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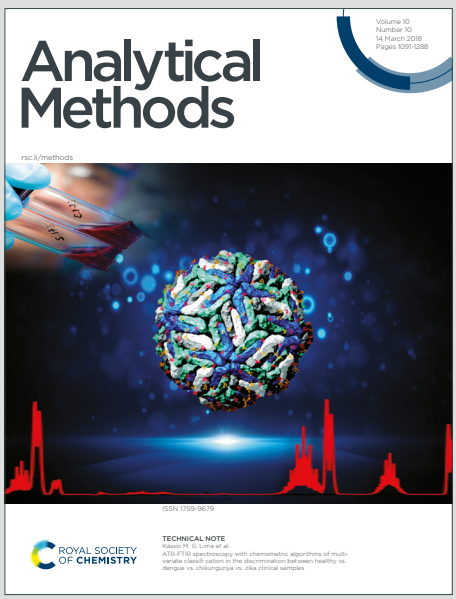

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Analysis of dithiocarbamates in berries and leafy vegetables by ultra-high performance liquid chromatography coupled with tandem mass spectrometry

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Environmental significance

Analysis of over 50 samples of berries and leafy vegetables purchased at the market showed the presence of dimethyl dithiocarbamates and ethylenebisdithiocarbamates in 96% of them, and 99% of these contained propineb.

Abstract

A sensitive method has been developed for the analysis of the three subclasses of dithiocarbamates (DTCs), (dimethyl dithiocarbamates (DMDs), ethylenebisdithiocarbamates (EBDs), propylenebisdithiocarbamates (PBD)) in berries and leafy vegetables using UHPLC/MS-MS. DTCs were extracted by first decomplexing metal ions using an alkaline solution (pH 9.8) of cysteine-EDTA. The second step was the methylation of the dithiocarbamic acids formed by dimethyl sulfate in acetonitrile to obtain the methylated dithiocarbamates. The method was validated using ziram, zineb, and propineb to represent DMDs, EBDs, and PBDs, respectively. In addition, spinach and

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blueberries were used as representative matrices for leafy vegetables and berries, respectively. The average recovery obtained ranged from 71.8% to 92.2% for methyl dimethyldithiocarbamate (DMD-Me) with an inter-day precision of 4.7% to 12.2%; from 30.8% to 62.2% for dimethyl ethylenebisdithiocarbamate (EBD-Me) with an inter-day precision of 4.5% to 8.9%. For dimethylpropylene bisdithiocarbamate (PBD-Me), they ranged from 6.3% to 8.2% with an inter-day precision of 0.8% to 1.1%. The limits of quantification (LOQ) expressed in µg/kg of carbon disulfide (CS_2) were low in berries and leafy vegetables, ranging from 0.14 µg/kg to 0.27 µg/kg for DMDs, 0.87 µg/kg to 1.27 µg/kg for EBDs, and 0.03 µg/kg for PBDs. Analysis of over 51 samples showed the presence of DMDs and EBDs in 96% of them, and 99% of these contained propineb. Furthermore, none of the concentrations detected in these samples exceeded the maximum residue limits (MRLs) set by the European Union, except for propineb, as its MRL has been lowered to the LOQ.

Keywords: Pesticides, methylation, decomplexation, LC-MS/MS, food residues.

1. Introduction

Dithiocarbamates (DTCs) are organosulfur chemicals like carbamates due to the replacement of two oxygen atoms by sulfur atoms, their general structure being: $(R_1R_2)N$-(C=S)-SX (X = metal) [1]. Due to the presence of two sulfur donor atoms in the DTC ligands and their lipophilic properties, these compounds have coordinating abilities with metals [2]. In addition, the resonance properties also make DTCs stabilizers of metals in different

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oxidation states [3]. The synthesis of this group of chemicals depends on the type of amine used (primary or secondary amines) during the process. This results in two types of DTCs: dialkyldithiocarbamates formed from primary amines and monoalkyldithiocarbamates synthesized from secondary amines [4]. Monoalkyldithiocarbamates include ethylenebisdithiocarbamates (EBDs, such as mancozeb, maneb, and zineb) and propylenebisdithiocarbamates (PBD, e.g., propineb). Dialkyl dithiocarbamates include dimethyldithiocarbamates (DMDs, e.g., ziram, ferbam, thiram, and nickel dimethyldithiocarbamate). In addition, polycarbamate is classified as both an EBDs and a DMDs. These classes were established based on the different carbon skeletons present in the various DTCs (Figure 1). DTCs form chelates with metals such as zinc (Zn), iron (Fe), manganese (Mn), and nickel (Ni).

