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Miniaturized and automated analysis of pesticides in *Cannabis sativa* L. flowering tops by means of a robotic platform coupled to liquid chromatography-tandem mass spectrometry

Danilo Donnarumma [a], Giuseppe Cirino Presti [a], Adriana Arigò [b], Alessandra Trozzi [a], Giuseppe Micalizzi [a,*], Luigi Mondello [a,c], Ivana Lidia Bonaccorsi [a]

[a] Messina Institute of Technology c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale G. Palatucci 13, Messina, 98168, Italy
[b] Department of Pure and Applied Sciences, University of Urbino Carlo Bo, Piazza Rinascimento 6, Urbino, 61029, Italy
[c] Chromaleont s.r.l., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale G. Palatucci 13, Messina, 98168, Italy

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ABSTRACT

Cannabis sativa L. plant has acquired significant attention in recent years considering that numerous countries around the world are legalizing it for medical uses or recreational purposes. Due to its increasing popularity, farmers are particularly prone to use pesticides such as insecticides, acaricides, and fungicides in order to eliminate, repel, or minimize aphids, spider mites, and thrips, respectively. Accurate determination of pesticide residues in cannabis plants is mandatory to safeguard consumer health. This research study is focused on the employment of a robotic platform online coupled to UHPLC-MS/MS instrument for the rapid screening of pesticides in *Cannabis sativa* L. flowering tops. The developed automated procedure required only 30 mg of dried sample and 200 μL of acetonitrile as unique extraction solvent. The total analysis time was 25 min per sample, including extraction cycle (10 min). The method was proved by defining the following figures of merit: intra- and inter-day repeatability, linearity range, limits of quantification (LoQs), recovery, and accuracy. The LoQs for all the analytes were of 0.005 μg g^{-1}, except for aldicarb and boscalid compounds (0.01 μg g^{-1}). Matrix-matched calibration curves showed good linearity over the range with coefficients of determination $\geq$ 0.9991. Recoveries ranged from 72.3 % to 116.2 % in accordance with 70-120 % allowed range by SANTE/11312/2021v2026 guidelines. The sample preparation greenness was assessed using sample preparation method of sustainability (SPMS) and analytical greenness metric (AGREEprep) tools, providing scores of 6.42 and 0.55 respectively, higher than those obtained for conventional QuEChERS workflow (3.79 and 0.16).

1. Introduction

Cannabis sativa L. is a dioecious, rarely monoecious, annual flowering herb belonging to *Cannabaceae* family [1]. Female inflorescences contain the resin particularly rich in glandular trichomes which enclose the phytocannabinoids, the main psychoactive and medicinal drugs of the cannabis plant, and terpenes [2]. The term "cannabis" refers to the flowering or fruiting tops of the cannabis plant from which the resin has not been extracted. Its non-medical use has been prohibited by the United Nations in the 1961 international drug control treaty [3] due to psychoactive effects of the delta-9-tetrahydrocannabinol (δ9-THC) which can cause irritability, anxiety, distorted perceptions, and dysfunctional behavior when it is administered at high doses [4]. Evidence on therapeutic benefits of cannabis has been also correlated with cannabidiol (CBD) [5–7].

Nowadays, *Cannabis sativa* L. cultivation affects almost all countries in the world (151 countries [8]), and the landscape of global legalization is constantly evolving. Some countries have fully legalized cannabis for medical and recreational uses, such as Canada, twenty-four states in the United States, Mexico, Uruguay, Germany, the Czech Republic, and South Africa. Other countries, such as most European nations, are showing more cautious progress and still limit cannabis to medical use

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* Corresponding author.
E-mail address: giumicalizzi@unime.it (G. Micalizzi).

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only. Globally, cannabis legalization is still in its early stages in many parts of the world, especially in Asia and Africa [9]. Apart from cannabis cultivation exclusively limited to medical and recreational uses, legitimate cultivations are also intended for industrial and horticultural purposes. Due to multiple application areas, cannabis crops are increasing worldwide, especially in countries and states that are legalizing it for medicinal and recreational uses [10].

Similarly to other plants, cannabis is susceptible to infestations of pests, fungi, infections, and diseases, consequently growers are particularly prone to use pesticides for eliminating, repelling, or minimizing insects, fungi, bacteria, virus and other microbes with the final objective to increase production yields. The most common pests associated with cannabis plants are aphides, spider mites, and thrips [11]. Fungal diseases can be also encountered, especially in indoor cultivations as lighting systems are typically employed. Consequently, insecticides, acaricides, and fungicides are frequently associated with cannabis plants cultivations [12]. Exposure to pesticide residues can occur via inhalation, ingestion, and dermal absorption. Inhalation of pesticides can occur when smoking dried cannabis flowering tops or inhaling vapors from processed cannabis products that are heated, melted, or burned [13]. As a result, pesticide residues laboratory testing for cannabis is mandatory in order to ensure high level of protection for consumers and medicinal cannabis users who already have adverse health complications.

Since the cannabis legalization continues to evolve with various scenarios around the world, no specific guidelines for pesticide use on cannabis cultivation have been established. This issue always seems to create confusion. In Europe, the Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 [14] governs the use of pesticides in agricultural products to ensure a high level of protection of both humans and animals health, and the environment. However, while for food and feed of plant and animal origin exist well-established rules setting maximum residue levels (MRLs) through Regulation (EC) No 396/2005 [15], cannabis is not yet specifically included in these lists. Therefore, in the absence of specific Community limits for pesticides residues in cannabis, it is recommended to apply the same rules as for food plants. However, these guidelines are associated with agricultural products intended for oral ingestion rather than inhalation. On the other hand, some European nations including Malta, Germany, Denmark, and Netherland follow the European Pharmacopeia guidelines as cannabis is classified as herbal active substances or herbal medicinal products. The lists of active ingredients and their limits are reported in the Ph. Eur. 2.8.13 guideline [16]. In United States, the use of pesticides is regulated by U.S. Environmental Protection Agency (EPA). At present, no pesticides have been registered or approved by EPA for use on cannabis specifically. However, each state has different regulations. California was the first state to develop action levels on allowable pesticide residues in cannabis products in accordance with public health concerns. In 2017, the California Department of Pesticides Regulation (CDPR) recommended the use of the term “action levels” to establish minimum testing criteria for pesticide residues remaining in cannabis products designated for the marketplace in California [13]. Due to the risks associated with inhalation exposure, specific action levels were established for cannabis products that are used or consumed via smoking or vaping such as dried flowering tops, concentrate and vape pens/cartridges [13]. The CDPR published a list of sixty-six pesticides recommend for analytical testing in legally processed cannabis products. The list was subdivided into two different categories: Category I included twenty-one components for which no detectable residues were admitted (zero tolerance); Category II included the remaining 45 pesticides which are currently registered by CDPR for food use in California. The Category II pesticides also have limits of quantification (LoQs) at variable maximum residue limits (MRLs) for “inhalables” or “Other Cannabis Goods.” Generally, inhalables have the lowest action limits at 0.1 $\mu g\ g^{-1}$. This means that very sensitive analytical approaches are mandatory for the pesticides screening in cannabis-based products.

