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Pesticide residues in dry herbs used for tea preparation by UHPLC-MS/MS: Method validation and analysis

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ABSTRACT

Dry herbs are used for tea preparation and as material for phytotherapy medicines, and both are widely used by the population. However, herbs may contain contaminants and residues that could pose a health risk to consumers, and their levels should be monitored. In this work, a multiresidue method was validated for the analysis of 65 pesticides in different dry herbs. The samples were extracted with acidified acetonitrile, $MgSO_4$ and CH_3COONa, purified by dispersive solid phase with PSA, and the pesticides were quantified by UHPLC-MS/MS. A mixture of seven herbs composed of different plant parts was used as a control for method validation. Recovery ranged from 70% to 120% with a few exceptions; repeatability and intermediate precision was below 20% for most compounds. Limit of quantification (LOQ) ranged from 0.005 to 0.100 mg kg^{-1}. The method was applied for the analysis of 75 samples of 33 different dry herbs. In total, 26 samples (34.6%) were positive for at least one pesticide (≥ limit of detection, LOD), from which 19 samples had residues at quantified levels (≥ LOQ; up to 1.60 mg kg^{-1}). Carbendazim and imidacloprid were the pesticides most detected (38.5% and 30.8% of positive samples, respectively). Only two of the analyzed pesticides are registered in Brazil for use in the investigated herbs, indicating that good agricultural practices are not being applied in herb cultivation in the country. A risk assessment for the consumption of chamomile tea containing fenpropathrin was conducted and did not indicate any health concern for consumers.

1. Introduction

Plants have been widely used for tea preparation and as nutritional supplements for disease prevention and treatment for thousands of years in many countries and cultures. They are easily accessible, have low adverse effects, and most people consider them harmless (Kosalec et al., 2009; Shaban et al., 2016). Many plants contain bioactive compounds with therapeutic properties, including anti-inflammatory, antiviral, antitumor and analgesic (Aye et al., 2019). Rahman et al. (2012) observed a decrease in the blood glucose level of rats with alloxan-induced diabetes mellitus when administered with gotu kola juice (*Centella asiatica*), which contains triterpene saponins (Gohil et al., 2010). Studies with rats have demonstrated antineoplastic and antioxidative activity of *Uncaria tomentosa* (cat's claw) and improvement in cognition, memory and learning (Castilhos et al., 2020; Dreifuss et al., 2013). Campos et al. (2011) studied the stimulant properties of guarana (*Paullinia cupana*) extracts on symptoms of fatigue, sleep quality, anxiety, depression and menopause in patients with breast cancer.

There are several contaminants and residues present in herbs and herbal medicines that may cause potential health risks for consumers, such as heavy metals, radioactive particles, mycotoxins and pesticides (Shaban et al., 2016). Thus, the safety and quality of these preparations have become a major concern for health authorities, pharmaceutical industries, and the public (Kosalec et al., 2009; WHO World Health Organization, 2007). Pesticides are widely used to control pests that can affect agricultural production, including herbs, but they need to be used properly to be economically viable, safe for human health and environmentally sustainable (Caldas., 2019). The World Health Organization (WHO) advises member countries to include the analysis of pesticides and contaminants in their regulations for medicinal herbs (WHO World Health Organization, 2007). In Brazil, RDC 26/2014 requires the analysis of pesticide residues, mycotoxins and heavy metals in medicinal herbs and their derivatives, to guarantee the quality and safety of these products (ANVISA, 2019a; BRAZIL, 2014).

There are several types of herbs used for tea preparation and for medicinal purposes, and different plant parts can be used (stem, flowers,

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stalks, leaves, bark, roots, seeds). Pesticide determination in dry herbs is challenging due to the low water content (<25%), and to the presence of a large number of co-extractives in the matrix, such as lipids, chlorophyll, sugars and natural pigments (Abbas et al., 2017; Ghani, 2014; Rutkowska et al., 2018). Determination of pesticides in dry medicinal herbs has been reported in the literature, mainly using the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, Safe) (Anastassiades et al., 2003) with some modifications (Besil et al., 2017; Ghani, 2014; Lozano et al., 2012; Rutkowska et al. 2018; Steiniger et al., 2010). However, most studies validate the method for a limited number of herbs, and analyses are performed only for the validated plants.

This work aimed to validate a multiresidue method applicable to different matrices to analyze pesticides in samples of different types and parts of dry herbs by LC-MS/MS (liquid chromatography coupled with triple quadrupole mass spectrometry). The method was satisfactorily applied in the analysis of 75 samples of 33 dry herbs collected in the local market. Additionally, a risk assessment from the consumption of chamomile containing fenpropathrin was performed.

2. Materials and methods

2.1. Reference materials and reagents

Certified reference standards of 66 pesticides (purity 95.50 – 100%) were purchased from Dr. Ehrenstorfer, Germany (zoxamide), Sigma-Aldrich, Germany (acetamiprid, atrazine, fenpyroximate, pencycuron, pyridaphenthion) and AccuStandard, USA (other compounds). The selection of analytes was based on the list in RDC 26/2014, which includes 250 pesticides registered or banned in the country. The most frequently detected pesticides in the Program for the Analysis of Pesticide Residues in Food (PARA - *Programa de Análise de Resíduos de Agrotóxicos em Alimentos*) were also taken into account (ANVISA, 2019b). In this work, only compounds amenable to liquid chromatography were analyzed.

Methanol (MeOH) and acetonitrile (ACN) HPLC grade or gradient grade were obtained from Merck (Darmstadt, Germany); ammonium formate (≥ 99.0%) and formic acid (98%) were obtained from Fluka (Buchs, Switzerland); magnesium anhydrous sulfate ($MgSO_4$, ≥ 99.5%) from Sigma-Aldrich (Missouri, USA); sodium acetate anhydrous (CH_3COONa, 99.5%) from J.T. Baker (New Jersey, USA); and primary secondary amine (PSA) was obtained from Supelco (Pennsylvania, USA).

2.2. Standard solution preparation

Stock solutions of each of the 66 analytes assessed in this study (Table S1) were prepared in MeOH, ethyl acetate, toluene or ACN at 1 mg mL^{-1}. Mixing working solutions were prepared in MeOH at concentrations of 5–150 pg μL^{-1} (acetamiprid, ametryn, atrazine, azoxystrobin, boscalid, buprofezin, carbendazim, carbofuran, carbofuran 3-OH, chlorfenvinphos, cyromazine, diazinon, dicrotophos, difenoconazole, dimethoate, epoxiconazole, fipronil, fluquinconazole, imazalil, imidacloprid, malaoxon, metalaxyl-M, methamidophos, methomyl, monocrotophos, omethoate, pencycuron, pyraclostrobin, pyridaphenthion, pirimicarb, pirimiphos-ethyl, pirimiphos-methyl, profenofos, pyrazophos, tebuconazole, thiabendazole, thiobencarb, thiophanate-methyl, triazophos, trifloxystrobin, zoxamide) or 20–800 pg μL^{-1} (2,4-D, acephate, carbaryl, chlorpyriphos, chlorpyriphos-methyl, ethion, fenitrothion, fenpyroximate, fenpropathrin, fenthion, flutriafol, heptenophos, linuron, malathion, methiocarb, metribuzin, methyl paraoxon, myclobutanil, phenthoate, prochloraz, propanil, prothiofos, quinalphos, thiamethoxam, trichlorfon). All solutions were stored in amber vials at ≤ −15 °C.