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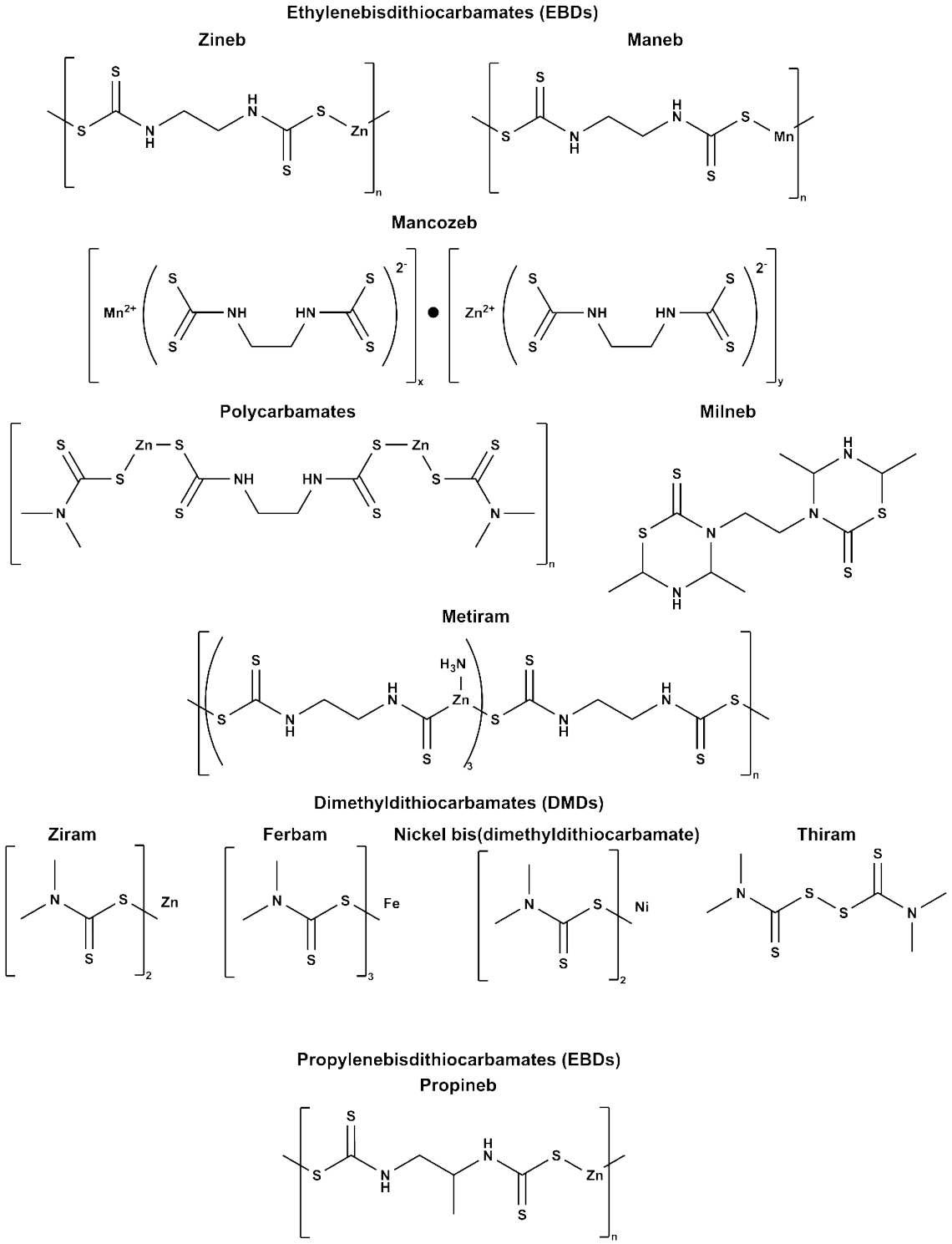

Figure 1: Chemical structures of the main DTCs. Compounds are classified into three subclasses based on their carbon skeletons.

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Introduced to the market around the 1930s [5], this group of organosulfur compounds has been used extensively worldwide. These compounds are widely produced and exported by China. For example, the value of DTCs exports increased exponentially from $3.02 million in 1995 to $194 million in 2022 [6]. This makes China the world's leading exporter of DTCs in recent years. These figures highlight the importance of DTCs on the global market, which leads us to question their applications. DTCs are mainly used as fungicides (they control fungal diseases such as mildew, scab, mold and leaf diseases), and insecticides (because DTCs can limit the development of nematodes and some parasitic larvae), but also as herbicides in fruit and vegetable production, for seed treatment, foliar treatment, and post-harvest treatment [7]. In addition, DTCs are also used in industry as antioxidants in rubber and as vulcanization accelerators. Furthermore, they are used as antimicrobial pesticides in water cooling systems and in paper manufacturing [8]. Furthermore, it has been discovered that some DTCs are pharmacologically active and can therefore be used for the treatment of alcoholism and Alzheimer's disease [9]. In this work, only the application of DTCs as pesticides will be considered. Thus, to date, detailed and accurate information on the mode of action of DTCs as insecticides is scarce in literature. However, as fungicides, their mode of action is documented as non-systemic, meaning that they are simply preventive, as the fungicide does not penetrate the cuticles of plants and therefore remains on their surface [10]. They protect host plants by inhibiting the germination of fungal spores and preventing the germination tubes of the spores from entering the host tissue. They must therefore be applied before infection by a pathogen. Since DTCs are compounds that degrade rapidly in the environment through hydrolysis, oxidation, photolysis, leaching, and

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metabolically, they must be reapplied weekly to ensure adequate protection for plants. As stated above, coordination between DTC ligands and metals stabilizes DTC fungicides. Their rate of decomposition therefore depends on the type of metal cations and the pH of the environment: dithiocarbamates are therefore unstable in acidic conditions [11,12]. Environmental factors and different degradation pathways reduce their persistence and expose them to different types of degradation leading to various transformation products.