In this respect, from an analytical point of view, the sample preparation for pesticide residues laboratory testing is extremely important, but it is also quite challenging, especially for cannabis herbal products as it is a very complex matrix. In fact, many pesticides are hydrophobic and adhere to the glandular trichomes on the leaves and flowers which are highly resinous, making them hard to isolate from the matrix [11]. As aforesaid, these structures abound in cannabinoids and terpenes, thus pesticides isolation and purification result particularly difficult. To date, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method is probably the most widespread sample preparation approach for multi-residual pesticide analysis due to its robustness and reliability. Nevertheless, it has several drawbacks consisting of i) multiple manual steps (centrifugation, homogenization, liquid handling) [17]; ii) reproducibility can be strongly associated with the analyst; iii) poor recovery of polar pesticides [17]; iv) high amount of consumable materials including salts, dispersive sorbent materials, acetate or citrate buffers, and extensive use of glassware [18,19], which affect the cost per analysis; v) large volumes of extraction solvents (e.g. 10 mL of water and 10 mL of acetonitrile [20]) affect the greenness and sustainability of the entire analytical procedure. Alternatively, solid-liquid extraction (SLE) followed by solid-phase extraction (SPE) method has been also experienced in literature for the removal and isolation of pesticides from cannabis inflorescences [21,22]. The major drawbacks are related to the large volumes of extraction solvent (total volume 25 mL for 1 g of sample [21]) and evaporative or concentration steps that affect the recovery of the most volatile components.

In accordance with recent trends in analytical chemistry towards automation to avoid or minimize analyst manipulation, to analyze the highest number of samples in as short a time as possible, to reduce costs per analysis and solvent volumes or, if possible, to eliminate toxic organic solvents, the present research project was focused on the employment of a robotic platform online coupled to high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) instrument for the rapid screening of pesticides in *Cannabis sativa* L. flowering tops. Among various figures of merit, intra- and inter-day repeatability, linearity range, limits of quantification (LoQs), recovery, and accuracy were assessed. During the final stage of the research, the developed analytical method was evaluated in terms of analytical sample preparation greenness using sample preparation method of sustainability (SPMS) [23] and analytical greenness metric (AGREEprep) tools [24].

2. Materials and methods

2.1. Sample and chemicals

A total of fifteen samples of dried flowering tops of cannabis (low THC contents according to Italian Regulation No 242/2016 [25]) produced in Italy, were selected for the analysis. The analytical laboratory was formally authorized by the Italian Ministry of Health to possess cannabis inflorescences with a low THC content for scientific research and educational/training purposes (authorization n° SP/77/2025). Pesticide standards (purity $\geq$ 98 %), acetonitrile (ACN, $\geq$ 99.9 %, gradient grade, suitable for HPLC), water (H_2O, suitable for LC/MS, LiChrosolv®), ammonium formate (eluent additive for LC-MS, LiChropur™, $\geq$ 99.0 %), formic acid (98 %-100 %, for LC-MS LiChropur), and triphenyl phosphate (TPP, $\geq$ 99 %) were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). The full list of pesticides is reported in Table S1. For the internal standard (IS) TPP, a stock solution was prepared at a concentration of 10,000 mg L^{-1} in ACN and stored at −20 °C until use. An intermediate TPP solution at a concentration of 100 mg L^{-1} was prepared by dilution of the stock solution in ACN and finally diluted to a final concentration of 1 mg L^{-1} used for sample spiking. A multi-analyte working mixture (3 mg L^{-1} in ACN) was prepared and stored at freezer temperature until use.

2.2. Automatic sample preparation method

An amount of 30 mg of grinded dried cannabis flowering tops was weighted directly in the filtration vial of the robotic preparative station (CLAM2030, Shimadzu Europe, Duisburg, Germany) and spiked with 5 µL of the IS solution (1 mg L^{-1}). The sample was then rehydrated by adding 50 µL of ultrapure H_2O and incubated at room temperature for 60 s. A preliminary rehydration step was included, as recommended by SANTE/11312/2021v2026 guidelines [26], since in dry plant materials, controlled hydration improves matrix wetting, solvent penetration, and analyte release, thereby enhancing extraction efficiency and recovery of pesticide residues [27]. A dried plant matter/rehydration water ratio ranging from 1:2 to 1:5 is conventionally applied [28,29]. However, 50 µL of water was used in this research in accordance with the limited volume of the filtration vial (max 350 µl) used by the CLAM2030 robotic preparative station. After the rehydration step, 200 µL of ACN were added, and vortexed for 5 min at 3000 rpm. After another incubation of 60 s at room temperature, the sample was filtered and 5 µL of the extract were directly injected into the UHPLC-MS/MS system. Such analytical workflow of rehydration, ACN extraction, and filtration steps is summarized in Fig. 1. For the matrix-matched calibration curve building, 30 mg of a blank hemp sample were spiked with 5 µL of the IS solution (1 mg L^{-1}), and 5 µL of the multi-analyte mix at different concentrations (0.005-0.5 µg g^{-1} final concentration in cannabis flowering tops). All the samples were submitted to three independent extractions.

2.3. Calibration and quantification

Pesticide residues quantification in cannabis flowering tops was performed using the IS method. Before being used for matrix-matched calibration and validation experiments, the blank cannabis flowering top sample was analyzed using the developed UHPLC-MS/MS method. No detectable signals were observed for the target pesticides at the corresponding retention times and MRM transitions. The sample was therefore considered suitable as blank matrix for matrix-matched calibration. Separate calibration curves for each pesticide present in the multi-analyte working mixture were used to evaluate MS/MS response linearity. Five-point matrix-matched calibration curves (five replicates for each point) at the concentration range of 0.005-0.5 µg g^{-1} were constructed spiking a blank cannabis flowering top sample (no detectable pesticide residues). The calibration curves were constructed using concentration ratio between pesticide analyte and IS along the horizontal axis *versus* peak area concentration ratio along vertical axis. To correct the data heteroscedasticity, four different weighting factors ($1/x$; $1/x^2$; $1/y$; $1/y^2$) were tested and evaluated considering the minimization of the relative error sum. All the calibration working mixtures were subject to automatic extraction process under the conditions described previously.

