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Uncertainty and associated risks in the analysis of pesticides in homogeneous paprika samples

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Highlights

- Risk estimation based on sampling and sub-sampling uncertainty was performed
- Saffron and paprika were selected to analyse pesticides in homogeneous spiced products
- Subsampling has more uncertainty than in-product sampling due to the lot homogeneity
- Uncertainty is lower for weights below 30 g and increases for 100 g samples.
- An ideal sample size can be between 20 and 30 g

Uncertainty and associated risks in the analysis of pesticides in homogeneous paprika samples

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Abstract

In this study, risk estimation based on sampling and subsampling uncertainty was performed for pesticide analysis in homogeneous spice products such as paprika. The results of the subsampling were also used to estimate the minimum weight necessary in subsampling to minimize overall uncertainty. The results show that subsampling has more uncertainty than sampling in the product due to high homogeneity in the manufacturer's batch. On the other hand, results using the Monte Carlo (MC) simulation on the size of the subsample indicate that uncertainty is lower for weights between 20 and 30 g and increases for sample sizes of 100 g. A sample size of 30 g was used for saffron, and the values simulated with the MC method were confirmed.

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1. Introduction

The use of pesticides in the agricultural sector is widespread, mainly for the protection of livestock and crops. Currently, however, pesticides are often overused, with more than 4.0 million tonnes of pesticides used worldwide each year to control a broad variety of pests (Liu, Fan, Li, Zhao, Luo, Wang, et al., 2021). Reports from the European Union (EU) indicate that around 333,000 tonnes of pesticides were used in 2021, with more than 400 new pesticides on the market (Eurostat, 2023).

The use of these pesticides on crops makes it necessary to ensure that a minimum level find their way into foodstuffs. In order to prevent toxicity problems associated with the excessive use of pesticides, Maximum Residue Limits (MRLs) have been set by various regulations. In the EU, for example, the European Commission (EC) has set MRLs for various foodstuffs in the consolidated version of Regulation (EC) No 396/2005 (EC, 2005). This Regulation applies to both food and feed of plant and animal origin as well as to unprocessed spices such as pepper, but not to some processed foods such as flour or paprika. In these cases, manufacturers act differently depending on the type of product in order to ensure its safety. For foods where extrapolation from the raw material is possible, the manufacturer uses these MRLs (e.g. wheat flour uses the MRL for wheat). However, there are other products that involve a dehydration treatment, and this extrapolation is not possible. In these cases, article 20 of Regulation (EC) No 396/2005 (EC, 2005) allows the modification of MRLs. For this reason, the European Spice Association (ESA) has established dehydration factors for different spices (ESA, 2008; Schaarschmidt, 2016) and, for example, as is the case for paprika, the MRLs for pepper are multiplied by a factor of 10. However, there isn't a drying factor for all products. For saffron, for example, the MRL for chlorpyrifos was changed from 0.05 mg/kg to 0.01 mg/kg in 2020.

The main physical characteristic of these processed foods is their high homogeneity, owing to the industrial grinding and homogenisation treatments they undergo (e.g. wheat flour, paprika or spices ground by the manufacturer, such as saffron). This is a great advantage for carrying out pesticide analysis, as the product is already homogeneous and the laboratory does not have to perform further processes such as quartering or unit selection, as is the case with fruit and vegetables (EC, 2005; ISO, 1980; RD, 2003). This minimizes error sampling of subsampling by the laboratory.

The sample size to be sent to a laboratory depends mainly on two factors: the minimum quantity required by the analysis method, and the minimum quantity required to accurately represent the starting batch. As for the second factor, Regulations 2002/63/EC (EC, 2002) and 152/2009 (EC, 2009) provide guidelines as to how sampling for official control should be carried out. However, the recommendations for homogeneous samples only refer to the minimum sample size, e.g. 0.5 kg for flours and 0.1 kg for spices. This sample size can pose a significant problem for the analysis of high-value spices such as saffron. In the case of this spice, whose market price fluctuates greatly depending on quality and country of origin, it can mean incurring costs of up to €1000 per sample (0.1 kg) (Azafrán, 2023; Holz, Illarionov, Wax, Schmidt, & Fischer, 2023; Quoc, 2023) for laboratory analysis.

As for analysis methods, the Quick, Easy, Cheap, Effective, Rugged & Safe (QuEChERS) (UNE, 2019) method has been the most widely used pesticide extraction method in laboratories for the last two decades. In QuEChERS extraction, a sample size of 5 g (for dry products such as spices) to a maximum of 10 g (for fresh products such as vegetables or fruit) is sufficient for simultaneous analysis by GC-MS/MS and LC-MS/MS (da Costa Morais, Collins, & Jardim, 2018; Jiang, Cheng, Chen, Dong, Xu, Liu, et al., 2021).

There are publications that have looked at how sampling and subsampling affects pesticide residue results (Á. Ambrus & Soboleva, 2004). However, all these studies focus on fresh products such as fruit and vegetables which, due to the variability in their size or number of pieces, have greater uncertainty associated with sampling and subsampling.

Uncertainty is defined in the Guide to the expression of Uncertainty of Measurement (JCGM, 2008) as a parameter associated with results which characterizes the dispersion of the values that could reasonably be attributed to the measurand. Uncertainty is related to risk, as defined by the standard ISO 31000 (ISO, 2018), where risk is defined as the effect of uncertainty on the achievement of objectives. Although there are differences between the two concepts (Toma, Chiriţă, & Şarpe, 2012) both are important. For laboratories, the results obtained might contain false positives or negatives due to the high uncertainty associated with subsampling or analysis, while manufacturers risk having their products returned due to incorrect results stemming from product inhomogeneity or the sample size of the product sent to the laboratory. As an example, the new MRL for chlorpyrifos in saffron (Mahdavi, Eslami, Golmohammadi, Tajdar-oranj, Keikavousi Behbahan, & Mousavi Khaneghah, 2021) could pose a risk of false negatives in the laboratory, especially when the limit of quantification is 0.01 mg/kg, as well as for the manufacturer if the portion analysed is negative for chlorpyrifos but not actually representative of the batch to be sold.

Due to problems like this, one tool that has become common in risk analysis (ISO, 2018) is the use of the Monte Carlo (MC) method. The method is a simulation method developed around 1944 and it is increasingly used in different fields such as measurement uncertainty (ISO/IEC, 2008) engineering (Platon & Constantinescu, 2014) risk management (ISO, 2018) or pesticides (Guo & Li, 2021).

Therefore, the aim of this study was to evaluate the uncertainty and risk associated with sampling and subsampling between customer and laboratory in multi-residue pesticide analysis by GC-MS/MS and LC-MS/MS in homogeneous spice samples. Paprika and saffron were used for this study. Both spices are homogeneous: paprika was chosen to evaluate uncertainty and risk due to its low cost and high availability, while saffron was chosen in order to validate the results obtained from the paprika in a high cost, and therefore low quantity, of product in multi-residue pesticide analysis.

2. Material and methods

2.1. Samples

Ground samples of paprika and saffron were selected for this study. The saffron and the paprika were supplied by two different factories located in the Region of Murcia. Both companies are Spanish with high production rates. They export their products both nationally and globally based on the traceability of the batches produced. The total weight of each batch may vary based on the crushing and grinding performed, but production batches of at least 1000 kg are common.

The samples used in this study came from a batch previously analysed by the supplier. In the case of the paprika, the original batch was positive for GC analysis in fludioxinil and cypermethrin, defined in Regulation (EC) No 396/2005 as cypermethrin including other mixtures of constituent isomers (sum of isomers). For LC analysis, it was positive in acetamiprid and spirotetramat, defined in Regulation (EC) No 396/2005 as spirotetramat and spirotetramat-enol (sum of), expressed as spirotetramat. In the case of the saffron, the batch was positive for the pesticide chlorpyrifos and the organic compound piperonyl butoxide (PBO) in GC analysis, which is used as a synergistic component in pesticide formulations.

2.2. Pesticide analysis

The solvents for GC and LC analysis were supplied by J.T. Baker (Center Valley, PA, USA). These included ethyl acetate, methanol, water and residual formic acid. The ammonium formate, formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl) 1 g, disodium hydrogen citrate sesquihydrate (DHS) 0.5 g, trisodium citrate dihydrate (THS) 1 g, sodium sulfate (Na_2SO_4) 4 g, primary Secondary Amine (PSA), and C18 (end-capped) for dispersive solid phase extraction (dSPE) were supplied in an extraction kit by Agilent Technologies (Santa Clara, USA). All pesticides had certified reference standards with a minimum purity of 95% and were purchased from LGC Standards (Teddington, UK).

2.3. Data analysis

The Rstudio IDE Desktop 2022.02.01 tool and R 3.3.0 were used for data analysis and graph generation (Aria & Cuccurullo, 2017). This software is free and can be used in both desktop and cloud versions. Rstudio allows scripts to be created using code written in R. This can be used to automate optimizing for future studies. Different R libraries were used depending on the analysis carried out: for example, the data import into R was done using the Readxl library, and graph analysis and ANOVA analysis were performed using ggplot2. The R-script code used in this paper is available on request from the corresponding author.

The MC simulation technique performs numerical analysis by generating a large number of artificial values of a probabilistic variable using a defined distribution (Rezaei Kalantary, Barzegar, & Jorfi, 2022). The probability distributions used in R were rlnorm (lognormal) and rnorm (normal).

166 **2.4. Initial sample preparation**

167 The batch of paprika was prepared in the factory using the traditional methodology
168 (Fernández-Trujillo & Escarabajal, 2006). This consists of cleaning, pre-crushing by
169 hammer, milling in serial with at least 6 mills, mixing in a 1000 kg blender finally passing
170 through a 2000 µm sieve.

171 The saffron samples (provided as powder) were prepared by the producer-trader and the
172 homogenization included separation of stigmas, drying of stigmas and grinding and
173 sieving (Europasaffron, s.f.). It was not possible to obtain information about the sieve size
174 or the grinding mechanism that was used.

175 For the paprika, eight samples from the same production batch were analyzed. For the
176 saffron, due to its high cost, only three samples were obtained, and these samples were
177 used for validation after results were obtained from the paprika.

178 Each sample was weighed prior to subsampling and the results are shown in
179 supplementary Table 1. The samples were not manipulated in any way other than being
180 placed on a tray for subsampling as described in the 2.7 Subsampling preparation section.

181 **2.5. Pesticide analysis**

182 The QuEChERS extraction, based on EN 15662, used 5 grams of paprika or saffron, to
183 which 10 ml of water was added. After manual homogenization, 10 ml of acetonitrile was
184 added (E7 EN 15662 extraction) and the mixture was shaken for 1 minute. After shaking,
185 the salts (NaCl, DHS, THS, Na_2SO_4) were added and it was centrifuged for 5 minutes at
186 3000 rpm. A clean-up process using PSA and C18 was carried out (based in C3 EN 15662
187 clean-up) on the organic aliquot obtained and finally it was centrifuged again at 3000 rpm
188 for 5 minutes to get the final aliquot for GC-MS/MS and LC-MS/MS analysis.