Once DTCs enter the body of a mammal (including humans), mainly via the respiratory tract (aerosols, dust), skin, mucous membranes (occupational exposure), and digestive tract, they undergo several metabolic transformations. This metabolic process also applies to plants. On the one hand, DMDs such as ziram, ferbam, etc. begin by losing their metals to form dimethyldithiocarbamic acid, then break down to form carbon disulfide and dimethylamine, etc [13,14]. On the other hand, the metabolic decomposition of EBDs such as mancozeb, zineb, etc. leads to the formation of numerous metabolites such as ethylene bisdithiocarbamic acid (which occurs when EBDs lose their metals upstream), carbon sulfide, ethylene diamine (EDA), etc. [15]. However, the main degradation products of EBDs are ethylene thiourea (ETU) [16]. A comparable metabolic degradation occurs with propineb, giving rise to propylene thiourea (PTU), which is the main degradation product [16]. The acute toxicity of DTCs on human health is low, and acute poisoning is unlikely to occur. On other hand, chronic exposure to DTCs through skin contact, ingestion, or inhalation can lead to cytotoxicity, immunotoxicity, hormonal and reproductive disorders, and functional changes in the hepatobiliary and nervous systems [17]. To date, there are no studies showing a correlation between cases of cancer (specifically thyroid cancer) and thyroid tumors in humans due to ETU and PTU transformation products [8]. Carbon sulfide is a general

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neuropathic agent, but according to the Canadian Environmental Protection Act (1999), carbon disulfide is not considered "toxic" to humans [18,19]. In view of the various disorders caused by DTCs in humans, it is imperative to control their use and consequently the quantities of residues left on food.

Despite their widespread use, direct detection of DTCs is difficult because many of these compounds are relatively unstable in the presence of moisture, oxygen present in biological systems, plant matrix, and aqueous solution. In addition, although DTCs are easy to synthesize, they are not easy to solubilize [4]. DTCs have low solubility in water and most organic extraction solvents. However, there is an official method for analyzing DTC residues in foodstuffs. This is based on hot acid digestion of the entire sample to release carbon disulfide (CS_2) which is then quantified by spectrophotometry or gas chromatography (GC-MS) [20,21]. Unfortunately, this method is laborious and can lead to false positives, as it does not distinguish CS_2 naturally present in the plant (due to the phytogenic effects of CS_2 in various crops of the Brassica family, such as papaya) and CS_2 from DTCs [22]. In addition to this limitation, it is also not possible to use this method to distinguish between different classes of DTCs. For this reason, numerous methods have subsequently been developed for analyzing DTC subclasses. For example, some DTCs are converted into water-soluble sodium salts by adding an alkaline solution of EDTA or $NaHCO_3$ to obtain the DTC ligand. Following this step, EBD-dimethyl, PBD-dimethyl, or DMD-dimethyl are produced by derivatization using methyl iodide or dimethyl sulfide [23–29]. The main method of samples processing is based on the QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) method. This is a simple and easy method for multi-residue analysis of pesticides in fruits and vegetables. It uses acetonitrile extraction and

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solid-phase dispersive extraction [30]. The second method is the simple extraction method, which uses an extraction buffer such as SHC-PA buffer ($NaHCO_3$ and DL-penicillamine) [31]. However, the use of liquid chromatography-ultraviolet absorption (LC-UV) for the analysis of extracts obtained following the methylation process is not very effective, as it results in insufficient sensitivity [32]. On the other hand, sensitive methods based on LC-MS or LC-MS/MS with APCI or ESI ionization have been developed [5].

The existing method for derivatization of DTCs using dimethyl sulfate was used in this study to detect DTCs (DMD, EBD, and PBD) due to its simplicity, selectivity, and suitability for many plant matrices [24–29]. However, its application to fruits and vegetables that are very rich in pigments, such as small fruits (mainly rich in anthocyanins) and leafy vegetables (rich in chlorophyll), remains limited due to analytical interference and the instability or degradation of DTCs in the presence of this type of matrix. To address these issues, the extraction step was first adapted by introducing an intermediate step consisting of extracting samples with a cysteine-EDTA extraction solution, then isolating a volume that would subsequently be treated according to the extraction method. In addition, a study was conducted to optimize the pH of the extraction solution, the concentration of dimethyl sulfate, and the reaction time to adapt this method to our matrices. These improvements aim to reduce interference by improving the sensitivity and performance (detection limits and quantification limits) of the method. Furthermore, knowing that DTCs mainly break down through enzymatic reactions, the samples were not ground prior to extraction, but rather cut up (for leafy vegetables) or left whole (for berries) to maximize recovery rates [33].

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Thus, the aim of this study was to detect DTCs in pigment-rich matrices (berries and leafy vegetables) purchased from various supermarkets and farms in the Montreal area (QC, Canada) and then analyze them using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC/MS-MS).

2. Materials and methods

2.1 Samples- collection and pre-treatment

A total of 51 samples of berries (blackberries, raspberries, blueberries, strawberries) and leafy vegetables (romaine lettuce, iceberg lettuce, curly lettuce, spinach, watercress, chopped kale, chinese spinach, chinese lettuce, and Taiwanese lettuce) used in this study were purchased fresh from various supermarkets in the Montreal area (IGA, Super-C, Maxi et Marché C&T), but some were sourced from farms in the greater Montreal area (details provided in Table SI-1a and SI-1b).

Upon arrival at the laboratory, the different lettuce samples were cut, weighed and stored in the freezer (-20 ºC) until the day of extraction. The berries were weighed directly in the appropriate tubes and stored in the same way.

2.2 Chemicals and standards

Certified standards for ziram, zineb, dimethylethylene bisdithiocarbamate (EBD-Me), dimethylpropylene bisdithiocarbamate (PBD-Me), dimethyldithiocarbamate methyl

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(DMD-Me) were purchased from Toronto Research Chemicals (TRC) and propineb from Sigma-Aldrich (St. Louis, MO, U.S.A.). Solvents such as acetonitrile and 0.1% formic acid with water, both of UHPLC quality, were obtained from Fisher Scientific (Whitby, ON, Canada). Various reagents such as dimethyl sulfate (purity ≥99.5%), ethylenediaminetetraacetic acid (EDTA, purity 99.4-100.6%), L-cysteine monohydrate hydrochloride (purity ≥98%), anhydrous sodium hydroxide (purity ≥98%), magnesium sulfate (($MgSO_4$) purity ≥99.5%) and sodium chloride ((NaCl) purity ≥99%) were all purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) as the secondary primary amine absorption sorbent (PSA SPE).