2.4. Method figures of merit

The method figures of merit were determined according to the SANTE/11312/2021v2026 guidelines [26]. The repeatability was evaluated in terms of intra-day precision, by performing five independent extractions and analyses of blank cannabis samples spiked at 0.01 µg g^{-1} within the same day (n = 5), and inter-day precision, by performing five independent replicate extractions and analyses at the same spiking level on each of three days (n = 15). Intra-day and inter-day precisions were expressed in terms of relative standard deviation (RSD %). For establishment of the LoQ, intended as the lowest concentration of the analyte that can be validated with acceptable accuracy by using the analytical method and identification criteria [26], ten replicates of the blank cannabis sample were analyzed. The lowest point on the calibration curves corresponding at the concentration of 0.005 µg g^{-1} and a signal-to-noise ratio (S/N) of at least 10, was used as the LoQ for individual analytes. The recovery of the extraction method expressed in terms of percentage (%) was evaluated by comparison of the normalized peak areas of a blank hemp sample spiked at 0.01 µg g^{-1}, with the values measured in a post-extraction spike at the same concentration. Accuracy was evaluated by retro-calculating the concentration of blank cannabis sample spiked before extraction at a nominal concentration of 0.01 µg g^{-1} using matrix-matched calibration curves. Accuracy was expressed as the percentage ratio between the measured and nominal concentrations.

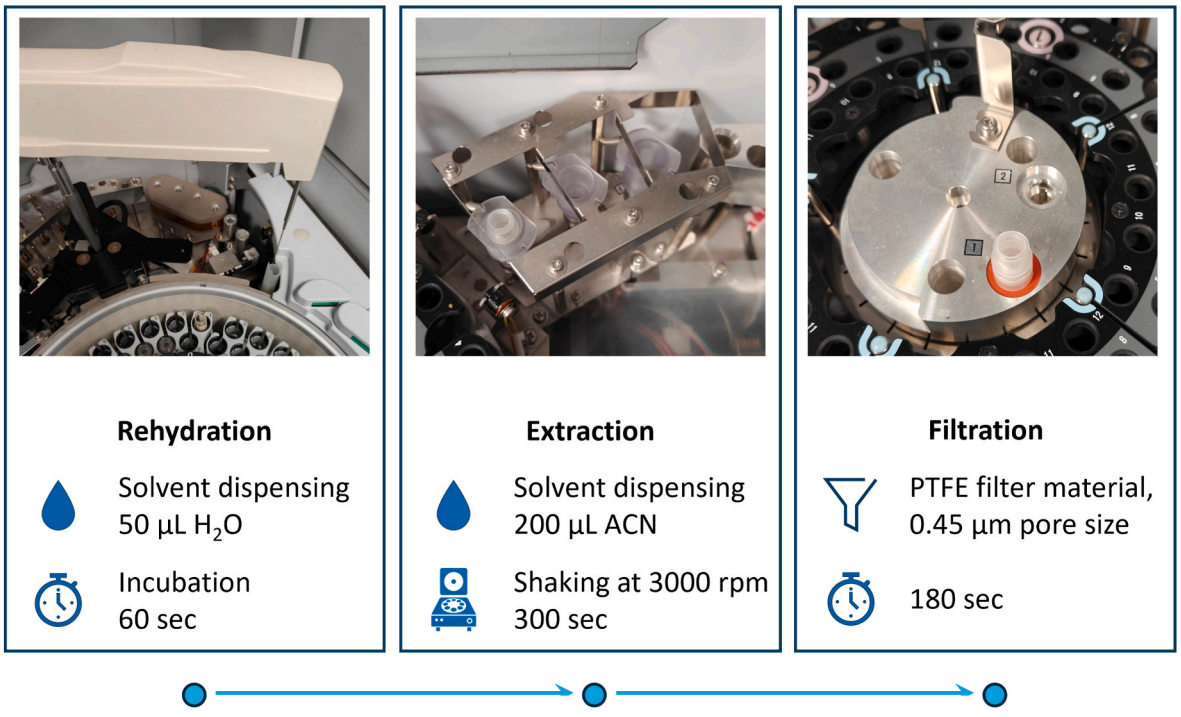

Fig. 1. Fully automated workflow of rehydration, acetonitrile (ACN) extraction, and filtration steps for the analysis of pesticide residues in cannabis flowering tops.

2.5. UHPLC-MS/MS method

For the UHPLC-MS/MS analysis, a Nexera X2 system (two LC-30AD pumps, a DGU-20A5R degassing unit, an autosampler SIL-30AC, a column oven CTO-20AC) coupled to a LCMS-8060 triple quadrupole mass spectrometer (Shimadzu Europe) was employed. As reported in Ferracane et al. [30], the separation was achieved by using an Ascentis Express 90 Å C18 column (10 cm × 2.1 mm ID × 2.7 μm dp) (Merck Life Science), at a temperature of 40 °C. The mobile phase, at a flow rate of 0.4 mL min^{-1}, was composed of H_2O (A) and ACN (B), both with ammonium formate (5 mM) and 0.1 % formic acid. The gradient program was as follows: 0 % B held for 1 min; 1-3 min, 0-42 % B; 3-15 min, 42-82 % B; 15-17 min, 82-98 % B (held 2 min); 19-20, 0 % B (held 1 min). The injection volume was 5 μL. The determination of the target analytes was performed in multiple reaction monitoring (MRM) acquisition mode (dwell time: 8.0 msec) using electron spray ionization source operating both in positive (+) and negative (−) ionization modes. MS/MS parameters were as follows: interface temperature 130 °C, desolvation line temperature 300 °C, heat block temperature 400 °C, nebulizing gas flow (N_2) 3 L min^{-1}, drying gas flow (N_2) 10 L min^{-1}, heating gas flow (dry air) 10 L min^{-1}. Two different MRM transitions (quantifier and qualifier) were monitored for each compound along with their ratio, as previously published [30]. System control and data handling were performed using the LabSolutions software (version 5.95, Shimadzu Europe).

3. Results and discussion

3.1. Automated pesticides extraction from cannabis flowering tops

The main objective of sample preparation procedures is to purify the analytes of interest, eliminating interfering agents that could compromise analyses. Depending on the matrix and the chemical-physical properties of the analyte(s) of interest, several extraction strategies can be employed. Focusing on pesticides, Anastassiades and Lehotay introduced QuEChERS in 2003 for the detection of pesticide residues in fruits and vegetables [31]. However, QuEChERS procedure has several drawbacks: homogenization, centrifugation, liquid handling steps performed manually by the analyst take time; less stable analytes may degrade due to pH change for the addition of salt and water; PSA as sorbent material could result in poorer recoveries of polar pesticides [22]; high amount of solvent, glassware, and consumables are typically required [18,19] affecting waste volumes, environment, and cost per analysis.