2.3. Sampling

Seventy-five samples of 33 different dry herbs were collected from 2018 to 2020 in different establishments and compounding pharmacies in the Federal District, Brazil: artichoke (*Cynara scolymus*, n = 4); black mulberry (*Morus nigra*, n = 2); angelica (*Angelica officinalis* L., n = 2); mountain arnica (*Arnica montana*, n = 1); "arnica-do-mato" (*Solidago microglossa* n = 2); "assa-peixe" (*Vernonia polyanthes*, n = 1); "barbatimão" (*Stryphnodendron barbatiman*, n = 1); boldo (*Peumus boldus*, n = 5); chamomile (*Matricaria chamomilla/Matricaria recutita*, n = 4); "canela-de-velho" (*Miconia albicans*, n = 2); "carqueja" (*Baccharis trimera*, n = 1); cascara buckthorn (*Rhamnus purshiana*, n = 2); horse chestnut (*Aesculus hippocastanum*, n = 1); horsetail (*Equisetum arvense/Equisetum hyemale*, n = 4); gotu kola (*Hydrocotyle asiatica*, n = 2); green tea (*Camelia sinensis*, n = 4); leather hat (*Echinodorus macrophyllus,* n = 3); chlorela (*Chlorella pyrenoidosa*, n = 2); comfrey (*Symphytum officinale*, n = 1); "espinheira santa" (*Maytenus ilicifolia*, n = 3); bladder wrack (*Fucus vesiculosus*, n = 2); ginkgo (*Ginkgo biloba*, n = 2); guarana (*Paullinia cupana*, n = 2); hibiscus (*Rosa sinensis/Hibiscus sabdariffa*, n = 2); Peruvian maca (*Lepidium meyenii*, n = 1); muira puama (*Ptychopetalum olacoides*, n = 2); mulungu (*Erythrina velutina/Erythrina mulungu*, n = 2); passion fruit (*Passiflora incarnata/Passiflora alata*, n = 2); myrcia (*Myrcia multiflora*, n = 1); senna (*Senna alexandrina/Cassia angustifolia vahl./Cassia acutifolia*, n = 5); spirulina (*Arthrospira (Spirulina) platensis*, n = 2); puncture vine (*Tribulus terrestris*, n = 1); and cat's claw (*Uncaria tomentosa*, n = 3).

Upon arrival at the laboratory, the samples were stored at room temperature and subsequently processed and homogenized in a blender (leaves, stems and flowers) or in a mill (stems, bark), with the exception of powdered products, which were only homogenized before analysis. The choice of dry herbs investigated was based on the Brazilian Herbal Medicines Memento (ANVISA, 2016), Brazilian National List of Essential Medicines (BRAZIL, 2020), Braga and Silva (2021) and an informal survey of the best-selling herbs in compounding pharmacies and establishments in the Federal District.

2.4. Instrumentation

This work was initiated using a QTRAP 4000 LC-MS/MS system (Applied Biosystem/MDS Sciex, MA, USA) to perform the gravimetric test of co-extractives and matrix effect of selected samples. The system consists of a UFLC Shimadzu (Kyoto, Japan), with binary pump (LC-20AD), degasser, automatic sampler, column oven (CTO-20AC) and controller (CBM-20A), coupled to a triple quadrupole mass spectrometer with TurboIonSpray source and electrospray ionization in positive mode (ESI+). Chromatographic separation was performed on a Synergi 4 μm Fusion RP 80 A column, 50 × 2.00 mm (Phenomenex) with a Fusion-RP 4 × 2.0 mm pre-column. MS/MS optimization, ionization source parameters and chromatographic conditions were described by Mozzaquatro et al. (2022). Data were acquired in scheduled Multiple-Reaction-Monitoring (MRM) mode and analyzed with Analyst® v. 1.5.2 (Sciex).

Further, a new LC-MS/MS system was acquired (QTRAP 6500+, MDS Sciex, MA, USA) and used for method validation and sample analysis. The system consists of a Exion LC Sciex AD Series UHPLC (Ultra-High Performance Liquid Chromatography) -system, with a binary pump, degasser, automatic sampler, column oven (AC) and controller, coupled to a triple quadrupole mass spectrometer with IonDrive™ Turbo V source and electrospray ionization in positive and negative modes. Data acquisition was performed using Analyst® v. 1.7.2 and processed in Sciex OS v. 1.6.2. MS/MS optimization for the 66 analytes studied was performed by direct infusion, at a flow rate of 10 μL min^{-1}, of solutions of the compounds in MeOH:water (1:1) with 5 mM ammonium formate and 0.1% acid formic, at concentrations of 50–100 pg μL^{-1}. Chromatographic separation was performed on a LUNA Omega Polar C18 1.6 μm≥ 100 A, 100 × 2.1 mm UHPLC column with Security Guard Ultra Cartridges UHPLC Fully Porous Polar C18 2.1 mm pre-column, both from Phenomenex (USA). The mobile phase consisted of water with 5 mM ammonium formate and 0.1% formic acid (phase A) and MeOH with 5 mM ammonium formate and 0.1% formic acid (phase

B). The flow rate was set at 0.3 mL/min, with gradient elution: 0–0.5 min 10% B, 0.5–10 min 10–100% B, 10–12 min 100% B, 12–15 min 10% B (run time: 15 min). The injection volume was set at 1 μL and the column oven temperature at 50 °C. Data were acquired in Scheduled MRM mode. The ionization source conditions were: temperature (TEM) at 450 °C, entrance potential (EP) of 10 eV and − 10 eV (ESI+ and ESI-, respectively), curtain gas (CUR) at 40 psi, collision gas (CAD) medium, ion spray voltage (IS) 5500 V and − 4500 V (ESI+ and ESI-, respectively), ion source gas of 65 psi and 50 psi (GS1 and GS2, respectively). With the exception of 2,4-D and fipronil, all analytes were analyzed in positive mode. The optimized parameters of the compounds in the QTRAP 6500+ LC-MS/MS system are shown in Table S1 (Supplementary Material).

2.5. Gravimetric test of co-extractives and matrix effects (QTRAP 4000 LC-MS/MS)

When developing the method, it was clear that it would be very time-consuming to determine the matrix effects of each one of the 33 different dry herbs to be included in the study. Furthermore, samples of the same dry herb type have a single part of the plant (e.g. leaves or flower) or include other parts (e.g. bark or stems). Hence, matrix effect (and validation) investigation using one kind of herb or plant part would not cover all possible samples of dry herbs available on the market. Taking a pragmatic approach, a co-extractive experiment was conducted to identify herbs that would potentially cover a large range of types and samples with different matrix effects to help decide which sample to use as a control in the method validation.