2.3 Preparation of solutions

The stock solutions (1000 mg/L) of EBD-Me, PBD-Me, and DMD-Me were prepared in HPLC grade acetonitrile and then stored in a freezer at -20ºC. The stock solutions (1000 mg/L) of the ziram, propineb, and zineb standards used to represent the DMD, PBD, and EBD subclasses, respectively, were prepared and diluted to the desired concentrations immediately before use in an aqueous EDTA solution (50 g/L) at pH 12. The L-cysteine-EDTA extraction solution was prepared by adding 50 g of EDTA (50 g/L) and 15.8 g of L-cysteine hydrochloride monohydrate (0.1 M) to 1000 mL. This solution was then alkalized to pH 9.8 using an aqueous solution of NaOH (10 M). Next, the reagent solution to be used as the methylating agent for the DTCs was prepared by solubilizing dimethyl sulfate (0.09 M) in acetonitrile.

2.4 Sample preparation and instrumental analysis

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A mass of 5 g of berries or leafy vegetable samples was weighed into 50 mL PTFE-capped centrifuge tubes and stored in a freezer at – 20 °C. On the day of extraction, 20 mL of the cysteine-EDTA solution was added to the centrifuge tube containing the sample, which were then vortexed for 1 min and centrifuged (5 min, 6000 rpm). Then, 4 x 2 mL of the extracted supernatant were removed and placed in new centrifuge tubes. To this volume, 10 mL of dimethyl sulfate solution in acetonitrile were added. The mixtures were then vortexed for 30 seconds at 3000 rpm, then placed in incubator shaker (Innova 4230; New Brunswick Scientific) at 400 rpm at room temperature for 30 minutes to allow the reaction to complete. After this step, 4 g of $MgSO_4$ and 1 g of NaCl were added to the reaction mixtures, which were then vortexed for 1 min and centrifuged (5 min, 6000 rpm). A volume of 4 x 2 mL of the organic phase was taken from each tube and transferred to 10 mL centrifuge tubes containing sorbents (900 mg of $MgSO_4$ and 200 mg of PSA SPE) to remove the organic acids and polar pigments contained in the mixture. The mixture was then vortexed (1 min, 3000 rpm) and centrifuged (10 min, 6000 rpm). 5 mL of supernatant from each tube were then transferred to new centrifuge tubes and evaporated to 300 µL. The evaporated extracts were then filtered (0.22 µm hydrophilic PTFE filter) and injected into the UHPLC-MS/MS. The main steps of the extraction are shown in Figure 2.

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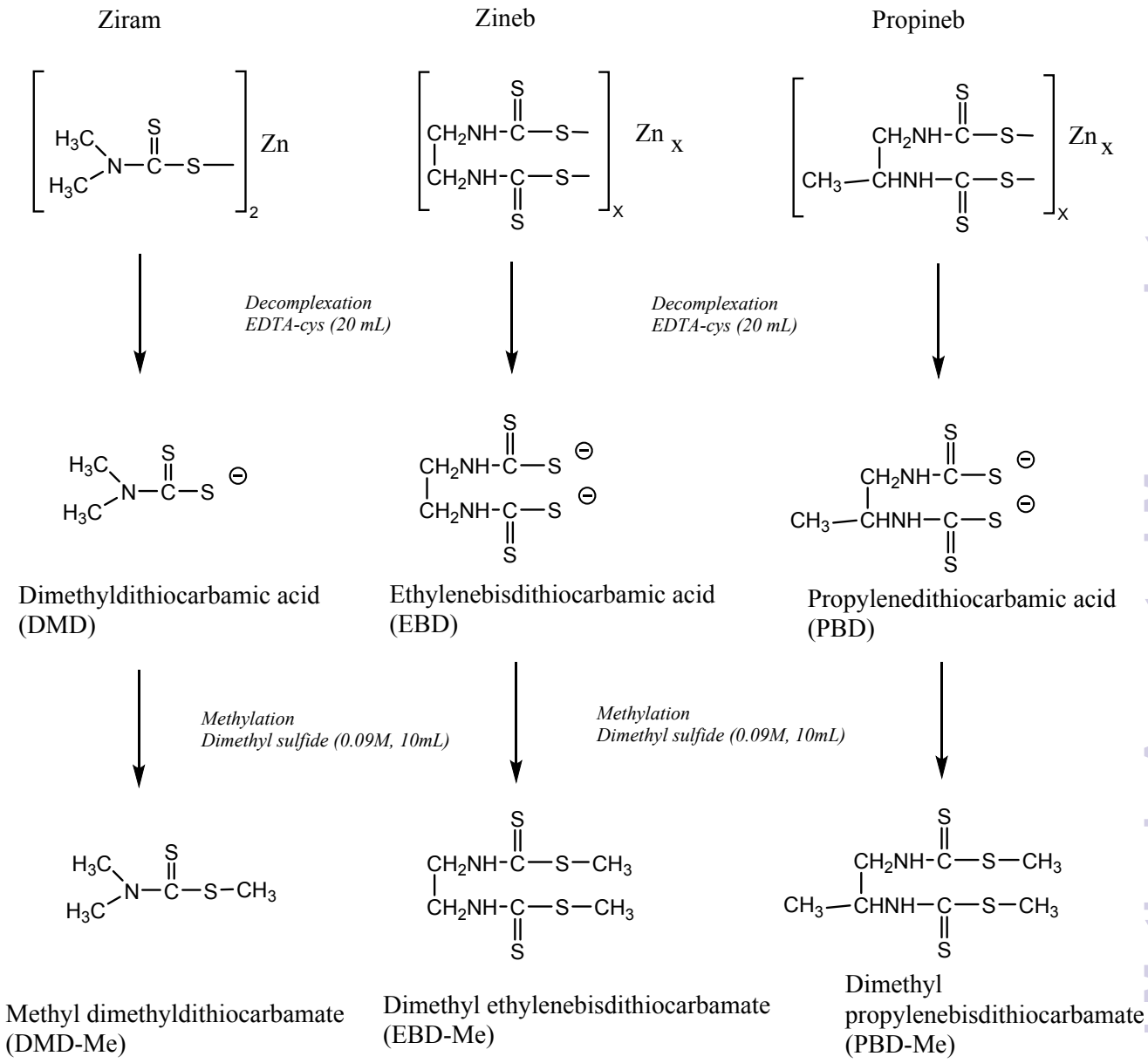