Alternatively, solid-liquid extraction (SLE) is a reliable alternative to QuEChERS for removing pesticide residues from cannabis flowering tops [21,22] and other matrices [32]. This strategy offers high extraction yields and sample cleaning efficiencies from solid material. However, several drawbacks are documented, including extensive use of organic solvents that may affect the safety of operators as well as cost per analysis. In addition to this limitation, preconcentration step is a critical element in SLE determining the sensitivity of the chromatographic technique (the lower the target analyte concentration the more sensitive the detection needs to be). In the last years, some efforts have been made to automate the extraction procedure and achieve higher throughput, but most of the reported approaches focus on the adaptation of existing QuEChERS or SLE methods without extensive modifications or reduction in solvent volumes and materials used [19,33]. On the other hand, both green analytical chemistry and green sample preparation principles push towards a miniaturization of the entire analytical workflow in order to significantly decrease solvents consumption and waste production [34,35]. To address this necessity and reduce the use of outdated, harmful and resource-intensive methods, a fully automated and miniaturized SLE extraction protocol was developed by using a robotic workstation with the objective of 1) accelerating and simplifying the sample treatment, 2) reducing the costs per analysis, 3) avoiding the analyst exposure to chemicals and reagents. For the automatic extraction of pesticide residues from cannabis flowering tops, the amount of homogenous sample was drastically reduced to 30 mg instead of the usual sample amount of 2 g [20,36]. Importantly, grinding of the cannabis sample before solvent extraction is a mandatory pretreatment as it promotes an increase of the contact surface between the solvent and the solid matrix and it ensures the transfer of pesticides. Extraction efficiency increases with decreasing of the particle size [37]. The first stage of the automatic extraction consisted in the rehydration of the dried cannabis material using 50 μL of water followed by 60 s of incubation. This step was particularly important because dried cannabis material is heterogeneous and highly resinous, which may otherwise reduce reproducibility of the extraction process [27]. After, the robotic platform dispensed ACN solvent as extractant for the recovery of analytes. Acetonitrile was selected as extraction solvent because it is widely used for pesticide residue extraction, is compatible with UHPLC-MS/MS analysis, and provides suitable extraction efficiency for compounds with different polarity. Considering the limited volume allowed by the automatic system (max 350 μl), and the presence of the dried matrix in the open extraction vials, further reducing the available space for the solvent, the volume of ACN was scaled down to 200 μL, the minimum amount of solvent that could be added to the vial without overspilling during the shaking step. This represents a marked reduction compared to conventional QuEChERS and SLE workflows or other published automated approaches [19–21,33]. To the author's knowledge, this is the lowest volume of extractant ever used in the extraction of pesticides from plant matter. Hence, the extraction mixture was then shaken in automatic manner for 300 s in order to facilitate the mass transfer from the solid particles to liquid solvent. Lastly, the cannabis extract was filtered (180 s) by using specialized propylene filtration vial equipped with a PTFE membrane filter with 0.45 μm pore size. Finally, cannabis extract was automatically injected onto UHPLC-MS/MS system for the separation and detection of the pesticide residues. No significant difference in terms of recovery was observed by increasing the rehydration time up to 5 min, while an overall reduction was observed by decreasing the shaking time. The automated workflow describing the rehydration, extraction, and filtration steps is illustrated in Fig. 1. Total extraction time was approximately 10 min. However, the robotic platform was capable to perform the pesticide extraction while UHPLC-MS/MS analysis was running, thus a neat gain in term of duration of the entire analytical workflow was obtained (total time 25 min per sample including sample treatment, chromatographic separation, and mass spectrometry detection).

3.2. Analytical method validation and performance

Linearity was determined using a multi-analyte working mixture containing forty-seven pesticides at different concentration levels ranging from 0.005 μg g^{-1} to 0.5 μg g^{-1}. Table 1 show the list of the pesticides as well as their respective retention times, MRM quantifier and qualifier transitions, calibration ranges, linear regression equations, and coefficients of determination (R^2) obtained for all the components present in the working mixture. Five-point matrix-matched calibration curves were constructed by spiking a blank cannabis flowering tops sample with no detectable levels of the investigated pesticides. Data scedasticity was tested by using the *F*-test (one tail), and all compounds resulted heteroscedastic. Therefore, different weighting factors were tested ($1/x$, $1/x^2$, $1/y$, $1/y^2$) to correct the bias induced by the high concentration points. For each pesticide, the relative error (*RE%*) using the different weighting factor was calculated with the formula reported in the Eq. (1):

$$RE\% = \frac{C_{exp} - C_{th}}{C_{th}} \times 100 \qquad \text{Equation 1}$$

where C_{exp} is the concentration calculated with the weighting factor and

Table 1
List of pesticide residues, retention times (RT), optimized MRM settings (quantifier MRM-1, and qualifier MRM-2 transitions), collision energies (CE, expressed in eV), calibration range, linear regression equation (corrected by weighting factors), coefficient of determination (R^2), LoQs, recovery (Rec., %), accuracy (Acc., %), and repeatability (intra- and inter-day precision, expressed as RSD %).