A total of 185 pesticides were analyzed, and only 2 were detected in paprika and 2 in saffron by GC-MS/MS using an Agilent (Santa Clara, USA) 7890A Gas Chromatography system coupled with a 7000A quadrupole tandem mass spectrometer. Chromatographic separation was performed on a RTx-5MS column (30 m, 0.25 mm, i.d., 0.5 µm) RESTEK. Helium was used as the carrier gas at a constant flow rate of 1.2 $mL \cdot min^{-1}$, while argon was used as the collision gas. The oven temperature was adjusted as follows: the initial temperature was set at 70 °C for 2 min, then increased to 150 °C at a rate of 25 °C min^{-1}, to 200 °C at 3 °C min^{-1} with a hold time of 1 min, and finally to 280 °C at 10 °C min^{-1} with a hold time of 10 min. The total run time was 45 min. The temperatures of the transfer line and ion source were set at 280 ºC and 250 ºC, respectively. The multiple reaction monitoring (MRM) mode was used to operate the mass spectrometer.

A total of 115 pesticides were analyzed, and only 2 were detected in paprika by LC-MS/MS using an Agilent liquid chromatography system (Santa Clara, USA) coupled with a 6470 triple quadrupole tandem mass spectrometer. The chromatographic separation was performed on a Poroshell C18 column (150 mm, 2.1 mm i.d., 2.7 µm) (Agilent, USA) with a flow rate of 0.1 ml at 40 ºC. The elution solvent used was water 5 mM ammonium formate with 0.01% formic acid (A) and methanol 5 mM ammonium formate with 0.01% formic acid (B). The gradient elution was carried out as follows: 40% solvent B for 0-5 min, changing to 60% solvent B for 6-12 min and finishing with 100% solvent B for 17-20 min. Pesticides were analyzed using programmed MRM in positive and negative modes simultaneously. Ion source parameters include optimized drying gas temperature, drying gas flow, nebulizer pressure, sheath gas temperature and flow, capillary voltage, nozzle voltage and high and low radio frequency voltage.

The analysis of pesticides by GC-MS/MS and LC-MS/MS has a quantification limit of 0.01 mg/kg in the case of paprika and saffron, as shown in the validation results according to SANTE 11312/2021 (SANTE, 2021) in supplementary Table 2.

All the samples were analyzed with quality control in each batch using spiked samples over a paprika and a saffron blank sample according to SANTE 11312/2021 (SANTE, 2021). The results of quality assurance for the spiked samples can be seen in supplementary Table 3.

2.6. Uncertainty in the sampling analysis

The estimation of uncertainty for the whole process of pesticide analysis, from sampling to analysis, is described in an excellent way elsewhere (Á. Ambrus, Zentai, Sali, & Ficzere, 2011; Eurachem, 2019) and can be estimated using Equation 1. This equation could be used to estimate the relative uncertainty (CV) (Valverde, Aguilera, & Valverde-Monterreal, 2017) of results in multi-residue analysis of pesticide.

$$CV_{res} = \sqrt{CV_s^2 + CV_L^2} \qquad \text{Equation 1}$$

Note that CV is the nomenclature for coefficient of variation or relative standard deviation, calculated as the quotient of standard deviation and mean.

Where CV_{res} is the relative standard uncertainty of the multi-residue analysis, CV_s is the relative standard uncertainty of sampling and CV_L is the relative standard uncertainty associated with the laboratory analysis. The relative standard uncertainty for the analytical procedure can be divided into two main components as shown in Equation 2.

$$CV_L = \sqrt{CV_{sp}^2 + CV_A^2} \qquad \text{Equation 2}$$

Where CV_{sp} is the relative standard uncertainty associated with subsampling, and sample preparation included an extraction and clean-up process (Lehotay, Han, & Sapozhnikova, 2018) in the laboratory and CV_A is associated with the specific analysis, in this case LC-MS/MS and GC-MS/MS.

Modelling the estimation of the CV of sampling can be complex due to the large number of errors that need to be considered (Gy, 2012) such as grouping and separation errors due to sample heterogeneity, or weighing errors due to errors in assigning weights to different parts of an unequal composite sample. There are several estimates of CV_s for different crops (A. Ambrus, 2004; Á. Ambrus, Zentai, Sali, & Ficzere, 2011) with values between 25 and 40%, depending on the crop. However, this estimate does not apply to homogeneous samples or to analyses performed using the QuEChERS method (Han, Lehotay, & Sapozhnikova, 2018; Lehotay, Han, & Sapozhnikova, 2018).

The Codex Alimentarius Commission established a CV_s between 8 and 10% (WHO, 2013), but there's no further information about the type of samples it could be used on.

However, an estimate of uncertainty can be made empirically using the duplicate method (Lyn, Ramsey, Coad, Damant, Wood, & Boon, 2007), as shown in Figure 1.

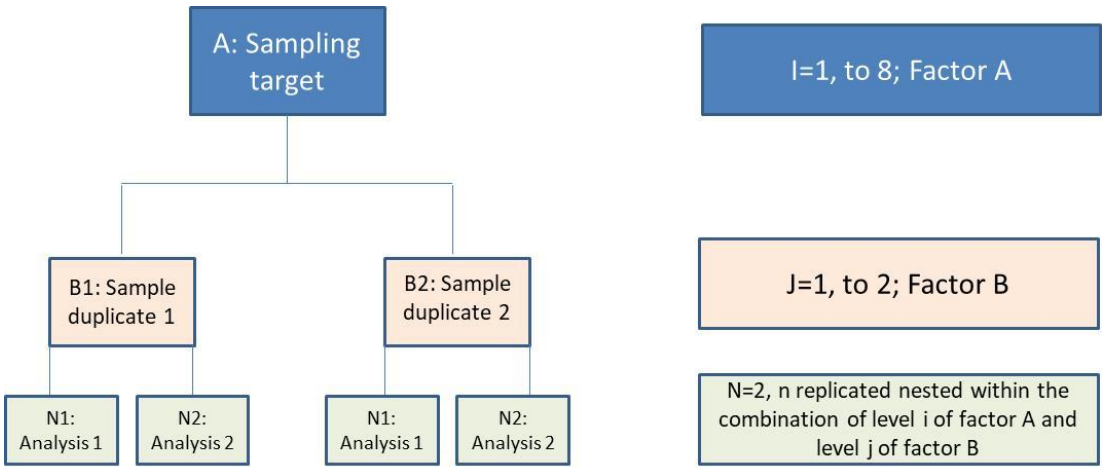

Figure 1. Duplicate design to estimation of the uncertainty.

This design is a two-way analysis of variance and is used to study the relationship between a quantitative dependent variable (pesticide concentration in the batch) and two independent variables (factors, subsampling and analysis), each with several levels. The two-way ANOVA allows us to study how each of the factors alone influences the dependent variable.

The associated variances for each of the sources of variability in a two-factor ANOVA are shown in supplementary Table 3.

Sample preparation is a source of uncertainty (Eurachem, 2019), mainly due to various errors associated with sample handling and heterogeneity. Some of the main sources are homogenisation and/or subsampling, drying, grinding or contamination. If the whole sample is prepared and analysed, the uncertainty of sample preparation, CV_{sp}, is zero, but in the QuEChERS method only 5 g are required. For an estimation of the CV_{sp} in different weights, a simplification of the equation of Gy's could be used (Gy, 2012) or the Ingamell´s sampling constant (Equation 3) (A. Ambrus, Solymosné, & Korsós, 1996).

$$CV_{sp} = \sqrt{\frac{Ks}{M_{tp}}} \qquad \text{Equation 3}$$

Where Ks is the Ingamell and Switzer sampling constant, defined as the weight of a single increment (w) that must be taken from a well-mixed material to maintain the relative subsampling uncertainty ($\%CV_{sp}$) at 1% with 68% confidence. M_{tp} is the weight of the sample, and this corresponds to the size of the subsample. Denoted that $\%CV_{sp}$ is the standard deviation relative of subsampling (A. Ambrus, Solymosné, & Korsós, 1996), divided by the median concentration.

From empirical CV_{sp} data, this equation can be used to estimate whether there are differences between CV_{sp} due to differences in K_s for the experimental weight used, or to

calculate the expected standard deviation for a given sample size, as shown in Equations 4 and 3.

$$Ks = M_{tp} \times CV_{sp}^2 \qquad \text{Equation 4}$$

Where M_{tp} is the subsampling size established as optimum after evaluation of the K_s values obtained.

Based on the evaluation of K_s (Equation 4), the duplicate design of Figure 1 is transformed to the design shown in Figure 2, where there are three types of weighted samples: 5 g for the minimum required by the QuEChERS method, 100 g to evaluate the normal minimum weight based on the R.D. 290/2003, and 20 g as an intermediate weight.

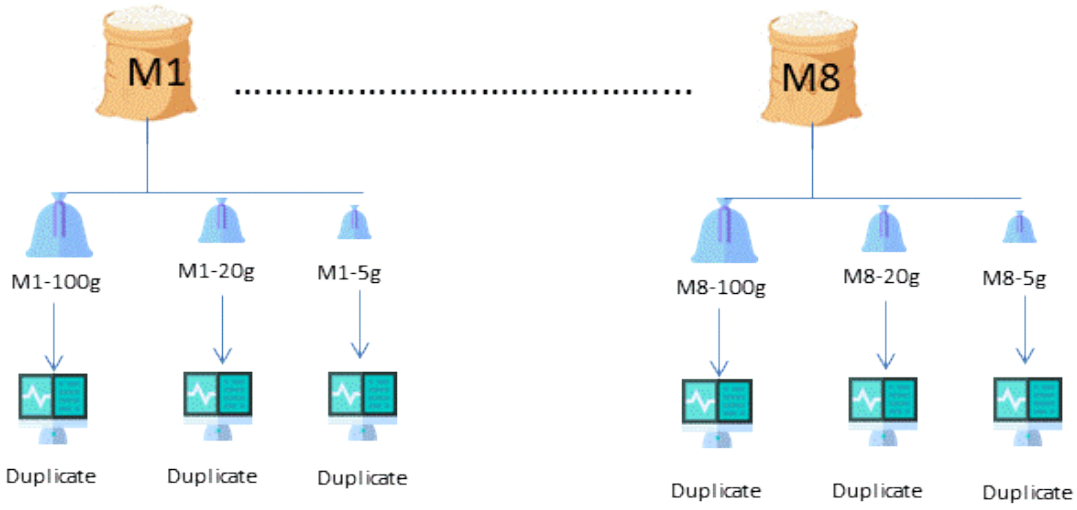

Figure 2. Modified design to compare variances and uncertainty.

Figure 2 shows the schema for the preparation of subsamples for the duplicate analysis in GC-MS/MS and LC-MS/MS. As 5 grams are used for the analysis of QuEChERS in spices, 5-gram samples M1 to M8 were prepared as duplicate samples in individual bags.

2.7. Subsampling preparation

290 To obtain each portion of the sample (5, 20 and 100 g) from the original sample (M1 to
291 M8, supplementary Table 1), a direct transfer was made into a plastic bag using a
292 disposable plastic spatula and weighed on a precision balance. The amount of sample was
293 weighed to exactly 5.0 grams, 20.0 grams and 100.0 grams.

294 For the duplicate analysis showed in Figure 2, 5.0 g of sample was weighed directly from
295 each heavier bag (20 g, 100 g) using a disposable plastic spatula to transfer to the
296 Eppendorf tube on a precision balance. In the case of the samples with 5.0 grams, the total
297 content of the bag was transferred directly to an Eppendorf tube. QuEChERS analysis
298 was then performed on the Eppendorf tube.

