calculated specially for those pesticides.

1. Introduction

Tea (Camellia sinensis L.) is consumed by over two-thirds of the world’s population due to its medicinal, refreshing and mild stimulant effects. There are mainly four types of tea: black or red, oolong, green and white which are used for tea infusion (water extract from fermented tea leaf) worldwide [1]. According to European Tea Committee (ETC) and European Herbal Infusions Association (EHIA) definition, herbal and fruit infusion (HFI) materials are plants or parts of plants that do not originate from the tea plant (Camellia sinensis L.) and are intended for food use by brewing with freshly boiling water. They also include blends of HFI with tea as a minor component [2]. Tea together with HFI is amongst...
the world’s most popular and widely enjoyed beverages, thanks to their almost unlimited variety and their convenience [3]. Cultivation of herbs and tea is quite sensitive because mites, winged insects, caterpillars, variety of diseases and weeds can cause huge problems. In order to minimise all the above problems, the most common practise in cultivation and production is use of pesticides [4]. Determination of pesticide residues is very important, especially for prevention in the field of public health safety [5] but it is a big challenge due to the complex matrix of herbs, consisting of polyphenol compounds, pigments, sugars and alkaloids [6]. Those components, by their nature, can influence the physical and chemical properties of the pesticide, and thus directly on the insulating cavity. With all these difficulties, there are also potential international trade barriers due to the maximum residue limit (MRL) in tea and HFI established by most countries and some international organisations. The current MRLs for some pesticides in tea occasionally changing and becoming more stringent [4]. Regarding to this, sample preparation and multiresidue method for pesticide residues are key steps for identification and quantification. In nowadays, there are a lot of different pesticides available and it is very hard to cover all of them by a single analytical method due to the differences in physical/chemical properties. From these reasons there is tremendous need of tea and HFI producers and other quality control laboratories worldwide for low-cost multiresidue method for a large number of pesticides in one method, reliable identification and quantification with the shortest possible analysis time [7]. Very important feature of analytical method must be robustness with the shortest possible downtime which impacts a lot on TCO (total cost of ownership) for analytical technique and at the end competitive analysis price.

Thus, the aims of this study were as follows:

1. Optimisation of extraction and cleanup step for the GC-MS/MS analysis of 300 pesticide residues in tea and HFI
2. Development of low cost, sensitive, selective and robust analytical method
3. Validation according to SANTE/11,813/2017 [8]
4. Application in routine for real samples
5. Risk assessment on human health for most presented pesticides in tea and HFI from region Balkans.

Sample preparation is one of the most important parts of analytical procedure and it is preferred that it would be fast, accurate, precise and economic. QuEChERS (quick, easy, cheap, effective, rugged and safe) method has already proved successful for the determination of pesticide residues in food samples [9–12]. For multiresidue method of large number of pesticides, a generic procedure of sample preparation is required. During the sample preparation, other presented organic matter and impurities may be extracted along with the targeted analyte so tea and HFI sample is considered to be a difficult task to extract and clean.

Initial investment in hardware and TCO including maintenance, spare parts and consumables are extremely high and most of laboratories have tendency for reduction of total costs in order to be more competitive on the market. To date, there have been reports on the multiresidue analysis of pesticide residues in tea using GC-MS/MS or LC-MS/MS techniques. Even if there are an increasing number of publications using LC-MS/MS for pesticide residues, the main goal of this work is low cost analysis. LC-MS/MS is
more than twice hardware, software and maintenance price comparing to GC-MS/MS. QuEChERS procedure in combination with GC-MS/MS is one of the preferred approaches at present for residue determination of GC suitable pesticides [13].

Most of analysts in laboratories worldwide are using standard procedures and applications given from instrument suppliers without any adjustment and optimisation. Such kind of approach can be fast in implementation but it is always better to do the optimisation on site regarding the instrument and matrix in order to have higher sensitivity and robustness of the analytical method. Analyst is able to achieve much better performance of the instrument with customised approach because all instrument types in the market are a bit different and need to be considered in individual way. So, different injection port parameters, columns and MS/MS parameters were compared in experimental part in order to create rapid, accurate multiresidue method for the analysis in tea and HFI samples with improved sensitivity, analysis time, TCO and instrument downtime.

Pesticides were selected on the basis of information on the frequent detection of their residues since 2010 in unknown samples by Quanta Company. Most of them were available in 203 compounds mix and all other were added in the mix as single standards. Final analytical procedure has been applied to 1,466 (Balkans) tea and HFI samples of different varieties in order to provide insight into pesticide residue levels, application and distribution in region Balkans. Risk assessment on human health has been calculated specially for the most common pesticides. For that purpose, the acute/short-term (aHI) and the chronic/long-term (hazard quotient, HQ) consumer health risk was calculated [14].