The gravimetric test was performed with 42 samples out of the 75 available for the study. Each sample (n = 2) was extracted with ethyl acetate + 1% acetic acid, $MgSO_4$ and CH_3COONa (Mozzaquatro et al., 2022), 5 mL of the extract transferred to a previously weighed test tube, the extract evaporated to dryness in CentriVap at 60ºC, the tube weighed again and the residual mass was calculated (Santos et al., 2019). Based on the results of this test, the matrix effect (ME) of selected dry herb types was investigated. The samples were extracted with ethyl acetate and cleaned up according to Mozzaquatro et al. (2022); the evaporated extract was fortified with a mix solution of 49 pesticides to a final concentration of 100 pg μL^{-1} (MeOH:water; 1:1) and injected in the QTRAP 4000 LC-MS/MS. A vial containing only the analytes at the same concentration in solvent was prepared and also injected in the LC-MS/MS. The matrix effect was calculated as [% ME = ((peak area of analyte in matrix/peak area of the analyte in solvent)− 1)x100] (SANTE, 2021).

2.6. Extraction and clean-up (QTRAP 6500+ LC-MS/MS)

Although in the preliminary study the extraction was conducted with ethyl acetate (Section 2.5), in the QTRAP 6500+ LC-MS/MS the method showed similar performance when using ACN (data not shown). As the objective was to have the same method for pesticide and mycotoxin determination (which performed better with ACN extraction, not covered here), ACN was used as extraction solvent. Briefly, 1 g of dry herb sample was hydrated with 6.5 mL of Milli-Q water, manually/vortex shaken, soaked for 15 min, 7.5 mL ACN with 1% formic acid was added, followed by manual shaking for 1 min, addition of 3 g of $MgSO_4$ and 0.75 g of CH_3COONa and centrifugation for 5 min at 3500 rpm. A 3 mL aliquot of the organic phase was transferred to a 15 mL falcon tube containing 450 mg of $MgSO_4$ and 150 mg of PSA, which was vortexed for 30 s and centrifuged (5 min, 3500 rpm). Then, 750 μL of the extract was evaporated to dryness at room temperature in a sample concentrator (CentriVap), resuspended in 500 μL of MeOH:water (1:1) and filtered through a PTFE hydrophilic syringe filter 0.45 μm before injection in LC-MS/MS.

2.7. Method validation (QTRAP 6500+ LC-MS/MS)

Method validation was performed using a control matrix sample prepared as a mixture of different dry herb types, selected based on the results of the tests for co-extractives and matrix effects (Section 2.5). The method was validated for 66 pesticides for selectivity, linearity, matrix effect, recovery, repeatability and intermediate precision (INMETRO, 2020).

Selectivity was assessed by checking the presence of interferents in the control matrix at the same retention time as the monitored ions.

Linearity was assessed through standard curve analysis prepared in control matrix (in-matrix post-extraction standard curve) at 5 calibration levels and 3 replicates for each level: 5, 10, 20, 40, 80 pg μL^{-1} for 2,4-D, acephate, carbaryl, chlorpyriphos, chlorpyriphos-methyl, ethion, fenitrothion, fenpyroximate, fenpropathrin, fenthion, phenthoate, flutriafol, heptenophos, linuron, malathion, methiocarb, metribuzin, myclobutanil, methyl paraoxon, prochloraz, propanil, prothiofos, quinalphos, thiamethoxam and trichlorfon; and 1, 3, 5, 7 and 15 pg μL^{-1} for the other analytes. The least squares method was used to estimate linear regression, the Grubbs test to verify the presence of outliers, the Cochran test for variance homogeneity, and ANOVA to determine the correlation coefficient (r) and significance of the regression (INMETRO, 2020). For heteroscedastic standard curves, $1/x$, $1/x^2$, $1/x^{0.5}$, $1/y$, $1/y^2$ and $1/y^{0.5}$ weightings were tested in order to determine the best regression fit.

Matrix effect was assessed to verify if matrix components interfere in the identification of the compounds under study, enhancing or suppressing the analytical signal. In-matrix standard curves were prepared for all analytes (5 levels and 3 replicates) and compared with standard curves prepared in MeOH:water (1:1) (5 levels and 3 replicates). The matrix effect was calculated as previously described. Values below or above 20% indicate signal suppression and enhancement, respectively, and those within the ± 20% range were considered acceptable.

Recovery (%) and repeatability (relative standard deviation, %RSD) were assessed by fortifying the control sample at three concentration levels (low, medium and high) with n = 5 for each level, on the same day, by the same analyst. The following levels were assessed: 0.025, 0.100 and 0.400 mg kg^{-1} for 2,4-D, acephate, carbaryl, chlorpyriphos, chlorpyriphos-methyl, ethion, fenitrothion, fenpropathrin, fenthion, phenthoate, flutriafol, heptenophos, linuron, malathion, methiocarb, metribuzin, methyl paraoxon, myclobutanil, prochloraz, propanil, quinalphos, thiamethoxam and trichlorfon; 0.025, 0.050, 0.100 and 0.400 mg kg^{-1} for fenpyroximate and prothiofos and 0.005, 0.025 and 0.075 mg kg^{-1} for the other analytes.

Intermediate precision (% RSD) was assessed by repeating the recovery experiment (same analyst) on a different day.

The method limit of quantification (LOQ) was defined as the lowest level with acceptable mean recovery (70–120%), and repeatability and intermediate precision less than or equal to 20% (SANTE, 2021). Mean recovery rates outside the range were accepted (not lower than 30% or higher than 140%) when repeatability was lower than 20% (SANTE, 2021). Limit of detection (LOD) was defined as 1/3 of the LOQ and samples were considered positive when at least one residue was present at the LOD or higher. Residues ≥ LOD but < LOQ were reported as traces.