299 No homogenization of the samples was performed during these steps in order to avoid
300 including any additional homogenization processes in the analysis.

301 **2.8. Monte Carlo simulation technique**

302 The MC model for each pesticide simulation was performed with a lognormal distribution
303 (Guo & Li, 2021; Hill & Reynolds, 2002; Horváth, Ambrus, Mészáros, & Braun, 2013)
304 in a loop of 10^5 simulations in R. The concentration and standard deviation required for
305 the simulation for each pesticide in the normal probability distribution were as follows:
306 for concentration, the value of the limit of quantification of each pesticide (0.01 mg/kg),
307 and for the standard deviation, the experimental values obtained for each pesticide and in
308 each of the subsamplings studied (100 g, 20 g and 5 g). The data obtained were used for
309 the modeling of CV versus sample weight (Supplementary Figure 2).

310 **3. Results and discussion**

311 The results of the analysis of pesticide residues in the paprika samples and the
312 corresponding CV and K_s are presented in Tables 1 to 4.

313 Table 1. Analysis of pesticide residues in samples of paprika for a portion of 5 g.

Sample	Fluodixinil		Cypermetrin		Acetamiprid		Spirotetramat	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
M1	0.095	0.096	0.012	0.013	0.071	0.074	0.014	0.015
M2	0.092	0.094	0.011	0.012	0.076	0.073	0.017	0.017
M3	0.082	0.084	0.01	0.011	0.077	0.078	0.016	0.016
M4	0.088	0.089	0.012	0.011	0.072	0.075	0.014	0.015
M5	0.093	0.090	0.013	0.013	0.075	0.075	0.016	0.015
M6	0.087	0.084	0.011	0.011	0.077	0.076	0.017	0.017
M7	0.093	0.093	0.012	0.011	0.075	0.074	0.017	0.017
M8	0.094	0.095	0.011	0.012	0.076	0.075	0.015	0.017

314 Rep: Repetitions in mg/kg with two significant figures as SANTE 11312/2021.

315

316 Table 2. Analysis of pesticide residues in samples of paprika for a portion of 20 g.

Sample	Fluodixinil		Cypermetrin		Acetamiprid		Spirotetramat	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
M1	0.094	0.094	0.012	0.013	0.068	0.072	0.016	0.015
M2	0.089	0.090	0.011	0.012	0.071	0.072	0.018	0.018
M3	0.080	0.081	0.010	0.011	0.069	0.067	0.015	0.015
M4	0.092	0.096	0.012	0.011	0.067	0.065	0.017	0.015
M5	0.085	0.083	0.011	0.013	0.065	0.072	0.017	0.018
M6	0.091	0.091	0.011	0.011	0.072	0.074	0.016	0.017
M7	0.089	0.080	0.012	0.011	0.065	0.067	0.018	0.017
M8	0.092	0.093	0.011	0.012	0.065	0.067	0.017	0.015

317 Rep: Repetitions in mg/kg with two significant figures as SANTE 11312/2021.

318 Table 3. Analysis of pesticide residues in samples of paprika for a portion of 100 g.

Sample	Fluodixinil		Cypermetrin		Acetamiprid		Spirotetramat	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
M1	0.110	0.110	0.013	0.012	0.061	0.064	0.015	0.015
M2	0.092	0.093	0.011	0.011	0.071	0.074	0.019	0.019
M3	0.079	0.079	0.014	0.013	0.082	0.085	0.015	0.015
M4	0.110	0.110	0.014	0.013	0.061	0.064	0.016	0.015
M5	0.092	0.093	0.013	0.012	0.071	0.074	0.015	0.015
M6	0.079	0.080	0.011	0.011	0.082	0.085	0.018	0.019
M7	0.088	0.081	0.013	0.012	0.061	0.064	0.017	0.018
M8	0.087	0.090	0.012	0.012	0.071	0.074	0.019	0.018

319 Rep: Repetitions in mg/kg with two significant figures as SANTE 11312/2021.

320

321

Table 4. CV sampling, subsampling and analysis for each pesticide.

Pesticide	C (mg/kg)	CV_s	CV_{sp}	CV_{an}	CV_{res}
Fludioxinil	0.090	5.9%	6.6%	2.2%	9.1%
Cypermetrine	0.012	3.7%	7.2%	5.9%	10.0%
Acetamiprid	0.072	4.2%	7.1%	2.7%	8.7%
Spirotetramat	0.016	5.9%	4.9%	4.2%	8.8%

CV_s: Calculated with the variance of supplementary Table 2 (S_s^2) dividing the square root of variance by the average of the concentrations obtained for all samples and multiplying by 100; CV_{sp}: Calculated with the variance of supplementary Table 2 S_{sp}^2 dividing the square root of the variance by the average of the concentrations obtained for all samples and multiplying by 100; CV_{sp}: Calculated with the variance of supplementary Table 2 S_{an}^2 dividing the square root of the variance by the average of the concentrations obtained for all samples and multiplying by 100; CV_{res}: uncertainty standard relative calculated with Equation 1.

The results of CV_{an} in Table 4 show that the relative standard uncertainty for the analysis (CV_{an}) is the lowest of all CV_s investigated. It should be noted that the CV_{an} value refers only to the relative standard uncertainty associated with the repeatability of the injection and does not take into account other important factors in pesticide analysis such as bias or reproducibility (SANTE, 2021). A better estimate of CV_{an} is based on the worst-case methodology (Sørensen, Giralt, Rallo, Espinosa, Münier, Gyldenkærne, et al., 2010). This methodology assumes a value of 20%,, a maximum expanded relative uncertainty of 50% and a maximum reproducibility of 20% according to the SANTE document (SANTE, 2021).

On the other hand, the results of sampling and subsampling (CV_s and CV_{sp}, respectively) show that the CV between sampling and subsampling is similar (for spirotetramat) or higher in subsampling (for the rest of the pesticides). This would indicate, on the one hand, the high homogeneity of the commercial product and, on the other hand, the existence of more differences in subsampling than in sampling by using different weights to analyse the pesticide using the QuEChERS method. The homogeneity of the paprika

345 product was confirmed by very low values of CV_s (<10%) compared to other values of
346 CV_s obtained in different crops with different sizes, units and weights (Á. Ambrus, Zentai,
347 Sali, & Ficzere, 2011) with CV_s values between 20% and 40%.

348 Supplementary Table 5 clearly shows that the highest CV associated with the weight in
349 the subsample is always at 100 g. This greater dispersion in the K_s (Equation 4) values
350 for the weight of 100 g is shown in Supplementary Figure 1.

351 The pesticide acetamiprid shows different orders of magnitude in the K_s value for 5 g and
352 20 g than the other pesticides. Since the concentration is similar for both pesticides, this
353 could be due to better extraction and chromatographic analysis by LC than for the
354 pesticide fludioxinil by GC.

355 According to the data obtained, the MC approach was applied for a worst-case scenario
356 in pesticide analysis. In this case, the limit of quantification (0.01 mg/kg) is used as the
357 mean and the highest value of CV in supplementary Table 5 for each weight.

358 Supplementary Table 6 shows the results of 10^5 iterations and Supplementary Figure 2
359 shows the CV estimate for different weights. Supplementary Figure 2 also shows that
360 there is an increase in CV from 30 g, where the red lines are crossed. For this reason, the
361 three saffron samples were prepared in a similar way as described in section 2.7 but only
362 with 30 g each (M1, M2 and M3) to verify the results obtained using paprika and the MC
363 method. The bags with 30 g were analysed using 5.0 g directly according to the laboratory
364 method used for paprika. The results are given in Supplementary Table 7.

365 The results in supplementary Table 7 show that the CV for the three samples is lower for
366 both compounds than the maximum value obtained from the data in supplementary Table
367 6 (6.8%). The mean value of each compound has been expressed, as usual in analytical

laboratories, with a maximum expanded uncertainty of 50% (Valverde, Aguilera, & Valverde-Monterreal, 2017).

Plotting the contributions to uncertainty (Supplementary Figure 3) according to the systematic approach established in the Quantifying Uncertainty in Analytical Measurement guide (Eurachem, 2019) and worst case (CV analysis 20% according to the SANTE 11312/2021 document, CV for sampling according to Table 4 and CV for subsampling according to the maximum value of supplementary Table 5) shows that subsampling is the second contribution to the uncertainty results.

5. Conclusions

The results of this study showed that the uncertainty associated with subsampling is greater than the uncertainty associated with sampling when the product is sufficiently homogeneous, as in the case of spices. The minimum sample size required for pesticide analysis using the QuEChERS method on this type of product is 5 g, but it has been shown that a better sample size is between 20 and 30 g. This weight, less than the 100 g established in various regulations such as R.D. 290/2003 (RD, 2003), can reduce the cost of sending products to the laboratory for products with high added value such as saffron, and keep the risk associated with subsampling uncertainty within the minimum values found in this study.

Declaration of Competing Interest

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Uncertainty and associated risks in the analysis of pesticides in homogeneous paprika samples

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Abstract

In this study, risk estimation based on sampling and subsampling uncertainty was performed for pesticide analysis in homogeneous spice products such as paprika. The results of the subsampling were also used to estimate the minimum weight necessary in subsampling to minimize overall uncertainty. The results show that subsampling has more uncertainty than sampling in the product due to high homogeneity in the manufacturer's batch. On the other hand, results using the Monte Carlo (MC) simulation on the size of the subsample indicate that uncertainty is lower for weights between 20 and 30 g and increases for sample sizes of 100 g. A sample size of 30 g was used for saffron, and the values simulated with the MC method were confirmed.

Keywords: Homogeneity; Pesticide residue analysis; Risk; Spice; Uncertainty

1. Introduction

The use of pesticides in the agricultural sector is widespread, mainly for the protection of livestock and crops. Currently, however, pesticides are often overused, with more than 4.0 million tonnes of pesticides used worldwide each year to control a broad variety of pests (Liu, Fan, Li, Zhao, Luo, Wang, et al., 2021). Reports from the European Union (EU) indicate that around 333,000 tonnes of pesticides were used in 2021, with more than 400 new pesticides on the market (Eurostat, 2023).

The use of these pesticides on crops makes it necessary to ensure that a minimum level find their way into foodstuffs. In order to prevent toxicity problems associated with the excessive use of pesticides, Maximum Residue Limits (MRLs) have been set by various regulations. In the EU, for example, the European Commission (EC) has set MRLs for various foodstuffs in the consolidated version of Regulation (EC) No 396/2005 (EC, 2005). This Regulation applies to both food and feed of plant and animal origin as well as to unprocessed spices such as pepper, but not to some processed foods such as flour or paprika. In these cases, manufacturers act differently depending on the type of product in order to ensure its safety. For foods where extrapolation from the raw material is possible, the manufacturer uses these MRLs (e.g. wheat flour uses the MRL for wheat). However, there are other products that involve a dehydration treatment, and this extrapolation is not possible. In these cases, article 20 of Regulation (EC) No 396/2005 (EC, 2005) allows the modification of MRLs. For this reason, the European Spice Association (ESA) has established dehydration factors for different spices (ESA, 2008; Schaarschmidt, 2016) and, for example, as is the case for paprika, the MRLs for pepper are multiplied by a factor of 10. However, there isn't a drying factor for all products. For saffron, for example, the MRL for chlorpyrifos was changed from 0.05 mg/kg to 0.01 mg/kg in 2020.