Figure 2: Illustration of the main steps in the extraction process (1st step: decomplexation of metals from the dithiocarbamates contained in the sample and 2nd step: methylation of dithiocarbamic acids in the presence of dimethyl sulfate).

Samples were analyzed using an UHPLC-MS/MS TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, U.S.A.). The chromatographic

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separation was performed using a Thermo Hypersil Gold C18 reverse phase column (100 x 2.1 mm, particle size 1.9 µm) thermostated at 50°C and a 20 µL injection volume. The mobile phases used for separation were acetonitrile (A) and a 0.1% aqueous solution of formic acid (B). Elution was performed at a flow rate of 0.45 mL/min, and the gradient was established as follows: (0 – 1.0 min), 10% A; (1 – 6.5 min), 100% A; (6.5 – 7.5 min), 100% A; (7.5 – 7.7 min), 10% A. All compounds were acquired within a single run of 9 minutes. Thermo Scientific TSQ Quantiva mass spectrometer was used in a positive mode at 3800 kV. To optimize the selectivity and sensitivity of the analytes of interest, the analyses were performed in multiple reaction monitoring (MRM) mode and the ionization technique used was electrospray ionization (ESI). The MS/MS acquisition parameters are detailed in Table SI-2. Compound-dependent MS/MS parameters of the methylated DTC derivatives are provided in the SI (Table SI-3).

2.5. Quantification and method validation

Peaks area corresponding to the target compounds was used for quantification. To do this, the intensities obtained were converted into methylated dithiocarbamates (µg/kg) using two external calibrations. In addition, each sample was extracted in triplicate, and their concentrations were calculated using these curves to determine the concentrations of DMD-Me, EBD-Me, and PBD-Me in these samples. As maximum residue limits (MRLs) are expressed in CS_2 equivalent for all active DTCs, apart from propineb and thiram, for which the MRLs have been lowered to the limit of quantification [34], the concentrations calculated for each group were therefore converted to CS_2 equivalents. DMD-Me concentrations were multiplied by a factor of 0.56 (1 mole of DMD-Me produce 1 mole of

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CS_2), EBD-Me concentrations by 0.63 (1 mole of EBD-Me produce 2 moles of CS_2) and PBD-Me concentrations by 0.60 (1 mole of PBD-Me produce 2 moles of CS_2). These concentrations were only calculated for the EBDs and DMDs subclasses, then summed for each sample to obtain a single concentration.

The performance of this method was evaluated using blueberries as the reference matrix for berries and spinach for broadleaf vegetables. In addition, ziram, propineb, and zineb standards were chosen to represent the DMDs, PBD, and EBDs subclasses, respectively. Validation was conducted to evaluate the method performance in terms of linearity, selectivity, detection limits (LOD), quantification limits (LOQ), matrix effect, intraday and interday precision, and accuracy [35].

Selectivity was evaluated by injecting 1 mg/kg standard solutions of methylated dithiocarbamates in solvent to determine the ability of this procedure to detect and identify the analytes. Further information on validation procedure such as matrix effects is available in supplementary material (SI).

Linearity was assessed by using calibration curves for EBD-Me, PBD-Me, and DMD-Me comprising ten calibrations levels from 0.02 ng/g to 1000 ng/g in the solvent (acetonitrile) and in the matrix. The solutions were prepared 24 hours before injection and stored in a freezer at -20 °C.

The limit of detection (LOD) and the limit of quantification (LOQ) determination was based on the analyte signal (n = 6) different from the blank and the standard deviation times 3 and times 10, respectively. These limits were calculated (µg/kg of CS_2) from samples of berries and leafy vegetables with low concentrations.

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3. Results and discussion

3.1 Optimization of UHPLC-MS/MS conditions

3.1.1. Optimization of mobile phase chromatographic conditions

Mobile phases were compared under the following chromatographic conditions (some test were based on literature): (A) aqueous solution of ammonium fluoride (NH_4F) at 0.1 mM and (B) methanol (MeOH) + 0.1 mM NH_4F (A) aqueous solution of ammonium acetate (AmAc) at 25 mM and (B) MEOH or (B) acetonitrile (ACN), (A) aqueous solution of 0.1% formic acid (FA) and (B) ACN, (A) aqueous solution of 0.1% FA and (B) MeOH or ACN or MeOH + 0.1% FA, (A) aqueous solution of 0.5% FA and (B) MeOH [24,26,27]. According to Table 1, the mobile phase composed of AmAc and CAN resulted in the lowest intensity (DMD-Me and EBD-Me) or no signal (PBD-Me) compared to other mobile phases tested. Higher intensities were obtained by using combination of NH_4F-MeOH and aqueous solution of NH_4F (for PBD-Me and EBD-Me) while 0.1% FA-MeOH generated better intensity for DMD-Me, thereby improving the ionization efficiency. NH_4F-MeOH and aqueous NH_4F solution mobile phase prior selected was chosen for further investigation, as it showed the best performance for the majority of DTC-Me. According to figure 3 (which shows chromatograms obtained with the mobile phase A: H2O + 0.1 mM and B: MeOH + 0.1 mM NH_4F) higher noise for m/z 241 and peak artifacts for m/z 255 were observed in its chromatogram (Figure 3) but the second transition was not affected. After a thorough analysis of the other chromatograms (Figure SI-1) higher intensities for DTC-Me were observed. Thus, ACN + 0.1% formic acid was selected as