3. Results and discussion

3.1. Gravimetric test of co-extractives and matrix effect

Fig. 1A shows the results of the co-extractive study for the 42 samples. A green tea sample had the highest residual mass (0.12 g), and a Peruvian maca sample, the lowest (0.0012 g). Different residual masses were obtained for samples of the same plant, such as “espinheira santa” and guarana, showing that the number of co-extractives also depend on other factors such as planting conditions, parts included and drying process. Nine representative samples of the 42 samples were selected to

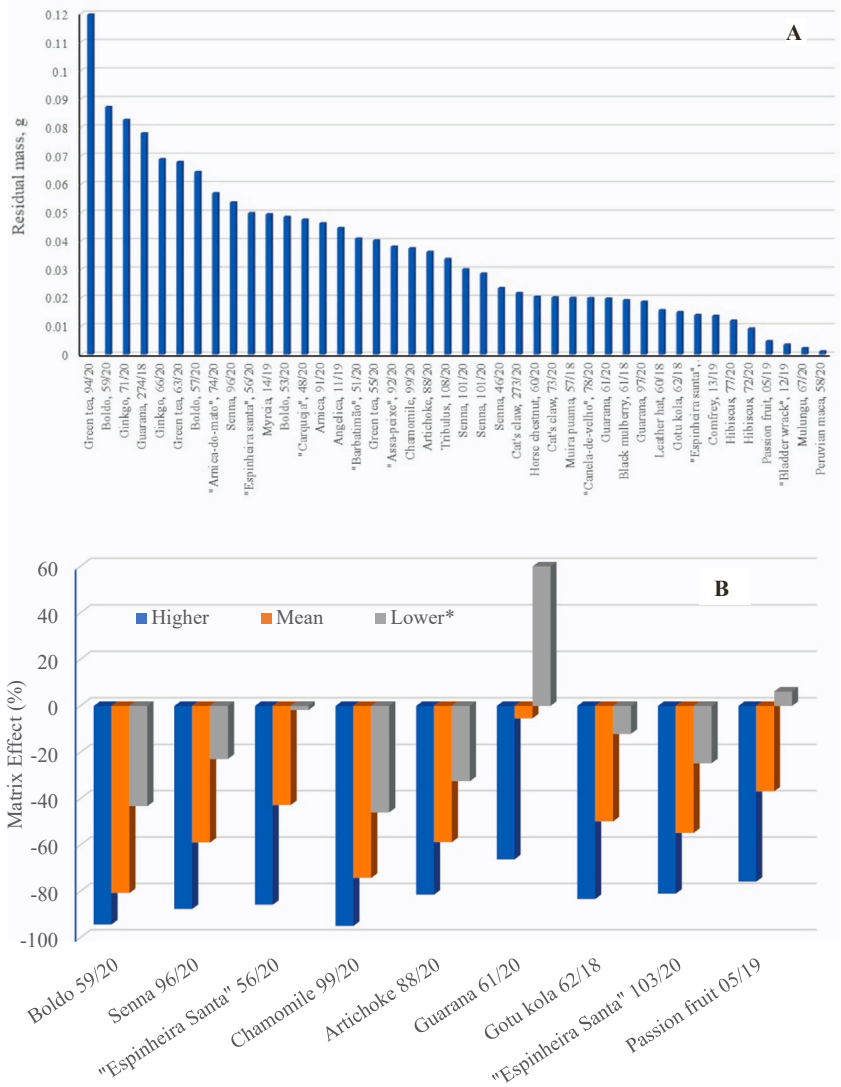

Fig. 1. A: Residual mass (g) of co-extractives of 42 dry medicinal herb samples B: Range and mean of suppression matrix effect (%) of all 49 analytes tested in selected samples (QTRAP 4000 LC-MS/MS). *For guarana and passion fruit samples, signal enhancement was also observed for some compounds.

determine matrix effect according to residual mass: high residual mass: boldo (0.1 g); medium residual masses: senna, artichoke, chamomile, and "espinheira santa" (0.05–0.04 g); low residual masses: gotu kola, guarana, passion fruit, and "espinheira santa" (0.02–0.005 g). A summary of the results is shown in Figure 1B, and the details in Table S2. All the samples showed a suppression matrix effect for all compounds, except for guarana and passion fruit, for which signal enhancement was observed for some compounds (23 pesticides for guarana, and 1 pesticide for passion fruit, highest of 62% for heptenophos). For all samples, the mean matrix effect was due to signal suppression (Fig. 1B).

The results shown in Fig. 1 indicated that a higher co-extractive mass does not always lead to a higher matrix effect, and vice-versa, and that samples from the same plant can generate different co-extractive and matrix effects, which supports the approach of using different herbs to compose a control sample to be used in the method validation. In addition to the fact that samples of the same herb can have different plant parts, as discussed previously, this could be due to other characteristics not related to the plant itself, such as its growing conditions, processing and drying methods.

Although the co-extractive and the preliminary matrix effect tests were performed using ethyl acetate extraction, the results were used to select a pool of dry herbs to be used as control in the method validation (ACN extraction), in an attempt to capture the dry herb matrix variability. This control sample was prepared with 19 samples of 7 herb types, to include different plant parts, previously confirmed using the 4000QTrap LC-MS/MS system as not containing the investigated analyte (49 analytes tested): leaves/barks of boldo (n = 4), artichoke (n = 3), "espinheira santa" (n = 2), cat's claw (n = 1) and senna (n = 5); flowers and stems of chamomile (n = 2) and bark of cascara buckthorn (n = 1) and cat's claw (n = 1). The control sample was prepared with 20 g of each sample, homogenized (850 μm, 20 mesh) and analyzed by the optimized method in the 6500 +QTrap system, where no residues were detected for all 66 pesticides.

3.2. Method validation

No interferents were observed at the same retention time for the ions monitored in the control matrix, indicating method selectivity. Fig. 2 summarizes the matrix effect data, and Table S3 shows the individual values for each analyte assessed. As matrix effect was greater than ± 20% for most compounds at all fortification levels, mainly with signal suppression effect (Table S3), the analytes were quantified against an in-matrix post-extraction standard curve (external calibration). Fig. 3A shows a chromatogram of a control matrix fortified with all 66 analytes.