The main physical characteristic of these processed foods is their high homogeneity, owing to the industrial grinding and homogenisation treatments they undergo (e.g. wheat flour, paprika or spices ground by the manufacturer, such as saffron). This is a great advantage for carrying out pesticide analysis, as the product is already homogeneous and the laboratory does not have to perform further processes such as quartering or unit selection, as is the case with fruit and vegetables (EC, 2005; ISO, 1980; RD, 2003). This minimizes error sampling of subsampling by the laboratory.

The sample size to be sent to a laboratory depends mainly on two factors: the minimum quantity required by the analysis method, and the minimum quantity required to accurately represent the starting batch. As for the second factor, Regulations 2002/63/EC (EC, 2002) and 152/2009 (EC, 2009) provide guidelines as to how sampling for official control should be carried out. However, the recommendations for homogeneous samples only refer to the minimum sample size, e.g. 0.5 kg for flours and 0.1 kg for spices. This sample size can pose a significant problem for the analysis of high-value spices such as saffron. In the case of this spice, whose market price fluctuates greatly depending on quality and country of origin, it can mean incurring costs of up to €1000 per sample (0.1 kg) (Azafrán, 2023; Holz, Illarionov, Wax, Schmidt, & Fischer, 2023; Quoc, 2023) for laboratory analysis.

As for analysis methods, the Quick, Easy, Cheap, Effective, Rugged & Safe (QuEChERS) (UNE, 2019) method has been the most widely used pesticide extraction method in laboratories for the last two decades. In QuEChERS extraction, a sample size of 5 g (for dry products such as spices) to a maximum of 10 g (for fresh products such as vegetables or fruit) is sufficient for simultaneous analysis by GC-MS/MS and ~~HP~~LC-MS/MS (da Costa Morais, Collins, & Jardim, 2018; Jiang, Cheng, Chen, Dong, Xu, Liu, et al., 2021).

There are ~~several~~ publications that have looked at how sampling and subsampling affects pesticide residue results (Á. Ambrus & Soboleva, 2004; ~~Balkan & Yılmaz, 2022~~). However, all these studies focus ~~on a small number of pesticides and~~ on fresh products such as fruit and vegetables which, due to the variability in their size or number of pieces, have greater uncertainty associated with sampling and subsampling.

Uncertainty is defined in the Guide to the expression of Uncertainty of Measurement ~~(GUM)~~ (JCGM, 2008) as a parameter associated with results which characterizes the dispersion of the values that could reasonably be attributed to the measurand. Uncertainty is related to risk, as defined by the standard ISO 31000 (ISO, 2018), where risk is defined as the effect of uncertainty on the achievement of objectives. Although there are differences between the two concepts (Toma, Chiriță, & Şarpe, 2012) both are important. For laboratories, the results obtained might contain false positives or negatives due to the high uncertainty associated with subsampling or analysis, while manufacturers risk having their products returned due to incorrect results stemming from product inhomogeneity or the sample size of the product sent to the laboratory. As an example, the new MRL for chlorpyrifos in saffron (Mahdavi, Eslami, Golmohammadi, Tajdar-oranj, Keikavousi Behbahan, & Mousavi Khaneghah, 2021) could pose a risk of false negatives in the laboratory, especially when the limit of quantification is 0.01 mg/kg, as well as for the manufacturer if the portion analysed is negative for chlorpyrifos but not actually representative of the batch to be sold.

Due to problems like this, one tool that has become common in risk analysis (ISO, 2018) is the use of the Monte Carlo (MC) method. The ~~Monte Carlo~~ MC method is a simulation method developed around 1944 and it is increasingly used in different fields such as measurement uncertainty (ISO/IEC, 2008) engineering (Platon & Constantinescu, 2014) risk management (ISO, 2018) or pesticides (Guo & Li, 2021).

Therefore, the aim of this study was to evaluate the uncertainty and risk associated with sampling and subsampling between customer and laboratory in multi-residue pesticide analysis by GC-MS/MS and ~~HP~~LC-MS/MS in ~~paprika and saffron as~~ homogeneous spice samples. Paprika and saffron were used for this study. Both spices are homogeneous, paprika was chosen to evaluate uncertainty and risk due to its low cost and high availability, while saffron was chosen in order to validate the results obtained from the paprika in a high cost, and therefore low quantity, of product in multi-residue pesticide analysis.

2. Material and methods

2.1. Samples

Ground samples of paprika and saffron were selected for this study. The saffron and the paprika were supplied by two different factories located in the Region of Murcia. Both companies are Spanish with high production rates. They export their products both nationally and globally based on the traceability of the batches produced. The total weight of each batch may vary based on the crushing and grinding performed, but production batches of at least 1000 kg are common.

The samples used in this study came from a batch previously analysed by the supplier. In the case of the paprika, the original batch was positive for GC analysis in fludioxinil and cypermethrin, defined in Regulation (EC) No 396/2005 as cypermethrin including other mixtures of constituent isomers (sum of isomers). For LC analysis ~~and for HPLC analysis,~~ it was positive in acetamiprid and spirotetramat, defined in Regulation (EC) No 396/2005 as spirotetramat and spirotetramat-enol (sum of), expressed as spirotetramat. In the case of the saffron, the batch was positive for the pesticide chlorpyrifos and the organic compound piperonyl butoxide (PBO) in GC analysis, which is used as a synergistic component in pesticide formulations.

2.2. Pesticide analysis

The solvents for GC and ~~HP~~LC analysis were supplied by J.T. Baker (Center Valley, PA, USA). These included ethyl acetate, methanol, water and residual formic acid. The ammonium formate, formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl) 1 g, disodium hydrogen citrate sesquihydrate (DHS) 0.5 g, trisodium citrate dihydrate (THS) 1 g, sodium sulfate (Na_2SO_4) 4 g, primary Secondary Amine (PSA), and C18 (end-capped) for dispersive solid phase extraction (dSPE) were supplied in an extraction kit by Agilent Technologies (Santa Clara, USA). All pesticides had certified reference standards with a minimum purity of 95% and were purchased from LGC Standards (Teddington, UK).

2.3. Data analysis

The Rstudio IDE Desktop 2022.02.01 tool and R 3.3.0 were used for data analysis and graph generation (Aria & Cuccurullo, 2017). This software is free and can be used in both desktop and cloud versions. Rstudio allows scripts to be created using code written in R. This can be used to automate optimizing for future studies. Different R libraries were used depending on the analysis carried out: for example, the data import into R was done using the Readxl library, and graph analysis and ANOVA analysis were performed using ggplot2. The R-script code used in this paper is available on request from the corresponding author.

The ~~Monte Carlo (~~MC~~)~~ simulation technique performs numerical analysis by generating a large number of artificial values of a probabilistic variable using a defined distribution (Rezaei Kalantary, Barzegar, & Jorfi, 2022). The probability distributions used in R were rlnorm (lognormal). ~~has been realized with R a loop of 10^5 simulations has been used to obtain the final values~~.

2.4. Initial sample preparation

The batch of paprika was prepared in the factory using the traditional methodology (Fernández-Trujillo & Escarabajal, 2006). This consists of cleaning, pre-crushing by hammer, milling in serial with at least 6 mills, mixing in a 1000 kg blender finally passing through a 2000 µm sieve.

The saffron samples (provided as powder) were prepared by the producer-trader and the homogenization included separation of stigmas, drying of stigmas and grinding and sieving (Europasaffron, s.f.). It was not possible to obtain information about the sieve size or the grinding mechanism that was used.

For the paprika, eight samples from the same production batch were analyzed. For the saffron, due to its high cost, only three samples were obtained, and these samples were used for validation after results were obtained from the paprika.

Each sample was weighed prior to subsampling and the results are shown in supplementary Table 1. The samples were not manipulated in any way other than being placed on a tray for subsampling as described in the 2.7 Subsampling preparation section.

2.5. Pesticide analysis

The QuEChERS extraction, based on EN 15662, used 5 grams of paprika or saffron, to which 10 ml of water was added. After manual homogenization, 10 ml of acetonitrile was added (E7 EN 15662 extraction) and the mixture was shaken for 1 minute. After shaking, the salts (NaCl, DHS, THS, Na_2SO_4) were added and it was centrifuged for 5 minutes at 3000 rpm. A clean-up process using PSA and C18 was carried out (based in C3 EN 15662 clean-up) on the organic aliquot obtained and finally it was centrifuged again at 3000 rpm for 5 minutes to get the final aliquot for GC-MS/MS and LC-MS/MS analysis.

A total of 185 pesticides were analyzed, and only 2 were detected in paprika and 2 in saffron by GC-MS/MS using an Agilent (Santa Clara, USA) 7890A Gas Chromatography system coupled with a 7000A quadrupole tandem mass spectrometer. Chromatographic separation was performed on a RTx-5MS column (30 m, 0.25 mm, i.d., 0.5 µm) RESTEK. Helium was used as the carrier gas at a constant flow rate of 1.2 mL·min^{-1}, while argon was used as the collision gas. The oven temperature was adjusted as follows: the initial temperature was set at 70 °C for 2 min, then increased to 150 °C at a rate of 25 °C min^{-1}, to 200 °C at 3 °C min^{-1} with a hold time of 1 min, and finally to 280 °C at 10 °C min^{-1} with a hold time of 10 min. The total run time was 45 min. The temperatures of the transfer line and ion source were set at 280 ºC and 250 ºC, respectively. The multiple reaction monitoring (MRM) mode was used to operate the mass spectrometer.

A total of 115 pesticides were analyzed, and only 2 detected in paprika by LC-MS/MS using an Agilent liquid chromatography system (Santa Clara, USA) coupled with a 6470 triple quadrupole tandem mass spectrometer. The chromatographic separation was performed on a Poroshell C18 column (150 mm, 2.1 mm i.d., 2.7 µm) (Agilent, USA) with a flow rate of 0.1 ml at 40 ºC. The elution solvent used was water 5 mM ammonium formate with 0.01% formic acid (A) and methanol 5 mM ammonium formate with 0.01% formic acid (B). The gradient elution was carried out as follows: 40% solvent B for 0-5 min, changing to 60% solvent B for 6-12 min and finishing with 100% solvent B for 17-20 min. Pesticides were analyzed using programmed ~~multiple reaction monitoring (MRM)~~ in positive and negative modes simultaneously. Ion source parameters include optimized drying gas temperature, drying gas flow, nebulizer pressure, sheath gas temperature and flow, capillary voltage, nozzle voltage and high and low radio frequency voltage.

The analysis of pesticides by GC-MS/MS and LC-MS/MS has a quantification limit of 0.01 mg/kg in the case of paprika and saffron, as shown in the validation results according to SANTE 11312/2021 (SANTE, 2021) in supplementary Table 2.

All the samples were analyzed with quality control in each batch using spiked samples over a paprika and a saffron blank sample according to SANTE 11312/2021 (SANTE, 2021). The results of quality assurance for the spiked samples can be seen in supplementary Table 3.