2. Experimental

2.1. Reagents and chemicals

Pesticide certified reference standards (purity higher than 99%): comprehensive 203-compound GC Multiresidue Pesticide Kit was obtained from Restek Corporation (Bellefonte, Pennsylvania, USA) and individual certified standards from Accustandard (New Haven, CT, USA). QuEChERS extraction salt packets (EN and AOAC method), dispersive solid phase extraction and SPE step were purchased from Restek Corporation (Bellefonte, Pennsylvania, USA). Stock standard solutions of the targeted compounds were prepared at default concentration 100 ng/mL in acetonitrile. These solutions were stored in a freezer at −20°C. Working solutions containing in total 300 compounds were prepared by appropriate dilution of the stock solutions with acetonitrile at concentration levels of 2, 5, 10, 20, 50 ng/mL and stored under refrigeration at 4°C and renewed monthly. Acetonitrile is pesticide residue grade and purchased from J.T. Baker.

2.2. Samples

The blank samples used for MMC and recovery experiments were uncontaminated samples verified to present residue below LOD. Around 137 different tea and HFI sorts and in total 1,466 samples were obtained from tea and HFI growers in region Balkans.
2.3. Sample preparation

Samples were extracted following by modified QuEChERS-based approach in two steps: extraction with salting-out and sample clean-up. Briefly, 10 mL of acetonitrile was added to 2 g of homogenised sample (diluted with 10 mL of water for matrix hydration) contained in a polypropylene centrifuge tube and the sample was shaken. Salting-out was performed with salt mixture 4 g MgSO$_4$, 1 g NaCl, 1 g TSCD (Trisodium citrate dihydrate) and 0.5 g DHS (Disodium hydrogen citrate sesquihydrate). Afterward clean-up step was performed with a mixture of salts; 250 mg of MgSO$_4$ (magnesium sulphate), 150 mg primary and secondary amine (PSA) and 45 mg graphitized carbon black (GCB).

2.4. Instrument and apparatus

GC-MS/MS analysis was performed using a Shimadzu GC-2010Plus gas chromatograph with SPL-2010Plus (Split/splitless injector), OCI/PTV-2010 (On column/Programmable Temperature Vaporization Injector) and Optic-4 (Multi mode Injector). GC is coupled with a AOC-20i+s autoinjector and autosampler, a GCMS-TQ8050 triple-quadrupole and a computer with GCMS solution software together with Insight software for data acquisition, processing and statistics (Shimadzu Corporation, Kyoto, Japan). Analytes were separated on the Rxi-5Sil MS capillary column from Restek (0.25 mm i.d., x 30 m, 0.25 µm film thickness), which is selected as most optimum column comparing to Rxi-5SilMS with precolumn, Rxi-5MS and InertCap-5. The column was set at a linear velocity of 47.2 mL/min using helium as carrier gas. The column temperature was programmed as follows: the initial temperature was 40ºC (for 1 min) and increased to 125ºC at 40ºC/min, ramped to 300ºC at 8ºC/min, then was held for 7.00 min. The total run time was 28 min. The injection volume was 0.6 µL in splitless mode at constant injector temperature of 250ºC. The three transitions were determined for each compound and the collision energy was optimised for each pesticide. Quantitation by GC-MS/MS was based on MMC using the GCMS solution software. Identification of pesticides in fortified samples by GC-MS/MS was determined by comparing expected retention time and the ratio of the two qualifier transition results to MMC.

2.5. Method validation

In the validation, the main parameters including limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision were evaluated. The method validation followed the EU guidelines on analytical quality control and validation procedures for pesticide residue analysis in food and feed described in SANTE [8]. The selectivity of the method was evaluated by injecting extracted blank samples. MMC was used in order to minimise the matrix effect because matrix constituents may increase or decrease the analytical signal. Matrix effects were assessed by comparison of the slopes of five-point MMC with the slopes of the calibration curves in solvent.

Recovery study was carried out to determine the method accuracy and precision. In order to avoid quantitative errors, MMC standards were used to calculate the analyte recoveries. Solvent-based standards were also analysed to assess the matrix effects. Linearity of the MS/MS system was evaluated by assessing the signal responses of the
target analytes from MMC solutions prepared by spiking blank extracts at 5 sample concentrations, from 2 to 50 ng/mL, which correspond in the sample to 10 to 250 µg/kg because of correction factor which is 5.

### 2.6. Risk assessment

Risk assessment on human health was calculated specially for the most common pesticides. For that purpose, the acute/short-term (aHI) and the chronic/long-term (hazard quotient, HQ) consumer health risk was calculated and impact on human health was estimated.

### 3. Results and discussion

#### 3.1. Optimisation of GC-MS/MS conditions

Identification of pesticides in fortified samples by GC-MS/MS was determined by comparing expected retention time and the ratio of the two qualifier transition results. MS/MS detector parameters (transitions and collision cell energies) were optimised together with fine adjustment of MRM transitions in order to get the best intensity and optimum parameters for all pesticides. Transitions with the highest intensities with related collision energies were selected for quantification analysis as confirmation together with retention times for all the pesticides.