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mobile phase (Figure 4-A which presents the mobile phase A: H2O + 0.1% FA and B: ACN. (A) methylated standards at 50 µg/kg). No spurious peaks were identified for EBD-Me and PBD-Me with this phase. In addition, after increasing the concentration of the DTC-Me standard mixture from 50 µg/kg to 1000 µg/kg injected, an improvement in the sensitivity of the compounds and a decrease in the baseline were observed with ACN + 0.1% formic acid mobile phase (Figure 4-B).

Table 1: Peak area obtained for the three DTC-subclasses in different mobile phases.

Phase A (H_2O)	Phase B	DMD-ME (m/z 136)		PBD-ME (m/z 255)		EBD-ME (m/z 241)	
		R_t	Area (10^5)	R_t	Area (10^5)	R_t	Area (10^3)
0.1 mM NH_4F	MeOH + 0.1 mM NH_4F	4.35	1.25	5.14	11.8	4.90	10.5
25 mM AmAc	MeOH	4.36	1.17	5.14	2.28	4.89	1.91
	ACN	3.95	0.02	4.62	0.02	NF	NF
0.1% FA	ACN	3.94	0.19	4.61	0.34	4.38	1.29
	MeOH	4.38	2.20	5.15	3.25	4.92	8.97
	MeOH + 0.1% FA	4.36	2.06	5.14	1.96	4.90	5.99
0.5% FA	MeOH	4.34	2.16	5.14	2.84	4.88	8.35

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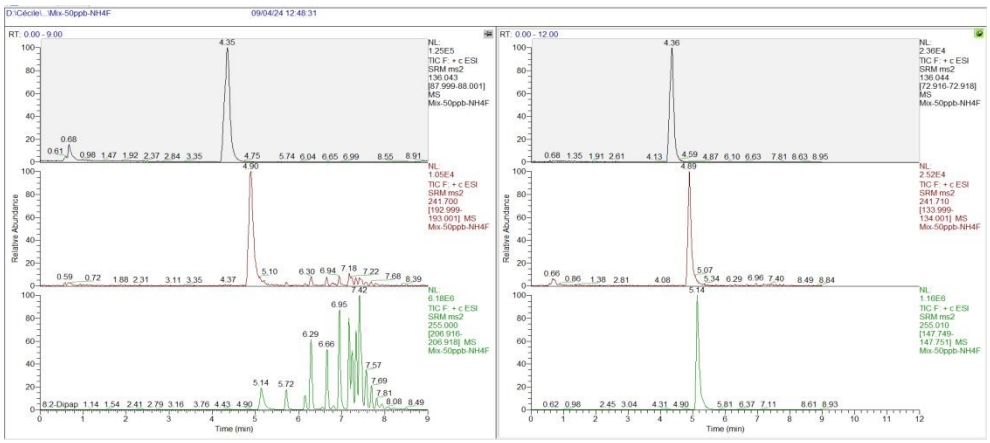

Figure 3: Chromatograms obtained with the mobile phase A: H_2O + 0.1 mM and B: MeOH + 0.1 mM NH_4F. Injection concentration: 50 µg /kg.