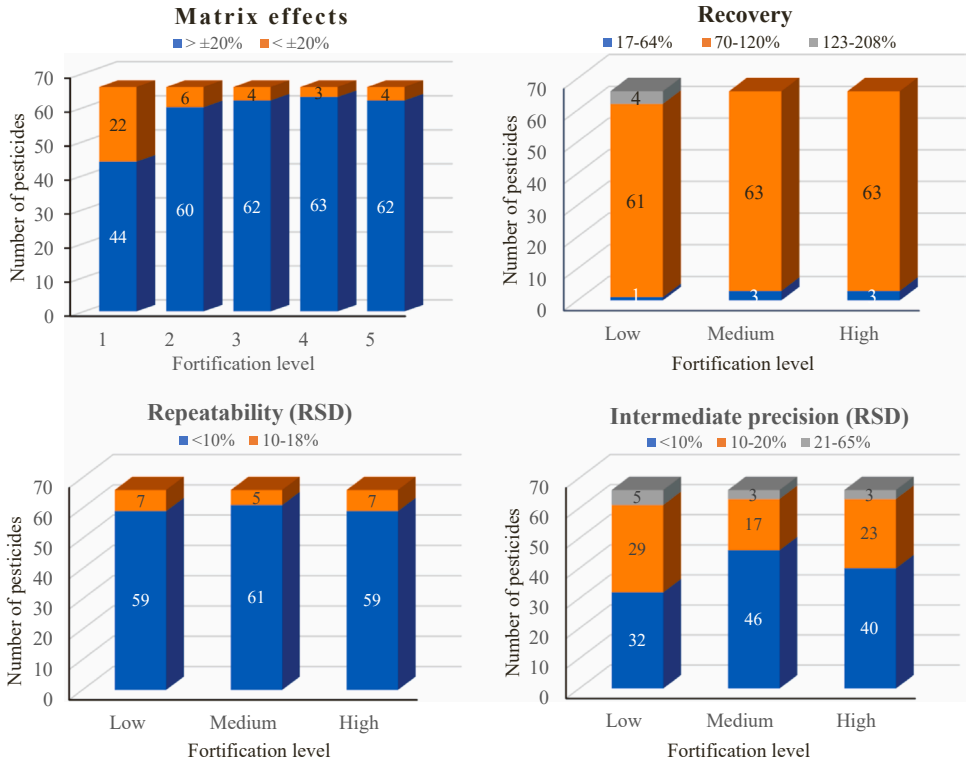

Fig. 2. Summary of validation data for the 66 pesticides in dry medicinal herbs conducted in the 6500 +QTrap UHPLC-MS/MS. Matrix effect fortification levels 1–5: 5, 10, 20, 40, 80 pg μL^{-1}, respectively, for 2,4-D, acephate, carbaryl, chlorpyriphos, chlorpyriphos-methyl, ethion, fenitrothion, fenpyroximate, fenpropathrin, fenthion, phenthoate, flutriafol, heptenophos, linuron, malathion, methiocarb, metribuzin, myclobutanil, methyl paraoxon, prochloraz, propanil, prothiofos, quinalphos, thiamethoxam e trichlorfon, and 1, 3, 5, 7 and 15 pg μL^{-1}, respectively, for the other analytes. Recovery, repeatability and intermediate precision fortification levels: low, medium and high: 0.025, 0.1 and 0.4 mg kg^{-1}, respectively, for 2.4-D, acephate, carbaryl, chlorpyriphos, chlorpyriphos-methyl, ethion, fenitrothion, fenpyroximate, fenpropathrin, fenpyroximate, fenthion, phenthoate, flutriafol, heptenophos, linuron, malathion, methiocarb, metribuzin, myclobutanil, methyl paraoxon, prochloraz, profenofos, propanil, prothiofos, quinalphos, thiamethoxam and trichlorfon, and 0.005, 0.025 and 0.075 mg kg^{-1}, respectively, for the other analytes.

The linearity of the in-matrix standard curve showed heteroscedastic behavior for 43 analytes ($C_{calculated} > C_{tabulated;5;3}$). For these compounds, weighted linear regression was used for quantification (Miller and Ambrus, 2000), and the following weights were selected: $1/x^2$ for pyridaphenthion and $1/x$ for 2,4-D, acephate, acetamiprid, ametryn, azoxystrobin, buprofezin, carbaryl, carbendazim, carbofuran, cyromazine, clorfenvinfos, chlorpyriphos-methyl, dicrotophos, difenoconazole, dimethoate, ethion, fenpyroximate, fenpropathrin, phenthoate, fipronil, fluquinconazole, flutriafol, imazalil, imidacloprid, malathion, metalaxyl-M, methamidophos, monocrotophos, omethoate, pencycuron, pyraclostrobin, pirimicarb, pirimiphos-ethyl, pirimiphos-methyl, prochloraz, profenofos, quinalphos, thiabendazole, triazophos, trichlorfon, trifloxystrobin and zoxamide. Homoscedastic behavior was observed for the other analytes ($C_{calculated} < C_{tabulated;5;3}$). Correlation coefficients (r) were equal to or greater than 0.99, except for diazinon (0.96) and dimethoate, malathion and prochloraz (0.98). All weighted regressions for their respective curves were significant ($p < 0.05$).

Fig. 2 also shows a summary of the results for recovery, repeatability, and intermediate precision, and Table S4 details the results for all analytes assessed. Repeatability was less than 20% for all analytes at all levels of fortification. Recovery values between 70% and 120% were obtained for most compounds, with the mean below 70% obtained for cyromazine at medium and high levels (60–64%, respectively) and for 2,4-D at all levels (27–33%), and above 120% at the lowest fortification level for fenpyroximate (125%), pirimiphos-ethyl (123%) and prothiofos (165%). Although recoveries were outside the acceptable levels, repeatability was less than 20%, and the method was considered validated for these compounds. Low recoveries of 2,4-D were also found by Lozano et al. (2012) when analyzing *Camellia sinensis* and chamomile (*Matricaria chamomilla),* probably due to the use of PSA for clean-up. The ability to remove interferents from the matrix by PSA is due to the presence of amine groups in its structure, which have basic properties, enabling hydrogen bond formation with matrix components. 2, 4-D has carboxyl groups, which can bind to PSA resulting in low recoveries (Lozano et al., 2012). One key issue in pesticide residue analysis of dry plants/herbs is the hydration step. Jadhav et al. (2017) showed no impact on cardamom powder incurred residues when the hydration step included soaking for 30 min before extraction or not, but when soaking was used, the RSDs were significantly lower (<10%) compared to the RSDs of when the soaking step was omitted (>20%). In the present study, the dry herb sample was soaked in water for 15 min before extraction, and probably was not an important factor to explain the higher RSD for some compounds.

Recoveries for thiophanate-methyl were 208%, 42% and 17% for the low, medium and highest fortification levels, respectively. Intermediate precision was higher than 20% for fenpyroximate (32–53%), prothiofos (40% at the lowest level, 21–25% at the others), thiophanate-methyl (58–65%), and fipronil and pencycuron at the low level (21%). Based on the overall results (Table S4) a LOQ of 0.025 mg kg^{-1} was established for 2,4-D, acephate, carbaryl, chlorpyriphos, chlorpyriphos-methyl, ethion, fenitrothion, fenpropathrin, fenthion, phenthoate, flutriafol, heptenophos, linuron, malathion, methiocarb, metribuzin, methyl paraoxon, myclobutanil, prochloraz, propanil, quinalphos, thiamethoxam and trichlorfon (23 compounds), and 0.005 mg kg^{-1} for the other analytes (40 compounds). The experiment was repeated for fenpyroximate and prothiofos at the 0.050 mg kg^{-1} level, giving unsatisfactory recoveries (126% and 136%) and the LOQ was defined as 0.100 mg kg^{-1} for both analytes (Table S4). The method was not validated for