Different approaches were obtained in order to achieve low cost, robust and high sensitivity method. On column injection (OCI) [15], programmed-temperature-vaporisation (PTV) injection [16] and high pressure splitless injection with split-splitless (SPL) [17] were tested. OCI is the simplest and most reliable of these techniques, however, contamination of the column inlet with non-volatile sample materials is frequent and it is not suitable for fast elution components. With programmed temperature sample introduction, the negative effect of column contamination can be more or less avoided, because nonvolatile products are retained in a vaporisation chamber without reaching the analytical column. Large volume injection (LVI) techniques have gained wide attention for lowering system detection limits to meet newer and more stringent regulations. By introducing more samples into the system, the mass of analyte reaching the detector will be proportionately increased. If the baseline noise stays constant, larger peak height means greater signal-to-noise ratios and lower system detection limits. Another advantage of LVI is the decrease in solvent that reaches the detector. In LVI, the solvent is carefully evaporated and vented from the inlet before the analytes are transferred to the analytical column. Disadvantages of the LVI technique are that sample-to-sample cycle time increases because the GC oven and inlet need to be cooled down to a lower initial temperature, technique requires clean samples (not suitable for dirty and complex matrices), it is less flexible technique and lower intensities have been noticed for late eluting peaks. Also, LVI causes that downtime of the instrument because of necessary maintenance is more frequent which directly increase total cost of ownership for the analytical instrumentation and decrease efficiency throughput of samples.
In Supplementary information, in Fig. S1, comparison of peak height for different injection techniques and liners for pesticides with the biggest difference in height is presented. Different injection techniques and temperature programmes used in optimisation are listed below:

- SPL injector with inert ‘Sky’ liner with improved deactivation for trace-level analysis at constant temperature of 250, 200 and 170°C
- SPL injector with single tapper with wool liner at constant 250°C temperature
- SPL injector with focused liner used for focusing a high pressure splitless injection at constant 250°C temperature
- OCI/PTV with standard PTV liner and two different temperature programmes (Programme 1 = 60°C–10°C/min–250°C and Programme 2 = 60°C–50°C/min–250°C)
- Multi-mode injector for LVI with standard LVI liner and temperature programme = 60°C–800°C/min–250°C

The highest sensitivity was obtained with high pressure injection with focused liner in high pressure splitless injection mode-SPL injector (Fig. S1). Result of obtained data showed that high pressure splitless injection is the most suitable for larger number of pesticides.

After optimisation of injection port conditions and techniques, high pressure splitless injection with focused liner using Shimadzu SPL-2010Plus injection port is taken for further method development and selection of suitable capillary column. Analytes were separated on the Rxi-5Sil MS capillary column from Restek (0.25 mm i.d., x 30 m, 0.25 µm film thickness) which is selected as most optimum column comparing with Rxi-5SilMS with precolumn, Rtx-5MS and InertCap-5 (Fig. S2) for pesticides with biggest difference between different capillary columns. MS/MS detector with scan rate of 20,000 units/second is fast enough so there is no need for perfect separation. MS/MS detector parameters (collision cell energies, dwell time) were optimised together with fine adjustment of MRM transitions in order to get the best intensity and optimum parameters for all pesticides.

Final GC-MS/MS parameters are listed below:

- SPL injector with focused liner used for focusing a high pressure splitless injection at constant 250°C temperature
- Linear Velocity mode with 100 kPa pressure and high pressure injection mode with 250 kPa in first minute
- Column oven programme: initial temperature of 50°C _25°C/minute up to 125°C _10°C/minute up to 300°C with hold time of 11 min (Rxi-5Sil MS capillary column from Restek with 0.25 mm i.d., x 30 m, 0.25 µm film thickness)
- Column flow: 1.7 mL/minute
- Interface temperature: 250°C/Ion source temperature: 230°C
- Transitions with highest intensities with related collision energies are given in Table S1.

Experimental part for GC-MS/MS parameters optimisation was carried out with 1 µL injection volume. Around 0.6 µL injection volume is used for method validation measurements because peak areas were higher than 10,000 for most of the analytes which helps in accurate and precise peak integration.
Splitless high pressure injection, high temperature isothermal programme of injection port together with inert liner have the biggest influence in better peak shape and higher response time for all pesticides. This could be a main reason for satisfying method validation results for most of target pesticides.

3.2. Sample preparation

The QuEChERS method includes simple extraction step with acetonitrile, salting-out and clean-up step. For products with water content lower than 25% and high content organic acids, fatty acids, pigments, caffeine and sugars such as tea and HFI samples which are considered as dry commodity, the QuEChERS method has to be modified [13]. Important key elements in QuEChERS theory is salting out effect with magnesium sulphate and adjusting the polarity of organic phase [18,19]. Based on recoveries alone MgSO₄ is the best choice, but selectivity of the extraction process has to be considered.