Pesticide Name	RT (min)	MRM (CE) 1	MRM (CE) 2	Linearity			LoQ (μg g^{-1})	Rec. (%)	Acc. (%)	Repeatability	
				Calibration range (μg g^{-1})	Linear Regression Equation	R^2				Intra-day (%)	Inter-day (%)
Abamectin B1	13.10	891 > 305 (−29)	891 > 567 (−15)	0.005-0.5	y = (0.147998 ± 0.002211)x - (0.000313360 ± 2.199e^{-05})	0.9995	0.005	82.0	101.2	2.4	10.6
Acephate	1.50	184 > 143 (−10)	184 > 49 (−22)	0.005-0.5	y = (4.25822 ± 0.069368)x + (0.0213409 ± 0.0006902)	0.9991	0.005	94.7	103.5	1.5	3.0
Acetamiprid	3.50	223 > 126 (−21)	223 > 56 (−16)	0.005-0.5	y = (4.60128 ± 0.13813)x + (0.000789408 ± 0.000137)	0.9991	0.005	109.6	101.5	6.3	6.9
Aldicarb	3.80	208 > 116 (−9)	208 > 89 (−18)	0.01-0.5	y = (0.100904 ± 0.006427)x - (0.000297098 ± 0.000012)	0.9992	0.01	113.4	97.5	10.8	10.9
Azoxystrobin	5.90	404 > 372 (−15)	404 > 329 (−31)	0.005-0.5	y = (64.8508 ± 2.23068)x + (0.0281768 ± 0.00219)	0.9995	0.005	82.9	106.8	11.9	13.5
Bifenazate	6.50	301 > 170 (−19)	301 > 152 (−38)	0.005-0.5	y = (9.18620 ± 0.17061)x + (0.00329850 ± 0.000169)	0.9992	0.005	85.7	103.8	5.4	9.1
Boscalid	6.20	343 > 307 (−21)	343 > 271 (−31)	0.01-0.5	y = (0.469611 ± 0.010604)x + (0.0121542 ± 0.000207)	0.9997	0.01	79.7	116.5	2.2	8.9
Carbaryl	4.40	202 > 145 (−12)	202 > 127 (−25)	0.005-0.5	y = (0.487337 ± 0.01242)x + (0.00261363 ± 0.00012)	0.9994	0.005	81.2	105.9	3.5	11.3
Carbofuran	4.30	222 > 123 (−21)	222 > 165 (−12)	0.005-0.5	y = (6.19831 ± 0.16422)x + (0.00837459 ± 0.001634)	0.9993	0.005	85.2	106.6	3.2	8.7
Chlorantraniliprole	5.10	484 > 453 (−19)	484 > 286 (−17)	0.005-0.5	y = (5.07574 ± 0.06244)x + (0.00738233 ± 0.00062)	0.9992	0.005	95.0	104.5	0.5	2.6
Clofentezine	8.70	303 > 102 (−37)	303 > 138 (−15)	0.005-0.5	y = (0.357531 ± 0.02558)x + (0.000185208 ± 0.000254)	0.9999	0.005	81.0	98.9	12.5	10.7
Coumaphos	8.70	363 > 227 (−26)	363 > 307 (−19)	0.005-0.5	y = (1.09768 ± 0.02225)x + (0.00174746 ± 0.00022)	0.9994	0.005	74.1	104.4	2.8	4.1
Diazinon	8.50	305 > 169 (−22)	305 > 153 (−21)	0.005-0.5	y = (22.5622 ± 0.9783)x + (0.0276188 ± 0.00973)	0.9998	0.005	82.6	107.5	6.0	11.0
Dimethoate	3.40	230 > 125 (−21)	230 > 199 (−10)	0.005-0.5	y = (3.23316 ± 0.1563)x + (0.00683963 ± 0.00155)	0.9992	0.005	77.5	112.2	5.6	14.2
Dimethomorph	5.20	388 > 301 (−21)	388 > 165 (−34)	0.005-0.5	y = (14.1641 ± 0.1911)x + (0.0283510 ± 0.0019)	0.9991	0.005	89.2	106.7	2.6	6.2
Ethoprophos	6.10	210 > 140 (−21)	210 > 98 (−27)	0.005-0.5	y = (7.13994 ± 0.099)x + (0.0100260 ± 0.00099)	0.9991	0.005	86.7	106.7	4.4	8.2
Etofenprox	15.40	394 > 177 (−14)	394 > 107 (−41)	0.005-0.5	y = (0.721250 ± 0.0112)x + (0.000690487 ± 0.000111)	0.9991	0.005	86.6	105.4	3.4	7.9
Etoxazole	12.20	360 > 141 (−30)	360 > 113 (−55)	0.005-0.5	y = (125.237 ± 5.0826)x + (0.128821 ± 0.0506)	0.9993	0.005	116.2	99.7	6.6	9.1
Fenhexamid	6.10	302 > 97 (−24)	302 > 55 (−41)	0.005-0.5	y = (2.70449 ± 0.04301)x + (0.00240253 ± 0.000428)	0.9993	0.005	73.8	104.9	3.0	3.6
Fenoxycarb	6.90	302 > 88 (−22)	302 > 116 (−11)	0.005-0.5	y = (3.06993 ± 0.1951)x + (0.0298250 ± 0.00194)	0.9994	0.005	111.2	84.9	7.7	8.3
Fenpyroximate	11.90	422 > 366 (−17)	422 > 215 (−27)	0.005-0.5	y = (45.5710 ± 1.6419)x + (0.0373610 ± 0.00164)	0.9993	0.005	83.0	106.9	6.6	11.0
Fipronil	7.80	435 > 330 (16)	435 > 250 (27)	0.005-0.5	y = (5.42437 ± 0.0772)x + (0.00360756 ± 0.000768)	0.9995	0.005	76.7	110.1	2.4	14.3
Fludioxonil	5.50	247 > 180 (28)	247 > 126 (29)	0.005-0.5	y = (0.211487 ± 0.01551)x - (2.06003e^{-05}±1.544e^{-06})	0.9992	0.005	76.8	95.9	3.3	9.5
Hexythiazox	11.50	353 > 228 (−16)	353 > 168 (−24)	0.005-0.5	y = (0.318309 ± 0.00545)x - (0.000638507 ± 5.422e^{-05})	0.9993	0.005	76.9	103.9	5.2	12.9
Imazalil	4.00	297 > 159 (−23)	297 > 41 (−31)	0.005-0.5	y = (3.47327 ± 0.0699)x + (0.0608912 ± 0.000696)	0.9993	0.005	96.0	100.9	1.2	2.3
Imidacloprid	3.40	256 > 175 (−20)	256 > 209 (−17)	0.005-0.5	y = (1.12609 ± 0.02627)x + (0.0261207 ± 0.000261)	0.9993	0.005	90.2	112.7	1.2	5.4
Kresoxim-methyl	7.80	314 > 267 (−8)	314 > 116 (−22)	0.005-0.5	y = (2.07272 ± 0.04114)x + (0.00281547 ± 0.000409)	0.9995	0.005	77.8	94.5	5.7	10.8
Malathion	6.80	348 > 127 (−17)	348 > 99 (−25)	0.005-0.5	y = (2.81408 ± 0.06101)x + (0.00131873 ± 0.000607)	0.9991	0.005	73.0	104.0	5.2	4.2
Metalaxyl	4.60	280 > 220 (−14)	280 > 192 (−18)	0.005-0.5	y = (19.5244 ± 0.22841)x + (0.0265950 ± 0.00227)	0.9997	0.005	81.4	108.6	2.1	10.9
Methiocarb	5.40	226 > 121 (−19)	226 > 169 (−10)	0.005-0.5	y = (1.01110 ± 0.02955)x + (0.000482110 ± 0.0000294)	0.9997	0.005	89.7	101.7	1.7	5.7
Methomyl	2.90	163 > 88 (−10)	163 > 106 (−11)	0.005-0.5	y = (0.689530 ± 0.03059)x + (0.000492270 ± 0.0000304)	0.9993	0.005	82.0	106.0	9.9	12.9
Mevinphos	3.30	225 > 127 (−12)	225 > 193 (−20)	0.005-0.5	y = (1.56372 ± 0.0907)x + (0.00715353 ± 0.000902)	0.9994	0.005	83.0	102.6	5.9	5.7