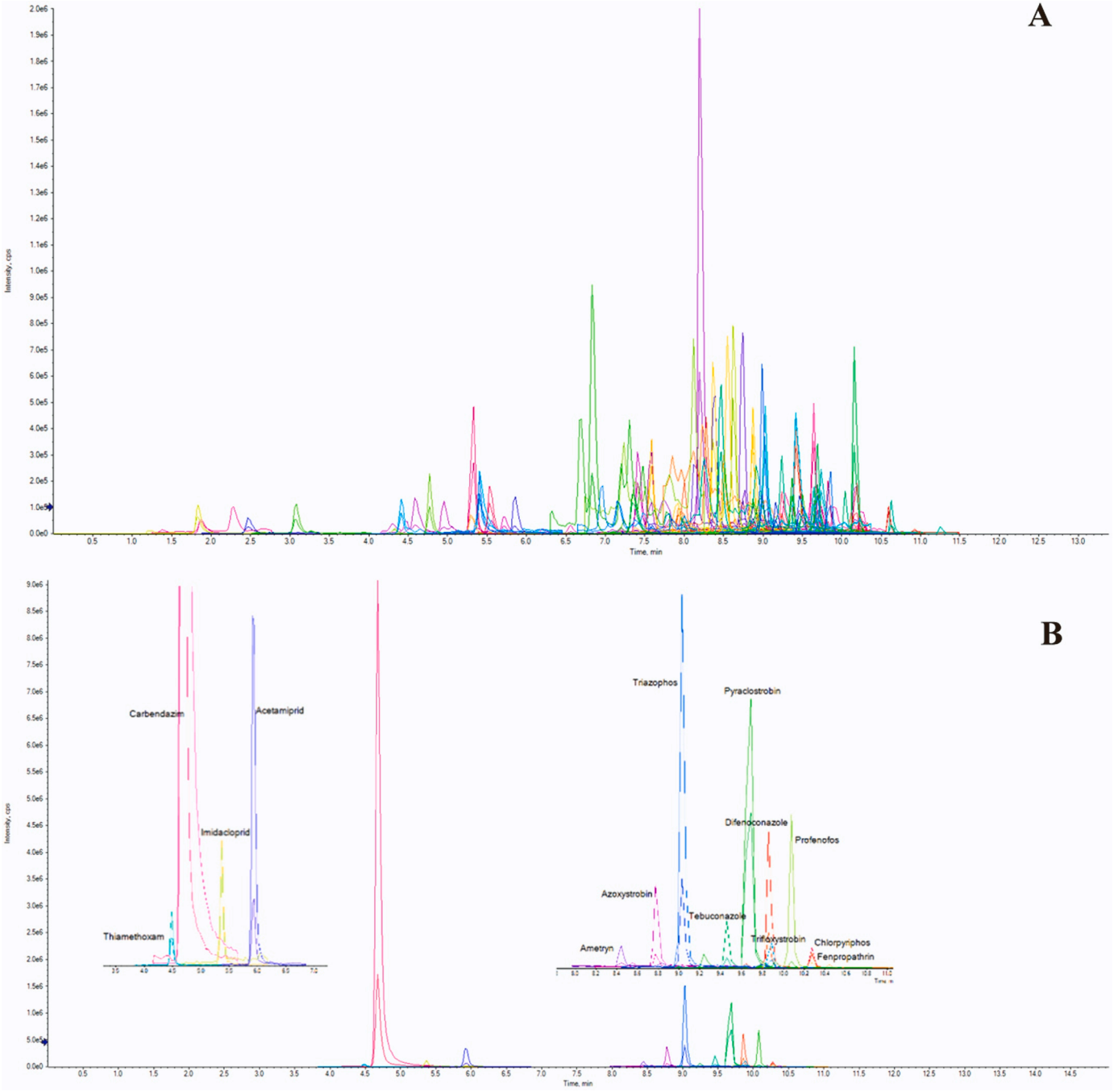

Fig. 3. QTRAP 6500+ UHPLC/MS/MS ion chromatogram of A: 66 analytes at 7 or 20 pg μL^{-1}; B: gotu kola sample 87/20, showing the 14 detected analytes in the inserts. With exception of ametryn (< 0.005 mg kg^{-1}), all the other compounds were present at quantified levels (Table S5).

thiophanate-methyl, and the results for this analyte were considered only qualitative. Table 1 shows the LOQs and LODs established for each of the 65 analytes validated in this study.

LOQs reported in this study were within the range of those reported in the literature for different dry herbs. Besil et al. (2017) validated a LC-MS/MS method for 24 pesticides on calendula at LOQs of 0.010–0.100 mg kg^{-1} (extraction with ACN, NaCl, $MgSO_4$, clean-up with PSA and $MgSO_4$). Chen et al. (2016), validated a GC-MS/MS method for 227 pesticides in green tea, ginseng, gingko leaves, saw palmetto, spearmint, and black pepper at LOQs of 0.01–0.03 mg kg^{-1} (extraction with ACN, $MgSO_4$ and NaCl, clean-up with solid phase extraction using carbon coated on alumina (CCA) and PSA or CCA/PSA/C18. Lozano et al. (2012) validated a method for 86 pesticides in green tea and chamomile at LOQs of 0.010–0.100 mg kg^{-1} (extraction with ACN and triphenyl phosphate, $MgSO_4$, NaCl, trisodium citrate dihydrate and disodium hydrogen citrate sesquihydrate, clean-up with $CaCl_2$ and PSA) and detection by LC-MS/MS and GC-MS/MS. A similar method was used by Machado et al. (2017) for 84 pesticides in artichoke at LOQs of 0.010 mg kg^{-1} (LC-MS/MS), or 0.005 and 0.010 mg kg^{-1} (GC-MS). Chen et al. (2013) achieved lower LOQs (0.00001–0.001 mg kg^{-1}) using ACN, $MgSO_4$ and NaCl extraction, homogenization with chloroform, followed by dispersive liquid-liquid microextraction for the determination of 39 pesticides in ginseng (UHPLC-MS/MS).

Table 1
Limit of Quantification (LOQ) and Limit of Detection (LOD) for the 65 pesticides in dry herbs validated in the QTRAP 6500+ UHPLC/MS/MS.

Pesticide	LOQ (LOD), mg/kg	Pesticide	LOQ (LOD), mg/kg	Pesticide	LOQ (LOD), mg/kg
2.4-D	0.025 (0.008)	Fenitrothion	0.025 (0.008)	Pencycuron	0.005 (0.002)
Acephate	0.025 (0.008)	Fenpropathrin	0.025 (0.008)	Phenthoate	0.025 (0.008)
Acetamiprid	0.005 (0.002)	Fenpyroximate	0.1 (0.03)	Pirimicarb	0.005 (0.002)
Ametryn	0.005 (0.002)	Fenthion	0.025 (0.008)	Pirimiphos-ethyl	0.005 (0.002)
Atrazine	0.005 (0.002)	Fipronil	0.005 (0.002)	Pirimiphos-methyl	0.005 (0.002)
Azoxystrobin	0.005 (0.002)	Fluquinconazole	0.005 (0.002)	Prochloraz	0.025 (0.008)
Boscalid	0.005 (0.002)	Flutriafol	0.025 (0.008)	Profenofos	0.005 (0.002)
Buprofezin	0.005 (0.002)	Heptenophos	0.025 (0.008)	Propanil	0.025 (0.008)
Carbaryl	0.025 (0.008)	Imazalil	0.005 (0.002)	Prothiofos	0.1 (0.03)
Carbendazim	0.005 (0.002)	Imidacloprid	0.005 (0.002)	Pyraclostrobin	0.005 (0.002)
Carbofuran	0.005 (0.002)	Linuron	0.025 (0.008)	Pyrazophos	0.005 (0.002)
Carbofuran 3-OH	0.005 (0.002)	Malaoxon	0.005 (0.002)	Pyridaphenthion	0.005 (0.002)
Chlorfenvinphos	0.005 (0.002)	Malathion	0.025 (0.008)	Quinalphos	0.025 (0.008)
Chlorpyriphos	0.025 (0.008)	Metalaxyl-M	0.005 (0.002)	Tebuconazole	0.005 (0.002)
Chlorpyriphos-methyl	0.025 (0.008)	Methamidophos	0.005 (0.002)	Thiabendazole	0.005 (0.002)
Cyromazine	0.005 (0.002)	Methiocarb	0.025 (0.008)	Thiamethoxam	0.025 (0.008)
Diazinon	0.005 (0.002)	Methomyl	0.005 (0.002)	Thiobencarb	0.005 (0.002)
Dicrotophos	0.005 (0.002)	Metribuzin	0.025 (0.008)	Triazophos	0.005 (0.002)
Difenoconazole	0.005 (0.002)	Monocrotophos	0.005 (0.002)	Trichlorfon	0.025 (0.008)
Dimethoate	0.005 (0.002)	Myclobutanil	0.025 (0.008)	Trifloxystrobin	0.005 (0.002)
Epoxiconazole	0.005 (0.002)	Omethoate	0.005 (0.002)	Zoxamide	0.005 (0.002)
Ethion	0.025 (0.008)	Methyl paraoxon	0.025 (0.008)		