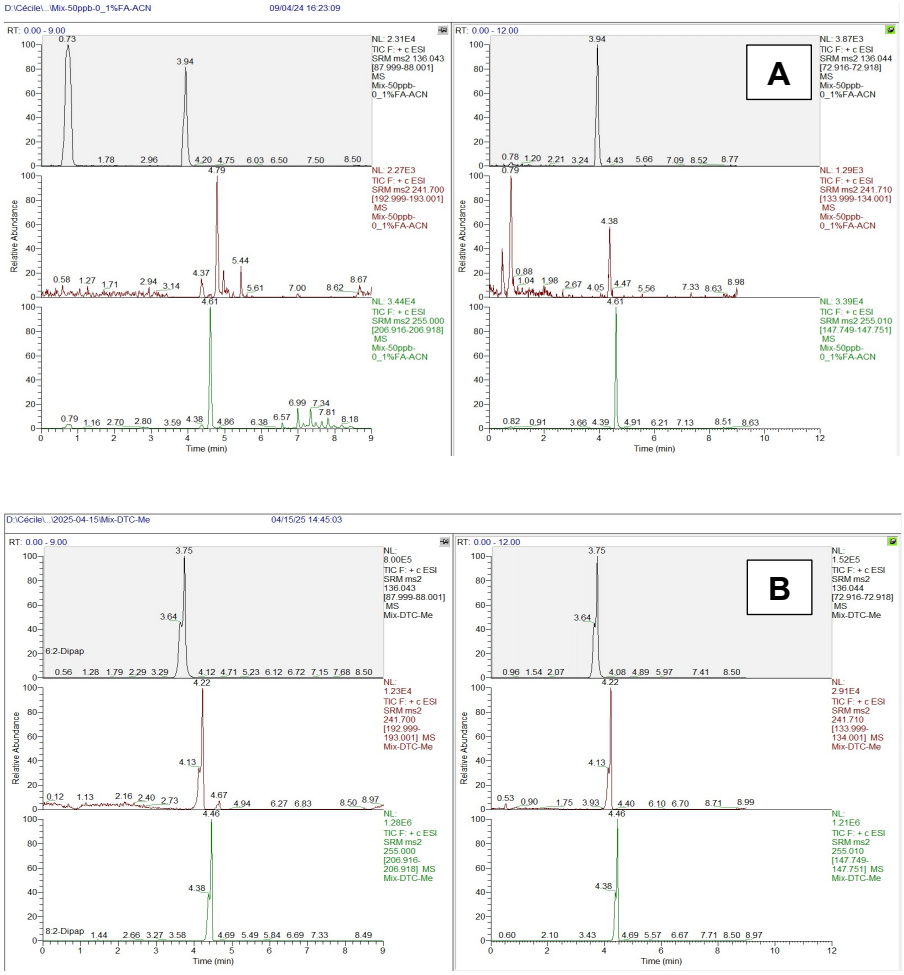

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Figure 4: Chromatograms obtained from the mobile phase A: H_2O + 0.1% FA and B: ACN. (A) methylated standards at 50 µg/kg, (B) methylated standards at 1000 µg/kg.

3.1.2. Optimization of MS conditions

Target compounds were infused to select suitable operating MS parameters (sheath gas, auxiliary gas, collision energy and tube lens voltage) and improve selectivity, sensitivity, resolution and MS/MS transitions such as lens voltage and collision energy (Table SI-2). Automatic optimization of the SRM transitions was also performed for each targeted analyte (Table SI-3).

3.2 Extraction method for DTCs and derivatization

The analysis of intact DTC molecules (Figure 1) poses a huge challenge due to the presence of metals in their structures, which makes them virtually insoluble in most organic solvents. To overcome this problem, the easiest solution was to separate the central ligand (DTC anion) from the metal to make the anion accessible for analysis. In this perspective, an aqueous solution of 50 g/L ethylenediaminetetraacetic acid (EDTA) was used as a chelating agent. This is the most used complexing agent in the literature for this type of situation [24,26]. Alkaline conditions (pH ≈ 9) were necessary to limit the degradation of DTCs and facilitate the solubilization of EDTA in water to increase the complexation efficiency [25]. This is why this parameter has been optimized (Figure 5-A). Best results were obtained with an extraction solution at pH = 9.8. These conditions exhibited the lowest variability, with %RSD values ranging from 2% to 31%. It has been shown by that the addition of a stabilizer such as L-cysteine to the extraction solvent (C ≈ 0.1 M) is essential [36,37]. Thiol group of L-cysteine (-SH) inhibits the conversion of DTCs into their main

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metabolites by neutralizing DTC free radicals (antioxidant role). This blocks the degradation pathways to metabolites during the formation of adducts with reagent intermediates (complexing agent role). In summary, an aqueous solution of cysteine-EDTA at pH 9.8 was used to complex metal ions of DTCs. Once the dithiocarbamic acids were formed, the next step was to make it to react with a methylation agent (S-methylation) to obtain the methylated derivatives (Me-DTCs). Previous works reported methyl iodide used for this derivatization [23,29]. However, studies showed that using dimethyl sulfate (DMS) as a methylation agent in ACN at concentrations between 0.05 M and 0.1 M resulted in a higher reaction yield (> 15%) compared to methyl iodide [26]. Reaction time effect on the yield was also assessed, and better results were obtained for 30 minutes at room temperature in both matrices (Figure 5-B) with a %RSD ranging from 10% to 30% for all targeted compounds and matrixes. The concentration of DMS was also optimized. Figure 5-C shows that at 0.0016 M, yields were very low for ziram, zineb, and (1 – 50%) but increased at 0.09 M (8.5% to 91.6%) with a low variability (% RSD ranging from 4% to 29%). Thus, this it was used in these conditions.

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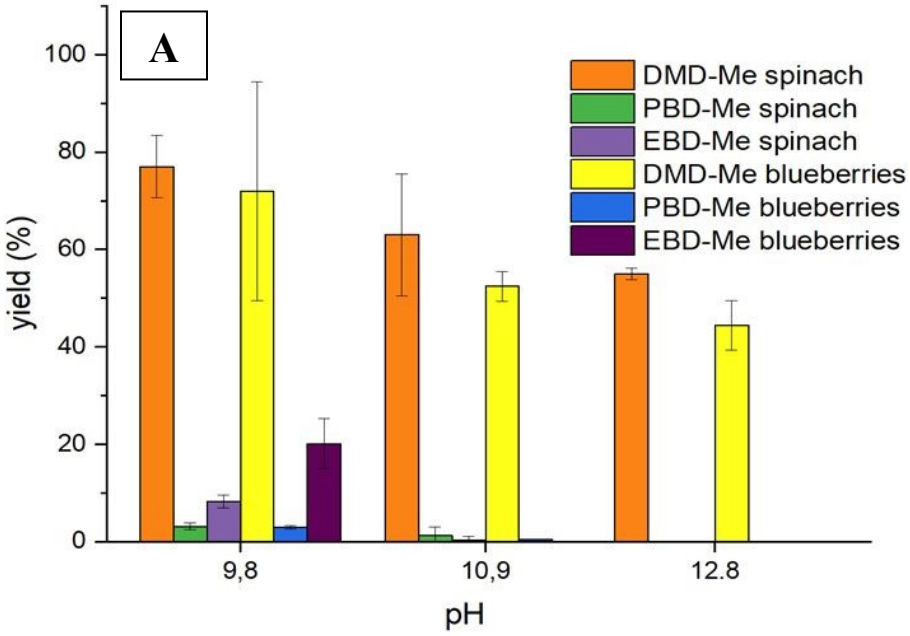