2.6. Uncertainty in the sampling analysis

The estimation of uncertainty for the whole process of pesticide analysis, from sampling to analysis, is described in an excellent way elsewhere (Á. Ambrus, Zentai, Sali, & Ficzere, 2011; Eurachem, 2019) and can be estimated using Equation 1. This equation could be used to estimate the relative uncertainty (CV) (Valverde, Aguilera, & Valverde-Monterreal, 2017) of results in multi-residue analysis of pesticide.

$$CV_{res} = \sqrt{CV_s^2 + CV_L^2} \qquad \text{Equation 1}$$

Note that CV is the nomenclature for coefficient of variation or relative standard deviation, calculated as the quotient of standard deviation and mean.

Where CV_{res} is the relative standard uncertainty of the multi-residue analysis, CV_s is the relative standard uncertainty of sampling and CV_L is the relative standard uncertainty associated with the laboratory analysis. The relative standard uncertainty for the analytical procedure can be divided into two main components as shown in Equation 2.

$$CV_L = \sqrt{CV_{sp}^2 + CV_A^2} \qquad \text{Equation 2}$$

Where CV_{sp} is the relative standard uncertainty associated with subsampling, and sample preparation included an extraction and clean-up process (Lehotay, Han, & Sapozhnikova, 2018) in the laboratory and CV_A is associated with the specific analysis, in this case ~~HP~~LC-MS/MS and GC-MS/MS.

Modelling the estimation of ~~relative~~ the CV of sampling ~~uncertainty (CV)~~ can be complex due to the large number of errors that need to be considered (Gy, 2012) such as grouping and separation errors due to sample heterogeneity, or weighing errors due to errors in assigning weights to different parts of an unequal composite sample. There are several estimates of CV_s for different crops (A. Ambrus, 2004; Á. Ambrus, Zentai, Sali, & Ficzere, 2011) with values between 25 and 40%, depending on the crop. However, this estimate does not apply to homogeneous samples or to analyses performed using the QuEChERS method (Han, Lehotay, & Sapozhnikova, 2018; Lehotay, Han, & Sapozhnikova, 2018).

The Codex Alimentarius Commission ~~(CAC)~~ established a CV_s between 8 and 10% (WHO, 2013), but there's no further information about the type of samples it could be used on.

However, an estimate of uncertainty can be made empirically using the duplicate method (Lyn, Ramsey, Coad, Damant, Wood, & Boon, 2007), as shown in Figure 1.

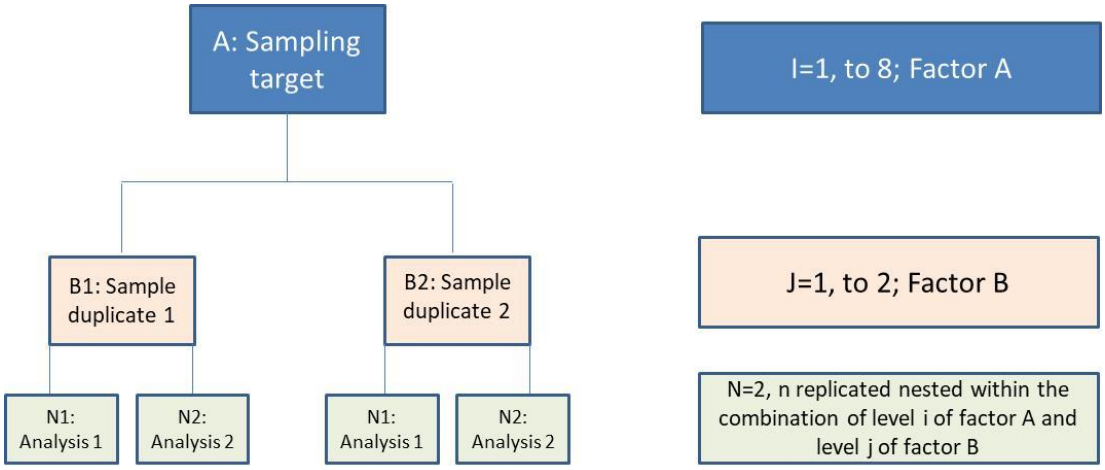

Figure 1. Duplicate design to estimation of the uncertainty.

This design is a two-way analysis of variance and is used to study the relationship between a quantitative dependent variable (pesticide concentration in the batch) and two independent variables (factors, subsampling and analysis), each with several levels. The two-way ANOVA allows us to study how each of the factors alone influences the dependent variable.

The associated variances for each of the sources of variability in a two-factor ANOVA are shown in supplementary Table 3.

Sample preparation is a source of uncertainty (Eurachem, 2019), mainly due to various errors associated with sample handling and heterogeneity. Some of the main sources are homogenisation and/or subsampling, drying, grinding or contamination. If the whole sample is prepared and analysed, the uncertainty of sample preparation, CV_{sp}, is zero, but in the QuEChERS method only 5 g are required. For an estimation of the CV_{sp} in different weights, a simplification of the equation of Gy's could be used (Gy, 2012) or the Ingamell´s sampling constant (Equation 3) (A. Ambrus, Solymosné, & Korsós, 1996).

$$CV_{sp} = \sqrt{\frac{Ks}{M_{tp}}} \qquad \text{Equation 3}$$

Where Ks is the Ingamell and Switzer sampling constant, defined as the weight of a single increment (w) that must be taken from a well-mixed material to maintain the relative subsampling uncertainty (%CV_{sp}) at 1% with 68% confidence. M_{tp} is the weight of the sample, and this corresponds to the size of the subsample. Denoted that %CV_{sp} is the standard deviation relative of subsampling (A. Ambrus, Solymosné, & Korsós, 1996), divided by the median concentration.

From empirical CV_{sp} data, this equation can be used to estimate whether there are differences between CV_{sp} due to differences in K_s for the experimental weight used, or to calculate the expected standard deviation for a given sample size, as shown in Equations 4 and 3.

$$Ks = M_{tp} \times CV_{sp}^2 \qquad \text{Equation 4}$$

Where M_{tp} is the subsampling size established as optimum after evaluation of the K_s values obtained.

Based on the evaluation of K_s (Equation 4), the duplicate design of Figure 1 is transformed to the design shown in Figure 2, where there are three types of weighted samples: 5 g for the minimum required by the QuEChERS method, 100 g to evaluate the normal minimum weight based on the R.D. 290/2003, and 20 g as an intermediate weight.

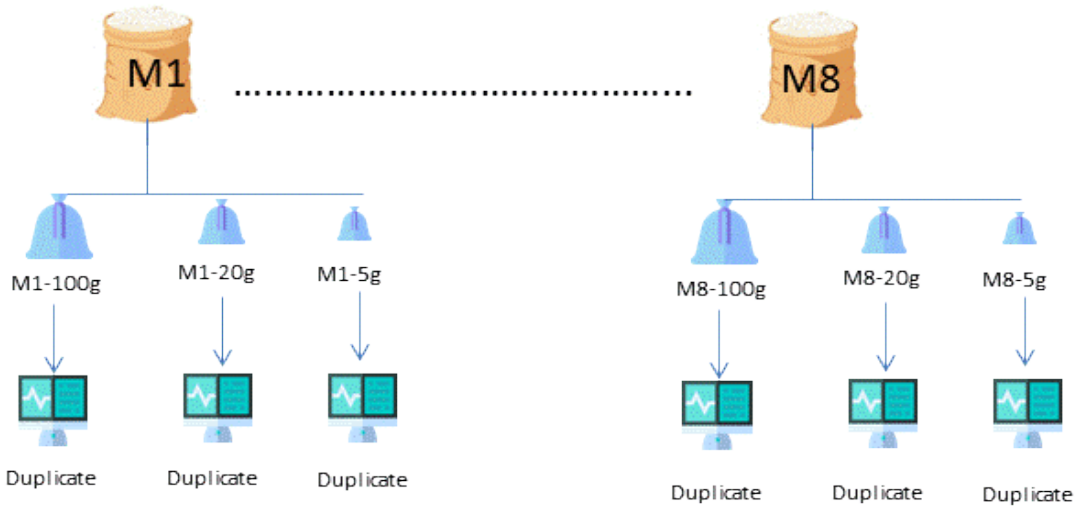

Figure 2. Modified design to compare variances and uncertainty.

Figure 2 shows the schema for the preparation of subsamples for the duplicate analysis in GC-MS/MS and LC-MS/MS. As 5 grams are used for the analysis of QuEChERS in spices, 5-gram samples M1 to M8 were prepared as duplicate samples in individual bags.

2.7. Subsampling preparation

To obtain each portion of the sample (5, 20 and 100 g) from the original sample (M1 to M8, supplementary Table 1), a direct transfer was made into a plastic bag using a disposable plastic spatula and weighed on a precision balance. The amount of sample was weighed to exactly 5.0 grams, 20.0 grams and 100.0 grams.

For the duplicate analysis showed in Figure 2, 5.0 g of sample was weighed directly from each heavier bag (20 g, 100 g) ~~with~~ using a disposable plastic spatula to transfer to the Eppendorf tube on a precision balance. In the case of the samples with 5.0 grams, the total content of the bag was transferred directly to an Eppendorf tube. QuEChERS analysis was then performed on the Eppendorf tube.

No homogenization of the samples was performed during these steps in order to avoid including any additional homogenization processes in the analysis.

2.8. Monte Carlo simulation technique

The MC model for each pesticide simulation was performed with a lognormal distribution (Guo & Li, 2021; Hill & Reynolds, 2002; Horváth, Ambrus, Mészáros, & Braun, 2013) in a loop of 10^5 simulations in R. The concentration and standard deviation required for the simulation for each pesticide in the normal probability distribution were as follows: for concentration, the value of the limit of quantification of each pesticide (0.01 mg/kg), and for the standard deviation, the experimental values obtained for each pesticide and in each of the subsampling studied (100 g, 20 g and 5 g). The data obtained were used for the modeling of CV versus sample weight (Supplementary Figure 2).

3. Results and discussion

The results of the analysis of pesticide residues in the paprika samples and the corresponding CV and K_s are presented in Tables 1 to 4.

Table 1. Analysis of pesticide residues in samples of paprika for a portion of 5 g.

Sample	Fluodixinil		Cypermetrin		Acetamiprid		Spirotetramat	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
M1	0.095	0.096	0.012	0.013	0.071	0.074	0.014	0.015
M2	0.092	0.094	0.011	0.012	0.076	0.073	0.017	0.017
M3	0.082	0.084	0.01	0.011	0.077	0.078	0.016	0.016
M4	0.088	0.089	0.012	0.011	0.072	0.075	0.014	0.015
M5	0.093	0.090	0.013	0.013	0.075	0.075	0.016	0.015
M6	0.087	0.084	0.011	0.011	0.077	0.076	0.017	0.017
M7	0.093	0.093	0.012	0.011	0.075	0.074	0.017	0.017
M8	0.094	0.095	0.011	0.012	0.076	0.075	0.015	0.017

Rep: Repetitions in mg/kg with two significant figures as SANTE 11312/2021.

Table 2. Analysis of pesticide residues in samples of paprika for a portion of 20 g.