(*continued on next page*)

Table 1 (*continued*)

Pesticide Name	RT (min)	MRM (CE) 1	MRM (CE) 2	Linearity			LoQ (μg g^{-1})	Rec. (%)	Acc. (%)	Repeatability	
				Calibration range (μg g^{-1})	Linear Regression Equation	R^2				Intra-day (%)	Inter-day (%)
Myclobutanil	5.80	289 > 70 (−22)	289 > 125 (−31)	0.005-0.5	y = (4.60577 ± 0.12991)x + (0.00323473 ± 0.00129)	0.9998	0.005	77.2	97.6	8.1	10.1
Oxamyl	2.90	237 > 72 (−22)	237 > 90 (−8)	0.005-0.5	y = (3.19434 ± 0.0816)x + (0.00523420 ± 0.000812)	0.9991	0.005	84.9	109.0	6.8	10.0
Paclobutrazol	5.30	294 > 70 (−22)	294 > 125 (−36)	0.005-0.5	y = (7.36246 ± 0.08667)x + (0.0155040 ± 0.00086)	0.9996	0.005	81.5	109.1	2.2	10.9
Phosmet	5.80	335 > 160 (−16)	335 > 318 (−8)	0.005-0.5	y = (0.283442 ± 0.0087)x + (0.000195421 ± $8.659e^{-05}$)	0.9994	0.005	74.4	96.6	2.3	2.8
Piperonyl-butoxid	10.50	356 > 177 (−14)	356 > 119 (−33)	0.005-0.5	y = (5.55421 ± 0.0984)x + (0.0120922 ± 0.000979)	0.9993	0.005	79.2	109.4	4.8	12.9
Propiconazole	7.10	342 > 159 (−28)	342 > 69 (−22)	0.005-0.5	y = (6.48331 ± 0.2442)x - (0.000592479 ± 0.0000243)	0.9997	0.005	80.7	105.4	3.6	11.6
Propoxur	4.20	210 > 93 (−24)	210 > 168 (−9)	0.005-0.5	y = (1.43348 ± 0.02145)x + (0.00589504 ± 0.000213)	0.9996	0.005	99.4	102.9	1.1	1.0
Pyridaben	13.40	365 > 147 (−25)	365 > 309 (−13)	0.005-0.5	y = (23.8371 ± 1.0037)x + (0.00432669 ± 0.00099)	0.9999	0.005	105.6	99.4	9.3	8.2
Spinosad	6.00	733 > 142 (−29)	733 > 98 (−55)	0.005-0.5	y = (16.5213 ± 0.4415)x + (0.00670557 ± 0.000439)	0.9992	0.005	102.6	103.1	1.6	3.5
Spirotetramat	5.60	374 > 216 (−33)	374 > 302 (−18)	0.005-0.5	y = (21.3792 ± 0.2331)x + (0.0969650 ± 0.00232)	0.9994	0.005	72.3	105.7	2.1	5.8
Spiroxamine	4.60	298 > 144 (−20)	298 > 100 (−30)	0.005-0.5	y = (18.3357 ± 0.4209)x - (0.00424781 ± 0.000419)	0.9991	0.005	112.9	99.9	9.3	9.8
Tebuconazole	6.30	308 > 70 (−22)	308 > 125 (−38)	0.005-0.5	y = (12.1280 ± 0.7039)x + (0.0347312 ± 0.007)	0.9995	0.005	84.3	107.8	1.8	9.0
Thiacloprid	3.70	253 > 126 (−21)	253 > 90 (−39)	0.005-0.5	y = (4.54123 ± 0.0862)x + (0.000991322 ± 0.000086)	0.9992	0.005	75.3	98.7	7.3	9.7
Thiamethoxam	3.10	292 > 181 (−22)	292 > 211 (−13)	0.005-0.5	y = (0.699152 ± 0.0084)x + (0.00172253 ± $8.395e^{-05}$)	0.9998	0.005	82.3	105.3	1.1	4.9
Trifloxystrobin	9.90	409 > 186 (−19)	409 > 145 (−44)	0.005-0.5	y = (7.39992 ± 0.4488)x - (0.00429415 ± 0.000446)	0.9996	0.005	93.1	99.6	8.7	7.8

C_{th} is the theoretical concentration. The factor that resulted in the lowest sum of RE% was $1/x^2$.

Least squares regression analysis showed good linearity within the evaluated range, with coefficients of determination (R^2) ≥ 0.9991 for all target compounds, after correction with weighting factors. The use of matrix-matched calibration compensates for any matrix effect induced by the sample, as required by SANTE/11312/2021v2026 guidelines [26]. UHPLC-MS/MS chromatogram, acquired in the MRM mode, of the spiked cannabis sample (0.5 μg g^{-1}) is shown in Fig. 2.

The lowest point on the calibration curve, corresponding to a signal-to-noise ratio (S/N) of at least 10, was used as LoQ for the individual pesticides. Most pesticides showed LoQ values of 0.005 μg g^{-1}, except for aldicarb and boscalid with values of 0.01 μg g^{-1}. The developed quantitative analytical method was sensitive enough considering that reference MRLs of pesticides in dried cannabis flowering tops are usually set at higher values. For instance, the lowest action level for pesticides in inhalable cannabis goods reported in the California Code of Regulation (0.1 μg g^{-1}) [13] was at least 10 times higher than the experimental LoQs. Similar considerations can be also made for dried cannabis guidelines reported in "Mandatory cannabis testing for pesticide active ingredients" by the Health Canada where the lowest LoQ value was set at 0.02 μg g^{-1} [38].

Recovery assessment was carried out by comparing normalized peak areas of a blank cannabis sample spiked at 0.01 μg g^{-1} with the values

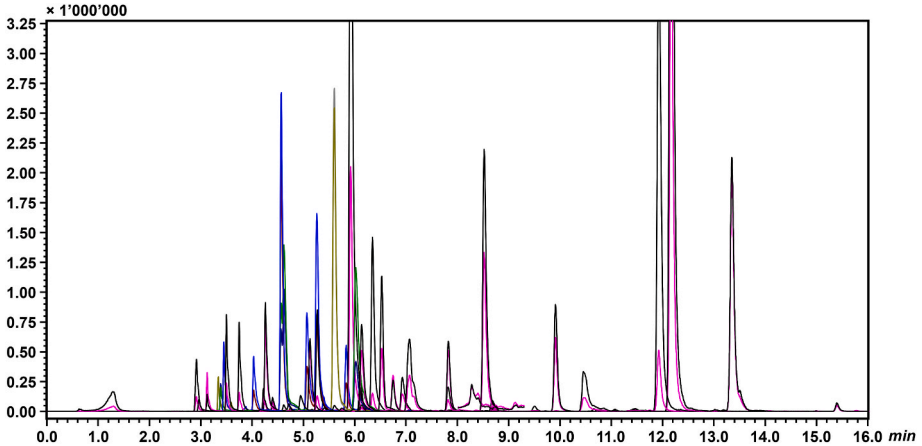

Fig. 2. UHPLC-MS/MS chromatogram, acquired in the MRM mode, of a blank cannabis flowering tops sample spiked with the multi-analyte working mixture at a concentration of 0.5 μg g^{-1}.