Like for other food matrices, a widely used sorbent (purification material) for the removal of free fatty acids, sugars and other polar compounds present in extracts of food samples is PSA. As a polar sorbent, PSA can form hydrogen bonds with polar compounds from the matrix, but retention of more polar analytes can also occur and, therefore, the amount of this sorbent was tested in the method development.

Tea and HFI samples besides PSA and MgSO₄ required GCB [4,20] in order to remove high levels of pigments from the matrix. GCB can remove sterols and pigments such as chlorophyll [19] and strongly retains planar pesticides [4].

In order to achieve better recoveries, a clean-up step was tested using mentioned salting-out mixtures so aliquot of the supernatant was transferred to a tube containing mixture of PSA, MgSO₄ and GCB. Three different producers of mixtures were tested in order to choose one with the best recovery (Figure 1). These mixtures were selected because of different amount of PSA and GCB content: Mixture 1 tube containing mixture of salts; 900 mg MgSO₄, 150 mg PSA and 15 mg GCB, Mixture 2 tube containing mixture of salts; 900 mg MgSO₄, 150 mg PSA and 45 mg GCB and Mixture 3 is handmade mix of adsorbents and salts in quantities: 900 mg MgSO₄, 300 mg PSA and 150 mg GCB. From the Figure 1 it is obvious that Mixture 2 (Restek salt mix) provides better recoveries for 85 pesticides comparing to results with Mixture 1 (VWR handmade mix) and 3 (Supelco bulk adsorbents and salts). Differences in recoveries were presented only for selected 85 pesticides which showed the biggest difference and influence of mixtures.

On the basis of the obtained results it is clear that the different mixture content affects the efficiency of removing pigments from the tea and HFI matrix and improving the recoveries of the investigated pesticides.

Final extraction, salting-out and clean-up process:

- Add 10 mL water in 2 g of sample and shake 1 min
- Add 10 mL of acetonitrile in hydrated sample and shake 1 min
- Add salt mixture 4 g MgSO₄, 1 g NaCl, 1 g TSCD and 0.5 g DHS, shake for 1 min and centrifuge at 4,000 rpm for 5 min
- Transfer 6 mL of supernatant to 15 mL sample tube add 900 mg MgSO₄, 150 mg PSA and 45 mg GCB, shake for 2 min and centrifuge at 4,000 rpm for 5 min
- Filter through 0.45µm syringe filter and inject to GC-MS/MS.
The purification materials are used are more than traditional QuEChERS method, which is usually added to 1 mL of supernatant \([4,20]\). However, there are also papers in which this supernatant volume is considerably greater than 1 mL (3 mL \([12]\) or even 8 mL \([13]\)) as in this work. Otherwise, there is a difference in comparison between the EN and AOAC standard method. This difference is based on the corresponding changes in the step with salt mixture and the volume of supernatant and purification salts \([13]\). So, 6 mL of supernatant was selected according to EN 15,662 standard method and different combinations of amounts for MgSO\(_4\)/PSA/GCB were tested for best recoveries.

3.3. Matrix effect

To evaluate the matrix effect for all analytes, the calibration curves of pure solvent standards and matrix-matched standards were measured in this work, and the calibration curve slopes of matrix-matched standards and pure solvent standards were compared subsequently. It is well known that matrix effects are one of the main drawbacks of MS/MS methods, making quantification in samples problematic in some cases \([4,21]\). In gas chromatography matrix-effect is attributed to the presence of active sites in the injector, which causes the differences in the observed response for the given analyte in solvent compared to response in sample matrix (signal suppression or enhancement). The presence of matrix in the injected sample extract can block active sites in the injector, reduce thermal stress for labile compounds, prevent thermal degradation/adsorption of analytes delivering more analytes to the column and can have significant effect on recovery values \([22]\). Matrix effect can be very variable, dependent on specific combination of an analytes in a particular matrix and concentration level. Because of its random nature, it cannot be predicted for a specific analyte-matrix combination, so available tools should be applied to minimise its effect on the results. Soft matrix effects

\[\text{Figure 1. Recoveries for pesticides (10 µg/kg) with Salt 1, 2 and 3.}\]
(suppression or enhancement of 0–20%) are negligible. However, if the pesticides suffer medium (suppression or enhancement of 20–50%) or strong (suppression or enhancement of >50%) matrix effects, it is necessary to use certain methods to overcome the influence of matrix. The matrix effect was calculated by the equation:

\[
ME(\%) = \left( \frac{\text{slope of calibration curve in matrix}}{\text{slope of calibration curve in solvent}} - 1 \right) \times 100
\]  

In view of the above equation, the negative and positive values of the ME signify matrix-induced suppression and enhancement, respectively.