Sample	Fluodixinil		Cypermetrin		Acetamiprid		Spirotetramat	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
M1	0.094	0.094	0.012	0.013	0.068	0.072	0.016	0.015
M2	0.089	0.090	0.011	0.012	0.071	0.072	0.018	0.018
M3	0.080	0.081	0.010	0.011	0.069	0.067	0.015	0.015
M4	0.092	0.096	0.012	0.011	0.067	0.065	0.017	0.015
M5	0.085	0.083	0.011	0.013	0.065	0.072	0.017	0.018
M6	0.091	0.091	0.011	0.011	0.072	0.074	0.016	0.017
M7	0.089	0.080	0.012	0.011	0.065	0.067	0.018	0.017
M8	0.092	0.093	0.011	0.012	0.065	0.067	0.017	0.015

Rep: Repetitions in mg/kg with two significant figures as SANTE 11312/2021.

Table 3. Analysis of pesticide residues in samples of paprika for a portion of 100 g.

Sample	Fluodixinil		Cypermetrin		Acetamiprid		Spirotetramat	
	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
M1	0.110	0.110	0.013	0.012	0.061	0.064	0.015	0.015
M2	0.092	0.093	0.011	0.011	0.071	0.074	0.019	0.019
M3	0.079	0.079	0.014	0.013	0.082	0.085	0.015	0.015
M4	0.110	0.110	0.014	0.013	0.061	0.064	0.016	0.015
M5	0.092	0.093	0.013	0.012	0.071	0.074	0.015	0.015
M6	0.079	0.080	0.011	0.011	0.082	0.085	0.018	0.019
M7	0.088	0.081	0.013	0.012	0.061	0.064	0.017	0.018
M8	0.087	0.090	0.012	0.012	0.071	0.074	0.019	0.018

Rep: Repetitions in mg/kg with two significant figures as SANTE 11312/2021.

Table 4. CV sampling, subsampling and analysis for each pesticide.

Pesticide	C (mg/kg)	CV_s	CV_{sp}	CV_{an}	CV_{res}
Fludioxinil	0.090	5.9%	6.6%	2.2%	9.1%
Cypermetrine	0.012	3.7%	7.2%	5.9%	10.0%
Acetamiprid	0.072	4.2%	7.1%	2.7%	8.7%
Spirotetramat	0.016	5.9%	4.9%	4.2%	8.8%

CV_s: Calculated with the variance of supplementary Table 2 (S_s^2) dividing the square root of variance by the average of the concentrations obtained for all samples and multiplying by 100; CV_{sp}: Calculated with the variance of supplementary Table 2 S_{sp}^2 dividing the square root of the variance by the average of the concentrations obtained for all samples and multiplying by 100; CV_{sp}: Calculated with the variance of supplementary Table 2 S_{an}^2 dividing the square root of the variance by the average of the concentrations obtained for all samples and multiplying by 100; CV_{res}: uncertainty standard relative calculated with Equation 1.

The results of CV_{an} in Table 4 show that the relative standard uncertainty for the analysis (CV_{an}) is the lowest of all CV_s investigated. It should be noted that the CV_{an} value refers

only to the relative standard uncertainty associated with the repeatability of the injection and does not take into account other important factors in pesticide analysis such as bias or reproducibility (SANTE, 2021). A better estimate of CV_{an} is based on the worst-case methodology (Sørensen, Giralt, Rallo, Espinosa, Münier, Gyldenkærne, et al., 2010). This methodology assumes a value of 20%, a maximum expanded relative uncertainty of 50% and a maximum reproducibility of 20% according to the SANTE document (SANTE, 2021).

On the other hand, the results of sampling and subsampling (CV_s and CV_{sp}, respectively) show that the CV between sampling and subsampling is similar (for spirotetramat) or higher in subsampling (for the rest of the pesticides). This would indicate, on the one hand, the high homogeneity of the commercial product and, on the other hand, the existence of more differences in subsampling than in sampling by using different weights to analyse the pesticide using the QuEChERS method. The homogeneity of the paprika product was confirmed by very low values of CV_s (<10%) compared to other values of CV_s obtained in different crops with different sizes, units and weights (Á. Ambrus, Zentai, Sali, & Ficzere, 2011) with CV_s values between 20% and 40%.

Supplementary Table 5 clearly shows that the highest CV associated with the weight in the subsample is always at 100 g. This greater dispersion in the K_s (Equation 4) values for the weight of 100 g is shown in Supplementary Figure 1.

The pesticide acetamiprid shows different orders of magnitude in the K_s value for 5 g and 20 g than the other pesticides. Since the concentration is similar for both pesticides, this could be due to better extraction and chromatographic analysis by ~~HP~~LC than for the pesticide fludioxinil by GC.

According to the data obtained, the MC approach was applied for a worst-case scenario in pesticide analysis. In this case, the limit of quantification (0.01 mg/kg) is used as the mean and the highest value of CV in supplementary Table 5 for each weight.

Supplementary Table 6 shows the results of 10^5 iterations and Supplementary Figure 2 shows the CV estimate for different weights. Supplementary Figure 2 also shows that there is an increase in CV from 30 g, where the red lines are crossed. For this reason, the three saffron samples were prepared in a similar way as described in section 2.7 but only with 30 g each (M1, M2 and M3) to verify the results obtained using paprika and the MC method. The bags with 30 g were analysed using 5.0 g directly ~~in the routine~~ according to the laboratory method used for paprika. The results are given in Supplementary Table 7.

The results in supplementary Table 7 show that the CV for the three samples is lower for both compounds than the maximum value obtained from the data in supplementary Table 6 (6.8%). The mean value of each compound has been expressed, as usual in analytical laboratories, with a maximum expanded uncertainty of 50% (Valverde, Aguilera, & Valverde-Monterreal, 2017).

Plotting the contributions to uncertainty (Supplementary Figure 3) according to the systematic approach established in the Quantifying Uncertainty in Analytical Measurement ~~(QUAM)~~ guide (Eurachem, 2019) and worst case (CV analysis 20% according to the SANTE 11312/2021 document, CV for sampling according to Table 4 and CV for subsampling according to the maximum value of supplementary Table 5) shows that subsampling is the second contribution to the uncertainty results.

5. Conclusions

The results of this study showed that the uncertainty associated with subsampling is greater than the uncertainty associated with sampling when the product is sufficiently homogeneous, as in the case of spices. The minimum sample size required for pesticide analysis using the QuEChERS method on this type of product is 5 g, but it has been shown that a better sample size is between 20 and 30 g. This weight, less than the 100 g established in various regulations such as R.D. 290/2003 (RD, 2003), can reduce the cost of sending products to the laboratory for products with high added value such as saffron, and keep the risk associated with subsampling uncertainty within the minimum values found in this study.

Declaration of Competing Interest

The authors declare that this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Facultad de Química

Dr. Pedro Andreo-Martínez

Editor-in-Chief
Food Chemistry
12th July 2023

Dear editor,

First, we want to thank you for the opportunity to revise our manuscript for possible publication in Food Chemistry journal. We also want to praise your choice of reviewers to review our manuscript. All of the reviewers were highly professional, and their comments were extremely valuable for improving our manuscript.

Thanks to the comments of the reviewers, we have conducted a deep revision of the manuscript and we have clarified some confused aspects and solved some errors.

Please find attached the full version of the revised manuscript entitled 'Uncertainty and associated risks in the analysis of pesticides in homogeneous paprika samples' and our reply to the reviewer's comments.

Yours sincerely,

The authors

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Answers to the reviewers' comments:

As a general remark, all the text introduced (or changed) into the revised version of the marked manuscript was highlighted in red.

Reviewer #2:

The manuscript (FOODCHEM-D-23-03405) "Uncertainty and risk associated in the analysis of pesticides in spices homogeneous samples" by José Manuel Veiga-del-Baño, Juan José Cuenca-Martínez, Pedro Andreo-Martínez, Miguel Ángel Cámara, José Oliva, Miguel Motas describes risk estimation based on sampling and sub-sampling uncertainty for the analysis of pesticides in homogeneous spice products such as saffron and paprika. It should be emphasized that such scientific papers are needed.

I have some comments which can help improve quality and value of the manuscript:

Thank you for taking the time to review the manuscript and for your comments, which will greatly improve our manuscript.

* Are pesticides used only to achieve a good insecticidal effect? Line 48, page 3

ANSWER:

Thanks for this comment. In response to the reviewer's question, the sentence has been rewritten in the revised version of the manuscript as follows:

"Currently, however, pesticides are often overused, with more than 4.0 million tonnes of pesticides used worldwide each year to control a broad variety of pests"

* Why the authors chose saffron and paprika for the study?

ANSWER:

Thank you very much for this comment. Paprika was chosen because it is a homogeneous and cheap product with pesticide positives (> 0.01 mg/kg) detected by our laboratory and because more than 10 kg of product were available. On the other hand, saffron was chosen because it is a spice that

is homogeneous but sent for analysis in very small quantities due to its high cost.

The explication of this fact has been added in the last paragraph of the introduction section of the revised version of the manuscript as follows:

"Paprika and saffron were used for this study. Both spices are homogeneous: paprika was chosen to evaluate uncertainty and risk due to its low cost and high availability, while saffron was chosen in order to validate the results obtained from the paprika in a high cost, and therefore low quantity, of product in multi-residue pesticide analysis."

* Are the authors sure they mean HPLC? Line 90 page 4, line 131 page 6, line 138 page 6, line 206 page 9 etc..

ANSWER:

Thanks for this comment. Yes, the authors are aware that HPLC stands for high performance liquid chromatography. But as we also know that the acronym "LC" is now more widely used to refer to liquid chromatography, "HPLC" by "LC" has been changed throughout the revised version of the manuscript.

* Please carefully check all the literature in the text as well as the references: for example the date of the article given is incorrect (line 93 page 4, line 351 page 17); Balkan, T., & Yılmaz, Ö. (2022) - in my opinion this paper does not describe how sampling and subsampling affects to pesticide residues (line 93 page 4), link is missing (line 332 page 16) and etc.

ANSWER:

Thanks for this comment. Following the reviewer recommendation, the date of the article given has been changed to 2004, the reference of Balkan, T., & Yılmaz, Ö. (2022) has been deleted and the link missing in line 332 of page 16 have been corrected in the revised version of the manuscript.

* Please correct the units in all manuscript: from mg/Kg to mg/kg

ANSWER:

Thanks for this comment. Following the reviewer recommendation, the units have been corrected in the whole revised version of the manuscript and in

Tables 3 and 4 of the supplementary.

* Please move -1 to the superscript for example: line 173 page 8.

ANSWER:

Thanks for this comment. Following the reviewer recommendation, -1 has been moved to the superscript 3 times in the revised version of the manuscript.

* Explanation of abbreviations appear only once in the text, check out all manuscript for example: line 176, 186 page 8.

ANSWER:

Thanks for this comment. Following the reviewer recommendation, all the abbreviations have been checked out in the revised version of the manuscript. Some of them (ie. CAC or QUAM) have been deleted as they appear only once in the text, others (ie. EU, EC, MC or CV) have been well defined the first time they appear in the text.

* Please, brief description of sample preparation for analysis.

ANSWER:

Thanks for this comment. Following the reviewer recommendation, the description of sample preparation have been added in the revised version of the manuscript (section 2.4) as follows:

> "The batch of paprika was prepared in the factory using the traditional methodology (Fernández-Trujillo & Escarabajal, 2006). This consists of cleaning, pre-crushing by hammer, milling in serial with at least 6 mills, mixing in a 1000 kg blender finally passing through a 2000 µm sieve."
>
> "The saffron samples (provided as powder) were prepared by the producer-trader and the homogenization included separation of stigmas, drying of stigmas and grinding and sieving (Europasaffron, s.f.). It was not possible to obtain information about the sieve size or the grinding mechanism that was used."