3.3. Sample analysis

In total, 75 samples of 33 different dry herbs were analyzed in the QTRAP 6500+ UHPLC-MS/MS, with 26 positive samples (34.6%; ≥ LOD) for at least one analyte assessed, of which 19 samples had quantified residues (≥ LOQ). Table 2 summarizes the results and Table S5 shows the data for all samples in detail. Thirty pesticides were found and carbendazim was the most detected analyte, present in 10 samples (38.5% of positive samples), followed by imidacloprid (8 samples, 30.8%). The highest concentrations found were 1.28 and 1.60 mg kg^{-1} of carbendazim in gotu kola and chamomile samples, respectively.

Fig. 3B shows the chromatogram of a sample of gotu kola (87/20), one of the samples with the highest number of pesticides detected (14). With exception of ametryn (< 0.005 mg kg^{-1}), all the other compounds were present at quantified levels, ranging from 0.009 mg kg^{-1} for azoxystrobin to 1.28 mg kg^{-1} for carbendazim (Table S5).

The validated method's performance during routine analysis was assessed through the inclusion of fortified samples with all the analytes at one level (0.100, 0.025 or 0.005 mg kg^{-1}) and two replicates (quality control samples). Mean recoveries were satisfactory for all analytes except for 2,4-D (38%), cyromazine (60%), fenpropathrin (147%), fenthion (50%), prothiofos (146%) and thiophanate-methyl (17%). Only fenpropathrin was detected in the analyzed samples (4 samples). Thiophanate-methyl (not satisfactorily validated) was monitored but not detected in any sample. Prothiofos and fenpyroximate had questionable intermediate precision results (Table S4), but they were not detected in any samples.

In principle, no pesticide should be detected in plants if no authorization is granted by the regulatory agency, although residues found at very low levels by very sensitive equipment may come from cross-contamination from nearby treated crops. Among the analytes found in the dry herb samples, only two have maximum residue levels (MRL) established in the Brazilian legislation (linuron for chamomile, MRL of 0.02 mg kg^{-1}, and methomyl for black mulberry, MRL of 0.05 mg kg^{-1}) (ANVISA, 2019a, 2022a). One black mulberry sample had trace levels of methomyl (< 0.005 mg kg^{-1}), and one chamomile sample contained 0.463 mg kg^{-1} of linuron. Considering that the MRL is established in the fresh material and that dry chamomile for tea preparation has about 10% water content (Misturi et al., 2020), the level found in the dry sample adjusted for water content (0.046 mg kg^{-1} of linuron) is over 2 times higher than the MRL.

Carbendazim was the most detected pesticide, found in 13.3% of analyzed samples, with concentrations varying between 0.005 and 1.60 mg kg^{-1} (chamomile). In 2022, ANVISA (Brazilian Health Regulatory Agency) determined the precautionary suspension of the importation, manufacture, commercialization, and distribution of carbendazim and the prohibition of the active ingredient due to its mutagenic potential, reproductive toxicity and effects on embryo-fetal

Table 2
Pesticides detected in dry herbs (UHPLC-MS/MS, QTRAP 6500+).

Pesticide (LOQ, mg kg^{-1})	Positive samples (n[a])	Concentration range, mg kg^{-1} (Traces, n[b])
Acetamiprid (0.005)	Chamomile[d] (1), Gotu kola (1), Green tea (1), Ginkgo (2), Tribulus (1)	0.005–0.068
Ametryn (0.005)	Gotu kola (1)	(1)
Atrazine (0.005)	Ginkgo (1)	(1)
Azoxystrobin (0.005)	Chamomile[d] (1), Gotu kola (1), Tribulus (1)	0.006–0.009 (1)
Buprofezin (0.005)	Green tea (1), Ginkgo (1), Spirulina (1), Tribulus (1)	0.006–0.007 (1)
Carbaryl (0.025)	Guarana (2), Hibiscus[f] (1)	(3)
Carbendazim (0.005)	Angelica (1), "Arnica-do-mato" (1), Chamomile[d] (1), Horsetail[e] (1), Gotu kola (1), Green tea (1), Ginkgo (2), Tribulus (1), Cat's claw (1)	0.005–1.602 (2)
Carbofuran[c] (0.005)	Tribulus (1)	(1)
Chlorpyrifos (0.025)	Chamomile[d] (1), Gotu kola (1), Ginkgo (1)	0.032–0.075
Difenoconazole (0.005)	Gotu kola (1), Ginkgo (1), Tribulus (1)	0.022–0.087
Epoxiconazole (0.005)	Ginkgo (1)	(1)
Fenitrothion (0.025)	Arnica (1)	0.486
Fenpropathrin (0.025)	Chamomile[d] (2), Gotu kola (1), Ginkgo (1)	0.042–0.198 (1)
Imazalil (0.005)	Artichoke (1), Chamomile[d] (1)	0.018–0.044
Imidacloprid (0.005)	Arnica (2), Arnica-do-mato (1), Chamomile[d] (1), Gotu kola (1), Green tea (1), Ginkgo (2), Tribulus (1)	0.006–0.081 (1)
Linuron (0.025)	Arnica-do-mato (1), Chamomile[d] (1)	0.463 (1)
Malaoxon (0.005)	Chamomile[d] (1)	0.006
Malathion (0.025)	Chamomile[d] (1)	0.255
Metalaxyl-M (0.005)	Chamomile[d] (1), Ginkgo (1), Tribulus (1)	0.017–0.051 (1)
Methomyl (0.005)	Artichoke (1), Black mulberry (1), "Canela-de-velho" (1)	0.007 (2)
Paraoxon-methyl (0.025)	Ginkgo (1)	0.031
Pirimiphos-methyl (0.005)	Green tea (1)	0.005
Profenofos (0.005)	Gotu kola (1), Green tea (1)	0.031–0.095
Pyraclostrobin (0.005)	Chamomile[d] (1), Gotu kola (2), Ginkgo (1)	0.066–0.386 (1)
Quinalphos[c] (0.025)	Boldo (1)	0.034
Tebuconazole (0.005)	Cáscara sagrada (1), Horsetail[e] (1), Gotu kola (1), Ginkgo (1), Tribulus (1)	0.004–0.101 (1)
Thiamethoxam (0.025)	"Canela-de-velho" (1), Gotu kola (1), Comfrey (1), Ginkgo (2), Tribulus (1)	0.005–0.026 (1)
Triazophos[c] (0.005)	Gotu kola (1), Green tea (1)	0.013–0.074
Trifloxystrobin (0.005)	Arnica (1), "Arnica-do-mato" (1), Gotu kola (1)	0.005–0.010 (3)
Zoxamide (0.005)	Angelica (1), Leather hat (2)	0.006–0.029