Values of ME are presented in Figure 2 and as can be seen most of the pesticides in tea and HFI samples suffer strong matrix effect.

Knowing that the nature of matrix effect is pretty varying, the percentage is just a relative indicator of the degree of suppression and enhancement. For most of the investigated pesticides, matrix affects the analyte signal in terms of enhancement, but it was not a general pattern because matrix effect is different for each analyte. Hexachlorobenzene and oxy-Clordane were not significantly affected by the matrix because change in signal is less than 20%. Pesticides like p,p'-DDE, trans-chlordane, cis-chlordane, heptachlor, o,p'-DDE, dichlobenil, beta-HCH, beta-endosulfan, biphenyl, chlorthal-dimethyl, alpha-endosulfan, pentachloroanisole and mirex show some degree (20%-50%) of medium signal enhancement. Eighty-nine per cent of the compounds like: bendiocarb, dichlofluanid, pyrethrin, fenoxycarb, famoxadone, folpet, etoxazole, bromuconazole-1, carbaryl, bifenox, fenvalerate-2 (esfenvalerate) and fluridone showed the highest signal enhancement. Only dichlorvos show signal suppression. This fact about signal enhancement goes in favour to lower detection limits for such small injection volume (0.6 µL).

The results proved that MMC is indispensable for accurate quantification by GC-MS/MS. Besides all mentioned results, more important is the fact that matrix effects for a given pesticide were similar in all matrices [12]. Since in this article the validated method will be applied to different types of tea and HFI samples, this fact greatly simplifies the procedures.

### 3.4. Method validation

The optimised methodology was validated in order to verify its applicability to the routine analysis of samples and to ensure the reliability of the results. Validation of
the method was based on the European Union guidelines [8]. The selectivity of the method was evaluated by injecting blank sample extracts. The absence of signal above a signal-to-noise ratio of three at the retention times of the target compounds showed that the method is free of interferences. The MMC curves for each compound were built using blank samples. For this, five concentrations levels were selected. The criteria adopted for the selection of the analytical curve levels were the signal to noise ratio and also the results of recovery studies. From this evaluation we selected the following concentration levels for the MMC curves: 2, 5, 10, 20 and 50 ng/mL. The LOD and LOQ for all pesticides, which were defined at a signal-to-noise (S/N) ratio of 3 and 10, were obtained, ranging from 0.018 to 40 µg/kg and 0.06 µg/kg and 135 µg/kg, respectively like it is shown in Figure 3.

Recovery study, inter-day and intra-day precision were carried out to determine the method accuracy and precision Table S2. In order to avoid quantitative errors, MMC standards were used to calculate the analyte recoveries. Solvent-based standards were also analysed to assess the matrix effects.

Method selectivity was assessed based on the presence of specific ion transitions (quantifier ion and two transitions for compound confirmation) at the corresponding retention time (Table S1), as well as the observed ion ratio values corresponding to those of the standards.

Method trueness was assessed by recovery studies using blank matrices spiked at concentration level 2 ng/mL which is equivalent to lowest possible maximum residue level (MRL) concentration and it is injected in five individually prepared replicates in Figure 4. Found concentrations, recovery and relative standard deviation (% RSD) were calculated. According to SANTE requirements recovery values are deemed acceptable if between 70% and 120% and relative standard deviation within ± 20%.

**Figure 3.** LOQ results for 300 pesticides.
Two hundred and sixty-three pesticides in concentration level of 10 µg/kg are within SANTE [8] recovery requirement range and only 1.3% of pesticides: difenoconazole-2, cypermethrin-2, terbacil and monocrotophos have recovery value higher than 120%. Pesticides like omethoate, thiabendazole, fluvinate-2, boscalid, fenvalerate, nicotine, propargite, pyrethrin and cyproconazole-2 have lower recovery in range between 28% and 58% and it can be compared to publication [22] where those pesticides were measured with liquid chromatography which is probably preferred technique for it. Only boscalide was measured in mentioned article with both, liquid and gas chromatography techniques and recovery of 75.6% was obtained (for a high concentration of 200 µg/kg) with gas chromatography which also proves together with our result that liquid chromatography would be better choice for boscalide. Based on obtained results, this GC-MS/MS multi residue method has proven to be very suitable for 87% of investigated pesticides, including pesticides like chlozolinate and fenobucarb which showed better recoveries comparing to other scientific publications. The triazole based pesticides (bitertanol, cyproconazole-1, myclobutanil, penconazole and triadimenol) showed nice recoveries comparing to very low recoveries in [23].

If there is a situation for the identification of pesticides in unknown sample whose recovery or RSD is outside the target range, accurate quantification will continue with standard addition method that is an alternative approach MMC method.