measured in a post-extraction spike at the same concentration. The analytical method was capable to provide acceptable mean recovery values (see Table 1) at the spiking level for all the analytes being within the range 70–120 % as reported in SANTE/11312/2021v2026 guidelines [26]. Recoveries for the forty-seven pesticide residues ranged from 72.3 % for spirotetramat to 116.2 % for etoxazole (average value of 86.8 %). Accuracy values for all the tested compounds ranged from 84.9 % to 116.5 % (average value of 103.8 %). Finally, repeatability expressed in terms of intra- and inter-day precision was evaluated by performing independent automated extractions of a blank cannabis flowering tops sample spiked at 0.01 μg g^{-1} with an associated repeatability RSD ≤ 20 %, for all analytes. In detail, intra-day precision ranged from 0.5 % to 12.5 % (average value 4.7 %), while inter-day precision ranged from 1.0 % to 14.3 % (average values 8.4 %).

3.3. Real sample analyses

Fifteen dried cannabis flowering tops samples (THC contents below 0.5 % according to Italian Regulation No 242/2016 [25]) was analyzed for determining the presence of the forty-seven pesticide residues. The fully automated and miniaturized extraction protocol followed by UHPLC-MS/MS analysis demonstrated that eight of the fifteen cannabis samples investigate (53.3 %) were affected by pesticide contaminations as reported in Table 2. In detail, propiconazole was found in six samples with concentrations ranging from 0.0055 ± 0.0003 μg g^{-1} to 0.0077 ± 0.0003 μg g^{-1}. Myclobutanil was detected in one cannabis sample at a concentration level of 0.0059 ± 0.0003 μg g^{-1}, while spiroxamine was quantified (0.0072 ± 0.0007 μg g^{-1}) in a single sample. It should be emphasized that the concentration levels reported in Table 2, especially for myclobutanil and propiconazole in S7 sample, were close to LoQ values. However, the detected values were well below the action limit at 0.1 μg g^{-1} provided for the targeted pesticides, therefore all the quantified pesticides were consistent with Regulation (EC) No 1107/2009 [14] and California Regulations [13]. Interestingly, all the quantified pesticide residues are classified as fungicide, reflecting the common use of such chemicals in the cultivation of *Cannabis sativa* L [12]. On the other hand, some insecticides were also detected at very low levels, below LoQs, such as chlorantraniliprole, fipronil, and thiacloprid. From an official Regulations point of view, all the quantified pesticides were consistent with Regulation (EC) No 1107/2009 [14], which sets their lowest MRL values in food and feed of plant and animal origin at 0.01 μg g^{-1}, except for fipronil 0.005 μg g^{-1}. With regard to other extra-European Regulations, some considerations can be made. For example, spiroxamine, fipronil, and thiacloprid are grouped in Category I under California Regulation [13], for which no detectable residues are admitted (zero tolerance). Regarding chlorantraniliprole (action levels: 10 μg g^{-1} for inhalable cannabis goods and 40 μg g^{-1} for other cannabis goods), myclobutanil (action levels: 0.1 μg g^{-1} for inhalable cannabis goods and 9 μg g^{-1} for other cannabis goods), propiconazole (action levels: 0.1 μg g^{-1} for inhalable cannabis goods and 20 μg g^{-1} for other cannabis goods), and trifloxystrobin (action levels: 0.1 μg g^{-1} for inhalable cannabis goods and 30 μg g^{-1} for other cannabis goods) residues, their contents were consistent with the admitted action levels for pesticides grouped in the Category II [13].

3.4. Greenness evaluation

In the latest years, several greenness metrics, such as red-green-blue (RGB) model, analytical greenness metric approach (AGREE), and blue applicability grade index (BAGI) [39–41], have been developed and freely available to assess the sustainability of the full analytical process, comprehensive of the sampling and instrumentation aspects. Since the main goal of this manuscript was the development of a fully automated and miniaturized sample preparation method for the analysis of pesticides in cannabis flowering tops, we decided to test the greenness only of the extraction part by using different tools, namely the sample preparation method of sustainability (SPMS) [23,42] and the AGREEprep [24,43] tools. The SPMS tool weights nine parameters grouped in four different categories (sample, extractant, procedure and energy & waste) reported in a diagram as coloured squares (from red to green) with the global score of the method, ranging from 1 (the worst case) to 10 (the best case), reported in the central square. The AGREEprep tool consider ten different criteria with more focus on safety, use of hazardous materials and waste generations, assigning scores ranging from 0 (worst case) to 1 (best case) for each of them. An overall score (0 to 1 range) is calculated considering the scores obtained for each criterion and their weight and reported in the center of a round pictogram, surrounded by 10 parts, corresponding to the different performance criteria, of different dimensions and colour, reflecting the weight assigned and the performance for each criterion, respectively.

The comparison between the proposed miniaturized and automated method against a conventional QuEChERS approach [36] using both greenness tools is reported in Fig. 3, while a detailed step-by-step calculation can be found in Table S2 (for SPMS) and Table S3 (for AGREEprep). As can be observed in Table S2, the significant differences regarded: i) volume of extractant ($0.1 < x \leq 0.5$ for developed automated SLE *vs* >1 for manual QuEChERS); ii) total waste (≤10 for developed automated SLE *vs* $10 < x \leq 50$ for manual QuEChERS). The SPMS score obtained for the proposed method (6.42) is much higher compared to the classical QuEChERS approach (3.79), with better results in extractant information and total waste according to the miniaturization and automation objectives. Similarly, the greenness profiles of the automated SLE and manual QuEChERS methods by AGREEprep tool (Table S3) highlighted main differences in terms of i) hazardous

Table 2
Concentration levels (μg g^{-1} ± standard deviation) determined in fifteen cannabis flowering tops samples (n = 3).