a: number of positive samples (at least traces); b: number of samples at trace levels; c: no registration in Brazil; d: *Matricaria* sp.; e: *Equisetum* sp.; f: *Hibiscus sabdariffa*.

development (ANVISA, 2022c). In 2021, carbendazim ranked 14th among the commercialized pesticides in Brazil (IBAMA., 2023), but it did not have an approved use for any herb analyzed in this work at the time of sample collection and is not included in the Brazilian Pharmacopoeia list.

Carbendazim is a metabolite of thiophanate-methyl and its residues may come from the use of this compound. Thiophanate-methyl is authorized in Brazil for passion fruit (foliar application), but no residues of this compound or its metabolite were found in the two passion fruit dry leaf samples analyzed, although the method was not considered validated for this pesticide due to poor recovery. Furthermore, malathion and its main metabolite malaoxon were found in a chamomile sample at concentrations of 0.255 and 0.006 mg kg^{-1}, respectively (Table S5). According to the Brazilian Pharmacopoeia, malathion plus its metabolite should not exceed 1 mg kg^{-1}. Quinalphos (boldo) and triazophos (gotu kola and green tea), found at levels above 0.010 mg kg^{-1}, are also not authorized for use in Brazil (ANVISA, 2022b). Traces of carbofuran (< LOQ), which is also no longer authorized in the country (ANVISA, 2022b), was detected in one tribulus sample.

The Brazilian Pharmacopoeia includes acceptable limits for 71 pesticides in herbal drugs at levels that vary from 0.01 to 2 mg kg^{-1} (ANVISA, 2019c), similar to the list published in the European Pharmacopoeia (2019). The list includes 21 pesticides investigated in the present study (not linuron or methomyl), and pesticides no longer registered in the country, such as quinalphos. In both cases (MRL and Pharmacopeia limits), the levels are above the LOQ obtained in this work, which also makes the method suitable for evaluating pesticides in herbs for compliance. The Codex Alimentarius has established MRL for 5 pesticides in herbs, with levels ranging from 0.01 (for abamectin) to 70 mg kg^{-1} (for azoxystrobin); MRLs for individual herbs are also established, including buprofezin and fipronil (1.5 mg kg^{-1}) and imidacloprid (20 mg kg^{-1}) in basil, but none of the herbs analyzed in the present study has Codex MRLs (CODEX, 2023).

Data from PARA, coordinated by ANVISA and the Brazilian National Plan for Residue and Contaminant Control (PNCRC - *Plano Nacional de Controle de Resíduos e Contaminantes*), coordinated by the Ministry of Agriculture (MAPA), show that irregular pesticide use in Brazil is common. Data from the two programs (2001–2010) showed that 72% of irregularities were due to illegal pesticide use for the crop (Jardim and Caldas, 2012). The 2017/2018 PARA report showed that carbendazim, imidacloprid and tebuconazole had the highest detection rates (11%, 16% and 12% of the 4616 fruit and vegetable analyzed samples, respectively) (ANVISA, 2019a). The herbs assessed in this work are not included in the Brazilian monitoring programs, and the results indicate illegal use of pesticides that should be investigated by national authorities and manufacturers of these products, as required by the RDC 26/2014 (ANVISA, 2019a; BRAZIL, 2014).

The potential risk to health from the dietary intake of pesticides present in crops, including dried herbs, can be assessed through a risk assessment process (Caldas & Velde-Koerts, 2017). Chamomile is one of the most popular plants used for tea preparation in Brazil, mainly for its anxiolytic effects (Zhang et al., 2022) and to treat sleep disorders (Lelli et al., 2021). In this study, two of the four chamomile samples analyzed contained pesticide residues (5 and 7 analytes), with the pyrethroid insecticide fenpropathrin the only one present in both samples (0.042 and 0.198 mg kg^{-1}). Assuming a daily chamomile consumption of 2 tablespoons (30 g) and a mean fenpropathrin concentration of 0.12 mg kg^{-1}, the daily intake of this insecticide through the consumption of chamomile tea by a person weighting 60 kg is 0.06 μg kg bw^{-1}. This intake accounts for 0.2% of the acceptable daily intake of fenpropathrin (ADI of 30 μg kg bw^{-1}; ANVISA, 2022a), not representing a potential risk to consumers. This conclusion most likely holds even when considering other dietary sources of exposure to fenpropathrin and other organophosphorus compounds (Jardim et al., 2018).

4. Conclusion

In this study, a method for the analysis of 65 pesticides in dry medicinal herbs by UHPLC-MS/MS was validated, using a mixture of 7 different plants as a control sample at LOQs ranging from 0.005 to 0.100 mg kg^{-1}. The method includes extraction with acidified acetonitrile, $MgSO_4$ and CH_3COONa and clean-up by dispersive solid phase with PSA/$MgSO_4$. To the best of our knowledge, this is the most

comprehensive study with respect to pesticides in dry herbs, as it covers 33 different plants (leaves, stems, seeds, algae, fruits, roots and/or flowers).

Of the 75 samples of dry medicinal herbs analyzed, 34.6% were positive for at least one pesticide, most at quantified levels. Only two of the 30 pesticides present in the samples are allowed in the analyzed herbs by ANVISA, indicating that good agricultural practices are not being respected by farms that grow these plants, which reinforces the importance of developing methods and analyzing pesticide residues in these plants to guarantee safe products for consumers.

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CRediT authorship contribution statement

Denise Carvalho Mello, Eloisa Dutra Caldas: Conceptualization, Writing – review & editing. **Denise Carvalho Mello, Nayara Luiz Pires, Camila Suguiura Evangelista**: Formal analysis, Methodology. **Eloisa Dutra Caldas**: Funding acquisition, Project administration, Supervision. **Denise Carvalho Mello**: Writing – original draft. All authors have read and agreed to the published version of the manuscript.

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.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105817.

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