The new references added are:

- Fernández-Trujillo, J. P., & Escarabajal, D. (2006). El proceso tradicional de elaboración del pimentón de Murcia y sus posibles innovaciones. Grasas y Aceites, 57(4), 433-442.
- Europasaffron. (s.f.). Regional saffron cultivation and harvesting techniques in Spain, Greece and Italy. Available on: http://www.europeansaffron.eu/archivos/Annex%20White%20book.pdf.

* Both analyses have a limit of quantification of 0.01 mg/kg in the case of spices (line 190 page 8) - this sentence is incorrect, change it.

ANSWER:

Thanks for this comment. In response to the reviewer's question, the sentence has been rewritten in the revised version of the manuscript as follows:

> *"The analysis of pesticides by GC-MS/MS and LC-MS/MS has a quantification limit of 0.01 mg/kg in the case of paprika and saffron, as shown in the validation results according to SANTE 11312/2021 in supplementary Table 2."*

Reviewer #3:

The measurement uncertainty in pesticide residue analysis is a quantitative indicator of the confidence describing the range around a reported or experimental result within which the true value can be expected. The paper entitled 'Uncertainty and risk associated in the analysis of pesticides in spices homogeneous samples' determined the measurement uncertainty of the QuEChERS-HPLC/GC-MS/MS method for paprika and saffron that were collected from two factories located in the Region of Murcia and sampling weight for best results were investigated. The results of this paper are important for market monitoring. However, the novelty of this paper is not enough for publication in Food Chemistry. Moreover, the paper in its current form has some problems which should be improved before being resubmitted to a journal.

Thank you for taking the time to review the manuscript and for your valuable comments, which will greatly improve our manuscript.

1. The concerns for readers are how big the measurement uncertainty is and which weight is the best for pesticide residue analysis in species homogeneous samples. However, it could be not found in the abstract.

ANSWER:

Thanks for this comment. In response to the reviewer's question, the sentence has been rewritten in the revised version of the manuscript as follows:

> *"On the other hand, results using the Monte Carlo (MC) simulation on the size of the subsample indicate that uncertainty is lower for weights between 20 and 30 g and increases for sample sizes of 100 g. A sample size of 30 g was used for saffron, and the values simulated with the MC method were confirmed."*

2. In section Introduction, several papers (such as 10.1007/s00769-022-01512-z; 10.1007/s11356-021-16042-3;10.1080/03601234.2012.676425; 10.1021/acs.jafc.9b07685) have investigated measurement uncertainty in pesticides residue analysis. However, the paper didn't cite and analyze them. Moreover, the topic of this paper is measurement uncertainty. But section introduction didn't summarize how many parameters affected the measurement uncertainty and why the sample size was selected. In previous studies, several parameters were investigated, such as the weight of the sample, calibration solutions, final volume of the sample, and intermediate repeatability.

ANSWER:

Thanks for this comment. the recommended articles are not referenced since they are related to the uncertainty of the pesticide analysis technique and our article does not evaluate the uncertainty of the analytical technique, but of the sampling and subsampling. Despite this, and to better explain the reason for this study, the following paragraphs in the introduction of the revised version of the manuscript have been modified as follows:

> *"As for the second factor, Regulations 2002/63/EC (EC, 2002) and 152/2009 (EC, 2009) provide guidelines as to how sampling for official control should be carried out. However, the recommendations for homogeneous samples only refer to the minimum sample size, e.g. 0.5 kg for flours and 0.1 kg for spices. This sample size can pose a significant problem for the analysis of high-value spices such as saffron. In the case of this spice, whose market price fluctuates greatly depending on quality and country of origin, it can mean incurring costs of up to €1000 per sample (0.1 kg) (Azafrán, 2023; Holz, Illarionov, Wax,*

Schmidt, & Fischer, 2023; Quoc, 2023) for laboratory analysis."
"Therefore, the aim of this study was to evaluate the uncertainty and risk associated with sampling and subsampling between customer and laboratory in multi-residue pesticide analysis by GC-MS/MS and LC-MS/MS in homogeneous spice samples. Paprika and saffron were used for this study. Both spices are homogeneous: paprika was chosen to evaluate uncertainty and risk due to its low cost and high availability, while saffron was chosen in order to validate the results obtained from the paprika in a high cost, and therefore low quantity, of product in multi-residue pesticide analysis."

3. In this manuscript, the samples are collected from two factories and are homogeneous according to the standard of the factories. How to ensure the homogeneity of samples? The spiked experiments are necessary.

ANSWER:

Thanks for this comment. One of the main ideas of this study is to measure the uncertainty involved in analyzing less sample amount than is possible for pesticide analysis, mainly because companies that produce spices very expensive as saffron, don´t have to send 100 g of sample to the laboratory.

The samples we have analyzed in the study come from two batches from two companies where natural pesticide contamination was detected. The companies immobilized the batches, and we had access to the samples to carry out the study. Therefore, these are real samples that the company sends to the laboratory for analysis (the homogeneity of the powder sample is assumed in its manufacturing process) and the batch did not have to be spiked since we previously detected that it was contaminated with the pesticides described in the manuscript.

4. Four analytes are determined and not adequate. Determining multi-class pesticide residues is necessary.

ANSWER:

Thanks for this comment. Four analytes were detected in the batch but a total of 115 and 185 pesticides were analyzed by LC-MS/MS and GC-MS/MS, respectively. The following sentences have been changed in the material and methods section of the revised version of the manuscript as follows:

"A total of 185 pesticides were analyzed, and only 2 were detected in

paprika and 2 in saffron by GC-MS/MS using an Agilent…"
"A total of 115 pesticides were analyzed, and only 2 detected in paprika by LC-MS/MS using an Agilent…"

5. Although the study investigated measurement uncertainty affected by sample weight, quality control and assurance data are essential.

ANSWER:
Thanks for this comment. In order to clarify this fact, the following sentence and a supplementary Table 3 have been included in the revised version of the manuscript as follows:

"All the samples were analyzed with quality control in each batch using spiked samples over a paprika and a saffron blank sample according to SANTE 11312/2021 (SANTE, 2021). The results of quality assurance for the spiked samples can be seen in supplementary Table 3."

Table 3. Results of quality assurance in batch M1 to M8 (paprika) and M1 to M3 (saffron).

Compound	Paprika	Saffron	%RSD
Fludioxinil	M1: 90; M2: 86; M3: 83 M4: 89; M5: 89; M6: 88 M7: 89; M8: 91	--	2.9
Cypermetrine	M1: 83; M2: 85; M3: 81 M4: 88; M5: 82; M6: 84 M7: 81; M8: 86	--	3.0
Chlorpyrifos	--	M1: 83; M2: 85; M3: 82	1.8
Piperonyl butoxide	--	M1: 87; M2: 81; M3: 80	4.6
Acetamiprid	M1: 86; M2: 91; M3: 82 M4: 93; M5: 89; M6: 92 M7: 89; M8: 94	--	4.4
Spirotetramat	M1: 91; M2: 92; M3: 88 M4: 92; M5: 86; M6: 86 M7: 81; M8: 81	--	5.1

Paprika: % Recovery in each batch (M1 to M8) of paprika. The batch is formed by all the samples analyzed for M1 to M8 in paprika; Safron % Recovery in each batch (M1 to M3) of saffron. The batch is formed by all the samples analyzed for M1 to M3 in saffron; % RSD: Relative standard deviation for all quality control.

6. Qualitative and quantitative ions for determination in GC/HPLC-MS/MS and other MRM parameters are not found.

ANSWER:

Thanks for this comment. The following sentence and a new supplementary Table 2 are included in the revised version of the manuscript as follows:

> "The analysis of pesticides by GC-MS/MS and LC-MS/MS has a quantification limit of 0.01 mg/kg in the case of paprika and saffron, as shown in the validation results according to SANTE 11312/2021 (SANTE, 2021) in supplementary Table 2."

Table 2. Pesticide validation summary.

Compound	Analysis	Numb	MRM	%Rec	%RSD	LoQ
Fludioxinil	GC	5	248>154 248>127	86	4.2	0.01 mg/Kg
Cypermetrine	GC	5	181>152 181>127	84	4.4	0.01 mg/Kg
Chlorpyrifos	GC	5	197>169 197>107	89	6.5	0.01 mg/Kg
Piperonyl butoxide	GC	5	176>131 176>115	81	5.2	0.01 mg/Kg
Acetamiprid	LC	5	223>126 223>90	87	5.5	0.01 mg/Kg
Spirotetramat	LC	5	374>330 374>302 302>216 302>115	86	5.7	0.01 mg/Kg

Analysis: GC corresponding to GC-MS/MS analysis, LC corresponding to LC-MS/MS analysis; Numb: Number of repetitions for the pesticide validation; MRM: precursor ion > product ion (quantitative), precursor ion > product ion (qualitative). In the case of spirotetramat, the table show the ions for spirotetramat and spirotetramat enol; % Rec: Average recovery percentage for each pesticide in the validation process. In the case of spirotetramat, the table show the result as sum of spritotetramat and spirotetramat enol; % RSD: Relative standard deviation for each pesticide; LoQ: Limit of Quantification calculated with 5 blank spike samples of paprika and saffron with a recovery between 70-120% and RSD<20% according to SANTE 11312/2021.

7. In supplementary Table 1, more than 200 g are collected for Paprika samples, but about 40 g are gathered for Saffron samples. And paprika was used for validation and investigation. The title is better changed to Uncertainty

and risk associated in the analysis of pesticides in homogeneous paprika samples. Moreover, 200 g is insufficient for three subsample sizes with two replications.

ANSWER:

Thanks for this comment. The title has been changed to:

"Uncertainty and associated risks in the analysis of pesticides in homogeneous paprika samples".

Further, the duplicate refers to 2 portions for analysis by QuEChERS. The following text has been changed in the revised version of the manuscript for clarification as follows:

"To obtain each portion of the sample (5, 20 and 100 g) from the original sample (M1 to M8, supplementary Table 1), a direct transfer was made into a plastic bag using a disposable plastic spatula and weighed on a precision balance. The amount of sample was weighed to exactly 5.0 grams, 20.0 grams and 100.0 grams.

For the duplicate analysis showed in Figure 2, 5.0 g of sample was weighed directly from each heavier bag (20 g, 100 g) with using a disposable plastic spatula to transfer to the Eppendorf tube on a precision balance. In the case of the samples with 5.0 grams, the total content of the bag was transferred directly to an Eppendorf tube. QuEChERS analysis was then performed on the Eppendorf tube.

No homogenization of the samples was performed during these steps in order to avoid including any additional homogenization processes in the analysis."

8. The detailed procedures of QuEChERS are not found throughout the paper.

ANSWER:

Thanks for this comment. The detailed procedures of QuEChERS method have been included in the revised version of the manuscript as follows:

"The QuEChERS extraction, based on EN 15662, used 5 grams of paprika or saffron, to which 10 ml of water was added. After manual homogenization, 10 ml of acetonitrile was added (E7 EN 15662 extraction) and the mixture was shaken for 1 minute. After shaking, the

salts (NaCl, DHS, THS, Na_2SO_4) were added and it was centrifuged for 5 minutes at 3000 rpm. A clean-up process using PSA and C18 was carried out (based in C3 EN 15662 clean-up) on the organic aliquot obtained and finally it was centrifuged again at 3000 rpm for 5 minutes to get the final aliquot for GC-MS/MS and LC-MS/MS analysis."