Pesticide Name	S1	S2	S3	S4	S5	S6	S7	S8
Chlorantraniliprole	-	-	-	< LoQ	-	-	-	-
Fipronil	-	-	-	-	-	-	< LoQ	-
Myclobutanil	-	-	-	-	-	-	0.0059 ± 0.0003	< LoQ
Propiconazole	-	< LoQ	-	0.0077 ± 0.0003	< LoQ	< LoQ	0.0055 ± 0.0003	-
Spiroxamine	-	-	-	-	-	-	-	-
Thiacloprid	-	-	-	-	-	-	< LoQ	-
Trifloxystrobin	-	-	-	-	-	< LoQ	-	< LoQ

Pesticide Name	S10	S11	S12	S13	S14	S15
Chlorantraniliprole	-	-	-	-	< LoQ	-
Fipronil	-	-	-	-	-	-
Myclobutanil	-	-	-	-	-	-
Propiconazole	0.0066 ± 0.0003	< LoQ	0.0068 ± 0.0001	0.0072 ± 0.0003	0.0064 ± 0.0005	< LoQ
Spiroxamine	-	-	-	0.0072 ± 0.0007	-	-
Thiacloprid	-	-	-	-	-	-
Trifloxystrobin	-	-	-	-	-	-

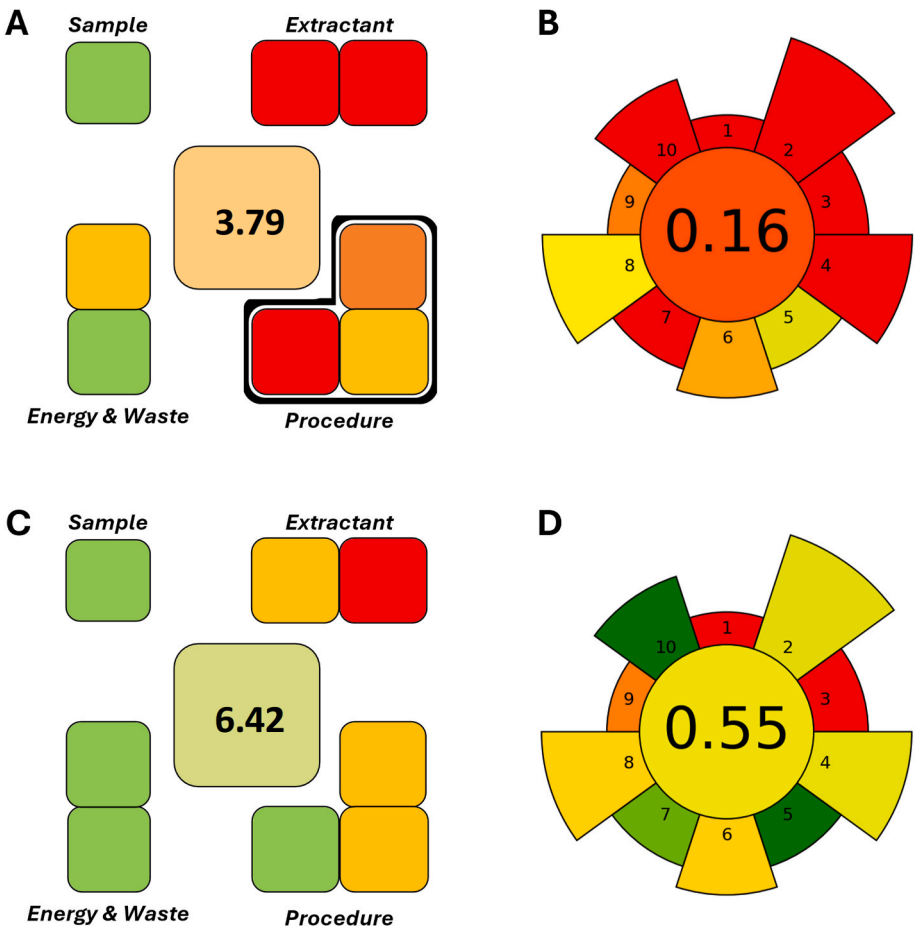

Fig. 3. Greenness profile of (A and B) QuEChERS method [36], and (C and D) of the proposed automatic SLE method by SPMS (A-C) and AGREEprep (B-D) tools.

materials (0.2 mL vs 46 mL); ii) waste, including not renewable materials such as solvent, mass of vegetable matter, and laboratory consumables (1.56 g *vs* 49 g); iii) energy consumption per analysis (93.3 Wh *vs* 75 Wh). The lowest scores obtained for the proposed method were in criterion 1 (score 0.0), 3 (score 0.0) and 9 (score 0.25). In particular, the necessity to perform the sample preparation in the laboratory far from the production site, criterion 1, and the complexity of the final determination step, criterion 9, are essential to perform a multi-residual pesticide analysis meeting the required analytical needs in terms of method performance and therefore cannot be avoided. On the other hand, the low score in criterion 3 depends on the use of non-sustainable/reusable solvents and materials. This aspect is strictly bound to fact that the commercial preparative station used for the proposed method automatically discard all the used consumables and materials used for the analysis, making impossible their reuse**.** Overall, the greenness profiles of the proposed automatic SLE and manual QuEChERS [36] methods by AGREEprep tool assigned a score of 0.55 and 0.16, respectively (Fig. 3).

4. Conclusion

The fully automated and miniaturized extraction protocol followed by UHPLC-MS/MS analysis developed in the present research has proved its suitability for the detection and quantification of pesticide residues in flowering tops of *Cannabis sativa* L. The employment of the robotic workstation allowed to accelerate and simplify the sample treatment, to reduce the solvent consumption (only 200 μL of ACN extract solvent) and cost per analysis, and to avoid the exposure of analyst to chemicals and reagents. The analytical method provided good recovery (72.3–116.2 %), accuracy (84.9–116.5 %), and intra- and inter-day repeatability (RSDs $\leq$12.5 % and $\leq$14.3 %). In addition, low LoQ values of 0.005 μg g^{-1} were obtained for forty-five pesticides, while LoQs of 0.01 μg g^{-1} were determined for aldicarb and boscalid. The method required only 30 mg of dried sample and 200 μL of acetonitrile for extraction, reducing solvent consumption, manual handling, and analyst exposure compared with conventional workflows. Greenness assessment using SPMS and AGREEprep tools confirmed that the proposed workflow improves the sustainability profile of sample preparation compared with a conventional QuEChERS approach, mainly because of the reduced sample amount, lower solvent consumption, and reduced waste generation. Future investigations will be devoted to including other pesticide residues to the present database in order to monitor the growers' usual practices in cannabis cultivations.

CRediT authorship contribution statement

Danilo Donnarumma: Formal analysis, Investigation, Methodology, Writing – original draft. **Giuseppe Cirino Presti:** Formal analysis. **Adriana Arigò:** Funding acquisition, Writing – review & editing. **Alessandra Trozzi:** Writing – review & editing. **Giuseppe Micalizzi:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **Luigi Mondello:** Conceptualization, Funding acquisition, Supervision. **Ivana Lidia Bonaccorsi:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2026.130154.

Data availability

Data will be made available on request.

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