3.5. Comparison of the proposed method with other works

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and GC-MS/MS methods in combination with QuEChERS sample preparation have been widely used for pesticide residue analysis. LC-MS/MS instrument cost and its TCO are almost double price comparing to GC-MS/MS technique so that is the main reason why GC-MS/MS has been chosen as target technique for method development. There are no many articles with GC-MS/MS determination of pesticides in tea and HFI samples because of matrix complexity and difficulties with extraction. In this study, a very simple and robust GC-MS/MS method was developed for the determination of 300 pesticides in tea and HFI samples taking special care of costs, a potential competitiveness of laboratory on the

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**Figure 4.** 2D plot representing the recovery percentages for all pesticides over the entire analysis time.
market. Compared to other works involving pesticide residue analysis with GC-MS/MS in tea samples and cereals, the proposed method has some superiorities in respect to the number of target pesticides in only one method, number of real samples, analysis runtime, injection volume and method validation parameters such as linearity and LOQ, demonstrating the suitability of the method for multiresidue analysis in tea and HFI samples for regulatory and routine residue qualification and quantification (Table 1). Total analysis time of proposed method is shorter even up to 30% comparing to other methods which means reduction in consumption of carrier/collision cell gases and longer lifetime for filaments. Injection volume of proposed method is smaller from 40% up to 88% comparing to proposed methods so this also leads to reduction in total cost of ownership because much longer lifetime of liner, capillary column, quadrupole and electron multiplier detector.

Although it may seem that large amounts of relatively expensive purification salts (MgSO$_4$ is quite cheap; PSA price is 7–8 times higher than MgSO$_4$; GCB price is 2–4 times higher than MgSO$_4$) are used for 6 mL of supernatant, the resulting method is compromise between standard method, number of pesticides analysed in one method, total analysis time, good recovery and the lowest possible amount of GCB/PSA, and as such is still, despite all above-mentioned, quite economical.

3.6. Sample analysis

The validated method was applied to detect and quantify residues of the pesticides in different sorts of tea and HFI samples acquired from region Balkans. In order to ensure the quality of the results and evaluate the stability of the method proposed, an internal quality control was carried out on every batch of samples. In order to test the feasibility of the proposed approach for routine identification and quantification of pesticide residues in real samples, 1,466 samples were analysed for the target compounds and 21 pesticides were detected in more than 3% of total analysed samples as shown in Figure 5.

Chlorpyriphos, DEET, tebuconazole, terbuthylazine, piperonyl butoxide, biphenyl, pendimethalin, pirimiphos-methyl and p,p’-DDE were detected more than 100 times so those pesticides are considered further. It is interesting to point out that in 1,466 analysed samples included 137 different tea and HFI sorts and only 4 of them are contaminated the most with mentioned 9 pesticides: 10.0% of total biphenyl and 19.9% of tertbuthylazine positive samples are chamomile samples; 10.9% of chlorpyrifos, 13.1% of pirimiphos-methyl, 16.4% of tebuconazole and 36.0 % of pendimethalin positive samples are nettle herb samples; 14.9% of DEET and 16.1% of piperonyl-butoxid positive samples are blue mallow samples and 16.0 % of tertbuthylazine positive samples are shavegrass samples as shown in Figure 6. This finding is confirmed by the fact that tea matrices have the highest percentage of the European Union Maximum Residual Limit and contain more than five pesticides per sample [30], which is the reason for constant need for a suitable, versatile and reliable extraction method that can be determined.