9. Detailed Monte Carlo method used in this study is not found. Was it used to simulate the lowest CV for different weights? In supplementary Figure 2, data from three points cannot indicate that an ideal sample size can be between 20 and 30 g. Setting more points or simulating the results based on the experimental data.

ANSWER:

Thanks for this comment. The MC method (ne section 2.8) and an explication in the supplementary Figure 2 has been included in the revised version of the manuscritpt as follows:

"2.8. Monte Carlo simulation technique
The MC model for each pesticide simulation was performed with a lognormal distribution (Guo & Li, 2021; Hill & Reynolds, 2002; Horváth, Ambrus, Mészáros, & Braun, 2013) in a loop of 105 simulations in R. The concentration and standard deviation required for the simulation for each pesticide in the normal probability distribution were as follows: for concentration, the value of the limit of quantification of each pesticide (0.01 mg/kg), and for the standard deviation, the experimental values obtained for each pesticide and in each of the subsamplings studied (100 g, 20 g and 5 g). The data obtained were used for the modeling of CV versus sample weight (Supplementary Figure 2)."

10. At least five replications for each level are set for method validation. Why are two replications set in this study? Can the results be applied for method establishment?

ANSWER:

Thanks for this comment. The validation of the pesticide analysis method was carried out with 5 repetitions as indicated in supplementary Table 2. However, the experimental design indicated in Figure 1 and 2 only indicates two replicates since we only wanted to compare variances and not validate the method. In any case, the introduction section and the title of the Figures have been modified in order to clarify these aspects.

Reviewer #4:

This article describe a research on the Uncertainty associated in the analysis of pesticides in spices homogeneous Samples. It is worthwhile to study the sampling or subsampling uncertainty associated in the residue analysis, since it is often ignored in the routine labs. However, some major information should be supplied in the manuscript.

Thank you for taking the time to review the manuscript and for your valuable comments, which will greatly improve our manuscript.

Detail comments:

1. line 84-85, Do you mean the cost of 0.1 kg of sample is 800-1000 eu, or it take 800-1000 eu to "send to the lab" ? Please clarify it.

ANSWER:

Thanks for this comment. In order to clarify the cost of the sample of saffron, the phrase has been rewritten in the revised version of the manuscript as follows:

> *"In the case of this spice, whose market price fluctuates greatly depending on quality and country of origin, it can mean incurring costs of up to €1000 per sample (0.1 kg) (Azafrán, 2023; Holz, Illarionov, Wax, Schmidt, & Fischer, 2023; Quoc, 2023) for laboratory analysis."*

In addition, two extra references have been added to the revised version of the manuscript to support the price of saffron:

> Azafrán. (2023). Price of saffron. Available on: https://azafranespecia.com/precio-azafran-%F0%9F%8C%BA/.
>
> Quoc, L. P. T. (2023). Saffron: A Look from the Customer in Vietnam. The Malaysian journal of medical sciences, 30(2), 180-181. https://doi.org/10.21315/mjms2023.30.2.17

2. line 131-132, For GC analysis and for HPLC analysis boscalid and spirotetramat. Were these two analytes also positive in paprika ?

ANSWER:

Thanks for this comment. It was a mistake in the boscalid pesticide, is spirotetramat and has been changed in the revised version of the manuscript.

3. line 167, Which pesticides were tested by GCMSMS。 The analytes and the MRM transitions should be listed, or give reference using the same condition. Similarly, line 178, Which pesticides were tested by LCMSMS。 The analytes and the MRM transitions should be listed, or give reference using the same condition.

ANSWER:

Thanks for this comment. In order to clarify this fact, the following sentences and supplementary Table 2 have been included in the revised version of the manuscript as follows:

"The samples used in this study came from a batch previously analysed by the supplier. In the case of the paprika, the original batch was positive for GC analysis in fludioxinil and cypermethrin, defined in Regulation (EC) No 396/2005 as cypermethrin including other mixtures of constituent isomers (sum of isomers). For LC analysis, it was positive in acetamiprid and spirotetramat, defined in Regulation (EC) No 396/2005 as spirotetramat and spirotetramat-enol (sum of), expressed as spirotetramat. In the case of the saffron, the batch was positive for the pesticide chlorpyrifos and the organic compound piperonyl butoxide (PBO) in GC analysis, which is used as a synergistic component in pesticide formulations."

Table 2. Pesticide validation summary.

Compound	Analysis	Numb	MRM	%Rec	%RSD	LoQ
Fludioxinil	GC	5	248>154 248>127	86	4.2	0.01 mg/Kg
Cypermetrine	GC	5	181>152 181>127	84	4.4	0.01 mg/Kg
Chlorpyrifos	GC	5	197>169 197>107	89	6.5	0.01 mg/Kg
Piperonyl butoxide	GC	5	176>131 176>115	81	5.2	0.01 mg/Kg
Acetamiprid	LC	5	223>126 223>90	87	5.5	0.01 mg/Kg
Spirotetramat	LC	5	374>330 374>302 302>216 302>115	86	5.7	0.01 mg/Kg

Analysis: GC corresponding to GC-MS/MS analysis, LC corresponding to LC-MS/MS analysis; Numb: Number of repetitions for the pesticide validation; MRM: precursor ion > product ion (quantitative), precursor ion > product ion (qualitative). In the case of spirotetramat, the table show the ions for spirotetramat and spirotetramat enol; % Rec: Average recovery percentage for each pesticide in the validation process. In the case of spirotetramat, the table show the result as sum of spritotetramat and spirotetramat enol; % RSD: Relative standard deviation for each pesticide; LoQ: Limit of Quantification calculated with 5 blank spike samples of paprika and saffron with a recovery between 70-120% and RSD<20% according to SANTE 11312/2021.

4. Line 190, How did you know the LOQ?. It should be in the method validation and put in the results and discussion. The method validation was ignored?

ANSWER:

Thanks for this comment. In order to clarify this fact, the following sentence and supplementary Table 2 have been included in the revised version of the manuscript as follows:

"The analysis of pesticides by GC-MS/MS and LC-MS/MS has a quantification limit of 0.01 mg/kg in the case of paprika and saffron, as shown in the validation results according to SANTE 11312/2021 (SANTE, 2021) in supplementary Table 2."

5. line 204, equation 2, in the CVsp , the "sp" may also be the sample preparation, including extraction and cleanup procedures. In this study, the CV of sample preparation was ignored, however, the sample preparation is also an source of uncertainty. As for the CVsp of the the sample preparation, and uncertainty measurement in pesticide analysis, I recommend the following related references to the authors:

[1] Steven J. Lehotay, Lijun Han, Yelena Sapozhnikova. Use of a quality control approach to assess measurement uncertainty in the comparison of sample processing techniques in the analysis of pesticide residues in fruits and vegetables. Analytical and Bioanalytical Chemistry (2018) 410:5465-5479. https://doi.org/10.1007/s00216-018-0905-1

[2] Lijun Han, Steven J. Lehotay,* and Yelena Sapozhnikova. Use of an efficient

measurement uncertainty approach to compare room temperature and cryogenic sample processing in the analysis of chemical contaminants in foods. J. Agric. Food Chem., 2018, 66, 4986-4996

ANSWER:

Thanks for this comment. In order to clarify this fact, one recommended reference has been mentioned and the following sentence has been included in the revised version of the manuscript as follows:

"Where CV_{sp} is the relative standard uncertainty associated with subsampling, and sample preparation included an extraction and clean-up process (Lehotay, Han, & Sapozhnikova, 2018) in the laboratory and CVA is associated with the specific analysis, in this case LC-MS/MS and GC-MS/MS."

6. Line 214-215, the above two references are recommended to be mentioned and cited.

ANSWER:

Thanks for this comment. Following the reviewer recommendation, the recommended references have been mentioned and cited in the revised version of the manuscript.

7. Line 261, Which version of QuEChERS METHOD did you use? The procedure should be briefly described. What sorbents did you use for the cleanup? and did you do the method validation of the method for the two spice samples?

ANSWER:

Thanks for this comment. Following the reviewer recommendation, the QuEChERS and validation method are included in the revised version of the manuscript as follows:

"The QuEChERS extraction, based on EN 15662, used 5 grams of paprika or saffron, to which 10 ml of water was added. After manual homogenization, 10 ml of acetonitrile was added (E7 EN 15662 extraction) and the mixture was shaken for 1 minute. After shaking, the salts (NaCl, DHS, THS, Na_2SO_4) were added and it was centrifuged for 5 minutes at 3000 rpm. A clean-up process using PSA and C18 was carried out (based in C3 EN 15662 clean-up) on the organic aliquot obtained and finally it was

centrifuged again at 3000 rpm for 5 minutes to get the final aliquot for GC-MS/MS and LC-MS/MS analysis."

"All the samples were analyzed with quality control in each batch using spiked samples over a paprika and a saffron blank sample according to SANTE 11312/2021 (SANTE, 2021). The results of quality assurance for the spiked samples can be seen in supplementary Table 3."

8. Line 261, "For the QuEChERS, 5g sample was usually used." How did you operate for the sample preparation for the 20g or even 100g samples using QuEChERS. This should be briefly described in specific.

ANSWER:

Thanks for this comment. Following the reviewer recommendation, the section 2.7 has been rewritten in the revised version of the manuscript as follows:

"To obtain each portion of the sample (5, 20 and 100 g) from the original sample (M1 to M8, supplementary Table 1), a direct transfer was made into a plastic bag using a disposable plastic spatula and weighed on a precision balance. The amount of sample was weighed to exactly 5.0 grams, 20.0 grams and 100.0 grams.
For the duplicate analysis showed in Figure 2, 5.0 g of sample was weighed directly from each heavier bag (20 g, 100 g) using a disposable plastic spatula to transfer to the Eppendorf tube on a precision balance. In the case of the samples with 5.0 grams, the total content of the bag was transferred directly to an Eppendorf tube. QuEChERS analysis was then performed on the Eppendorf tube.
No homogenization of the samples was performed during these steps in order to avoid including any additional homogenization processes in the analysis."

9. Table 1-3, acetamiprid? It was boscalid in line 132. Please check.

ANSWER:

Thanks for this comment. There was an error in the line 132, the phrase has been rewritten with acetamiprid in the revised version of the manuscript.

10. Table 4, how did you get the C, CVs, CVsp, CVan, and CVres using the 16

numbers in Table 1-3? Which groups of numbers did these CV come from? I think they are the RSDs of some data. This should be indicated clearly.

ANSWER:

Thanks for this comment. The legends in Table 4 have been rewritten to clarify values and calculations used.

11. English language need to be polished.

ANSWER:

Thanks for this comment. The language of the manuscript has been reviewed by a native English speaker with extensive experience editing manuscripts.

Author Contributions

José Manuel Veiga-del-Baño: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing; Juan José Cuenca-Martínez: Conceptualization; Data curation; Formal analysis; Writing – original draft; Pedro Andreo-Martinez: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing; Miguel Ángel Cámara: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; José Oliva: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; and Miguel Motas: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft.

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.