In addition to the sorts of tea and HFI, it is important to emphasise the geographic origin of plants which are contaminated the most with these pesticides. More than 90% of measured nettle samples grew in Bulgaria, more than 50% of chamomile and shavegrass samples grew in Croatia and 100% of blue mallow flower samples grew in Croatia.
<table>
<thead>
<tr>
<th>No. of pesticides</th>
<th>Method</th>
<th>Matrix</th>
<th>Linearity</th>
<th>Concentration; Recovery (RSD)</th>
<th>LOQ</th>
<th>Analysis time</th>
<th>Injection volume</th>
<th>Number of real samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 pesticides in one method file</td>
<td>GC-MS/MS</td>
<td>137 different dried tea sorts</td>
<td>≥0.999</td>
<td>10 µg/kg; 70%-110% (≤20%)</td>
<td>≤ 50 µg/kg (for 90% of pesticides)</td>
<td>28 min</td>
<td>0.6 µL</td>
<td>1466 samples</td>
<td></td>
</tr>
<tr>
<td>200 pesticides</td>
<td>GC-MS/MS</td>
<td>3 different cereals</td>
<td>&gt;0.99</td>
<td>100 µg/kg; 70%-120% (&lt;20%)</td>
<td>5–50 µg/kg</td>
<td>38 min</td>
<td>1 µL</td>
<td>10 samples</td>
<td>[13]</td>
</tr>
<tr>
<td>8 pesticides</td>
<td>GC-MS</td>
<td>Green tea</td>
<td>&gt;0.995</td>
<td>100–500 µg/kg; 77–114% (≤16%)</td>
<td>15–30 µg/kg</td>
<td>40 min</td>
<td>1 µL</td>
<td>N/A</td>
<td>[24]</td>
</tr>
<tr>
<td>8 pesticides</td>
<td>GC-MS</td>
<td>5 different tea sorts</td>
<td>N/A</td>
<td>60 µg/kg; 92.1–99.6% (&lt; 6%)</td>
<td>N/A</td>
<td>40 min</td>
<td>1 µL</td>
<td>10 samples</td>
<td>[25]</td>
</tr>
<tr>
<td>78 pesticides</td>
<td>GC-MS/MS</td>
<td>N/A</td>
<td>&gt;0.99</td>
<td>50–100 µg/kg; 70%-120% (&lt;20%)</td>
<td>N/A</td>
<td>60 min</td>
<td>5 µL</td>
<td>55 samples</td>
<td>[26]</td>
</tr>
<tr>
<td>33 pesticides</td>
<td>GC-MS</td>
<td>1 tea</td>
<td>N/A</td>
<td>50 µg/kg; 70%-120% (&lt;20%)</td>
<td>N/A</td>
<td>18 min</td>
<td>1 µL</td>
<td>1 tea sample</td>
<td>[27]</td>
</tr>
<tr>
<td>490 pesticides in six methods (approximately 90 compounds in each group)</td>
<td>GC-MS</td>
<td>4 different tea sorts</td>
<td>N/A</td>
<td>10–1000 µg/kg; 60%-120% (for 94% pesticides)</td>
<td>N/A</td>
<td>40 min</td>
<td>1 µL</td>
<td>N/A</td>
<td>[28]</td>
</tr>
<tr>
<td>101 pesticides</td>
<td>GC-MS/MS</td>
<td>Fresh tea leaves</td>
<td>&gt;0.99</td>
<td>50 and 1000 µg/kg; 70%-120% (&lt;20%)</td>
<td>1.1–25.3 µg/kg</td>
<td>40 min</td>
<td>5 µL</td>
<td>N/A</td>
<td>[29]</td>
</tr>
</tbody>
</table>
3.7. Risk assessment on human health for most presented pesticides

Whereas the aforementioned pesticides (chlorpyriphos, DEET, tebuconazole, terbuthylazine, piperonyl butoxide, biphenyl, pendimethalin, pirimiphos-methyl and p,p'-DDE) mostly detected in 1,466 real samples, we decided to calculate risk assessment on human health especially for those pesticides. Exposure to certain harmful substances is a function of the share of the food consumed and the concentration of harmful substances and can be chronic or acute. The obtained data are compared with the toxicological reference values. In terms of food safety, certain types of foods considered safe for consumers if the estimated intake of pesticide residues does not exceed the ADI (Acceptable Daily Intake) or ARfd (Acute Reference Dose) [31] (Table S3).

To assess the acute or short-term consumer health risk (aHI) it is important to know estimated short-term intake (ESTI) and ARfd value, while estimation of the chronic or long-term consumer health risk (health hazard quotient, HQ) was based on the estimated daily intake (EDI) and ADI. The relevant formulas are following [32]:

**Figure 5.** Maximum residue level, average level of all measured real samples and count of positive measured samples for pesticides presented in more than 3% of analysed samples.
For the precise evaluation, the ARfD and ADI are expressed as a percentage of daily intakes for a 60 kg person. HQ was used to assess the non-carcinogenic health risk to hazard materials in food consumption. The HQ level lower than 100% is acceptable risk for human health unlike the case when it is higher than 100% [33]. For risk assessment of consumer’s exposure to pesticide residues, the estimated intakes on the daily basis are expressed as percentages of the ArfD and ADI values for the tested pesticides [34] and evaluations [35]. Food consumption of tea in Croatia is 0.18 g/day [36].

The dietary exposure to pesticides (mgkg\(^{-1}\)bw\(^{-1}\)day\(^{-1}\)) was calculated based on consumption data and individual body weights, and residue monitoring data (the highest residue and mean residue). The results are shown in Table 2. For short-term risk assessment, all the ESTI values were much less than the ARfD values. Furthermore, the highest risk was from chlorpyrifos with aHI 0.6598%. All the aHIs were little, which meant there was a negligible short-term or acute risk with the exposure to the tested pesticides.
pesticides via tea consumption. However, in the long-term risk assessment, the risk indexes (HQs) were notable lower than aHIs, which indicated the chronic risk from pesticide exposure via tea consumption do not need to be considered further.

Water solubility is one of the crucial physical and chemical parameters that influences the transfer rates of pesticides from herb to infusion because water is the most important carryover of pesticides during brewing. In this article worst case scenario calculated although it will be more precise or more preferable to determine the transfer rate, especially for more water soluble pesticides. Chlorpyrifos, detected in real samples in highest residue level, has solubility in water < 1 mg/l and octanol/water partition coefficient (log \( K_{ow} \)) = 4.7 [37]. Less water soluble and high \( K_{ow} \) pesticides do not infuse into tea brew from prepared tea [24] so this is additional indicator of negligible chronic risk from detected pesticide exposure via tea consumption.

### 4. Conclusions

A low cost, fast, simple and high-throughput multiresidue pesticide analysis method was developed and validated for 300 pesticides in tea and HFI samples based on QuEChERS procedure combined with GC-MS/MS. Method with total analysis time of 28 min with only 0.6 µL injection volume has been successfully applied to the analysis of commercial tea and HFI samples, such novelty of an analytical method without the need for continual, high-cost and time-consuming maintenance influence a lot on TCO in terms of reduction of maintenance costs especially regarding to sample preparation, injection port, columns, filaments, quadrupole and detector. Accurate retention time information combined with fragmentation was used for accurate analyte detection and quantification and MMC was applied to compensate matrix effect. The overall performance of the method was satisfactory for most of targeted compounds. The coefficient of determination (\( R^2 \)) for 91% pesticides were > 0.999 within the calibration linearity range of 2 µg/kg–50 µg/kg and limits of quantification (LOQ) were ranged between 0.05 µg/kg and 135 µg/kg. Recoveries for 88% of pesticides at 10 µg/kg were in the range of 60%–120% indicating satisfactory accuracy. Around 174 pesticides were detected in 1,466 real tea and HFI samples but chlorpyrifos, DEET, tebuconazole, terbuthylazine, piperonyl butoxide, biphenyl, pendimethalin, pirimiphos-methyl and p,p’-DDE were most presented pesticides. Regardless of the large number of pesticides

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>ESTI (mg kg(^{-1}) day(^{-1}))</th>
<th>ARFD (mg/kg bw)</th>
<th>aHI (%)</th>
<th>EDI (mg kg(^{-1}) day(^{-1}))</th>
<th>ADI (mg/kg bw per day)</th>
<th>HQ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>3.2988E-05</td>
<td>0.005</td>
<td>0.6598</td>
<td>7.7288E-07</td>
<td>0.001</td>
<td>0.0773</td>
</tr>
<tr>
<td>DEET</td>
<td>0.2697E-05</td>
<td>-</td>
<td>-</td>
<td>0.7390E-07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td>0.3638E-05</td>
<td>0.030</td>
<td>0.0121</td>
<td>1.2872E-07</td>
<td>0.030</td>
<td>0.0004</td>
</tr>
<tr>
<td>Terbuthylazine</td>
<td>0.1241E-05</td>
<td>0.080</td>
<td>0.0016</td>
<td>0.5862E-07</td>
<td>0.040</td>
<td>0.0001</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>0.0971E-05</td>
<td>-</td>
<td>-</td>
<td>0.4660E-07</td>
<td>0.200</td>
<td>0.0000</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>0.0281E-05</td>
<td>-</td>
<td>-</td>
<td>0.1905E-07</td>
<td>0.038</td>
<td>0.0000</td>
</tr>
<tr>
<td>Pendimethalin</td>
<td>0.0620E-05</td>
<td>1.000</td>
<td>0.0001</td>
<td>0.3781E-07</td>
<td>0.125</td>
<td>0.0000</td>
</tr>
<tr>
<td>Pirimiphos-methyl</td>
<td>0.1878E-05</td>
<td>0.150</td>
<td>0.0013</td>
<td>1.2066E-07</td>
<td>0.040</td>
<td>0.0003</td>
</tr>
<tr>
<td>p,p’-DDE</td>
<td>0.0734E-05</td>
<td>-</td>
<td>-</td>
<td>0.5080E-07</td>
<td>0.010</td>
<td>0.0005</td>
</tr>
</tbody>
</table>
detected and especially for the most presented pesticides, the calculation of short-term and long-term estimates of exposure to human health does not give worrisome information. All the aHIs were tiny, which meant there was a negligible short-term or acute risk with the exposure to the tested pesticides via tea and HFI consumption. In the long-term risk assessment, the HQs were notably lower than aHIs, which indicated the chronic risk from pesticide exposure via tea and HFI consumption do not need to be considered further.

Concentrations of pesticides found in tea and HFI samples confirmed the global concern regarding HFI and tea contamination with pesticide residues. Large number of pesticides, high precision of the method and simple sample preparation together with certificate of accreditation makes proposed method a valid candidate for economic quality control of tea and HFI samples.

Acknowledgments

The authors gratefully acknowledge use of the instruments, standards, real samples and overall support services of Shimadzu Ltd and Quanta Ltd.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References