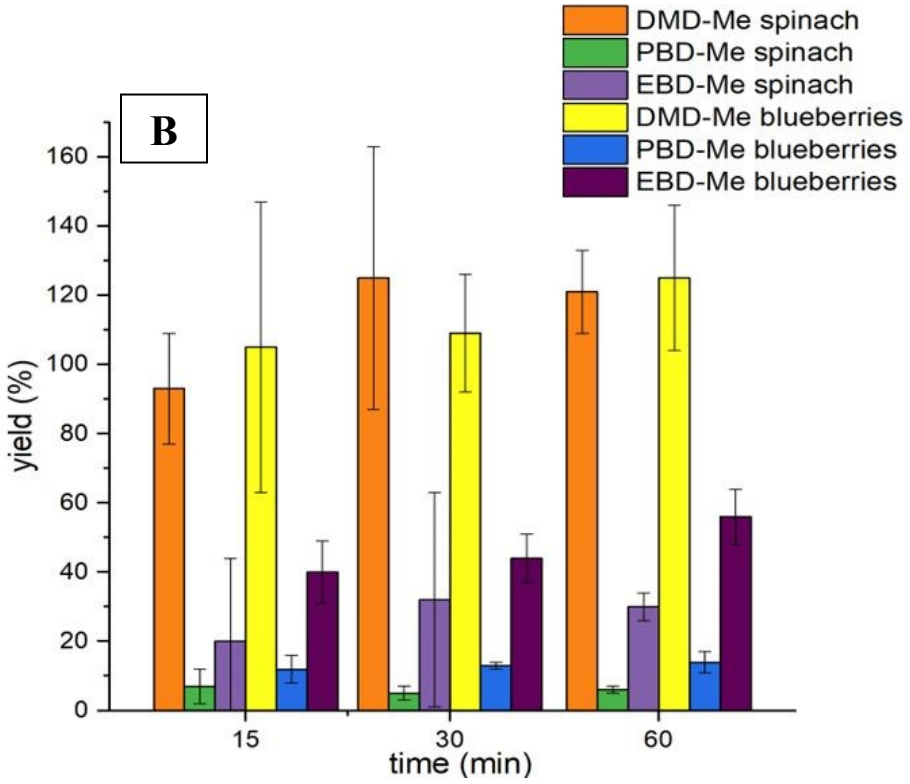

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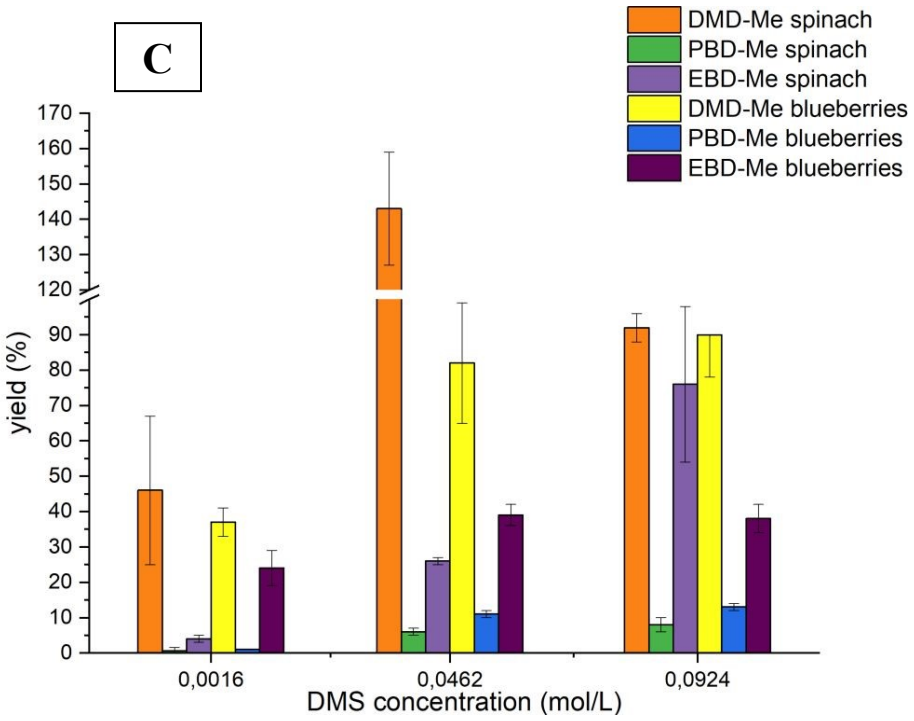

Figure 5 : (A) Effect of cysteine-EDTA extraction solvent pH on reaction yield (conditions: cysteine C = 0.1 M, EDTA C = 50 g/L, DMS C = 0.0016 M and reaction time: 60 min); (B) effect of reaction time on yield (conditions: cysteine C = 0.1 M, EDTA C = 50 g/L, cysteine-EDTA solution pH: 9.8 and DMS C = 0.0924 M); (C) effect of DMS concentration on yield (conditions: cysteine C = 0.1 M, EDTA C = 50 g/L, cysteine-EDTA solution pH: 9.8 and reaction time: 60 min). Each parameter was measured in triplicate (n = 3).

3.3. Validation of the UHPLC/MS-MS method

No interference was observed following the injection of a solution of DTC-Me standards (Figure 4.b), and the different DTC-Me (DMD-Me, EBD-Me, and PBD-Me) were easily identified at specific retention times (Figure 4.b). This confirms the selectivity of the

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method. The linearity range extended over 0.02 – 1000 µg/L (Table 2). Satisfactory linearities (R^2 = 0.9849 – 1.0000) were obtained for all curves. Matrix effects were determined by using the ratio of the slopes in the matrix and solvent (Table 2). It showed a signal increase in (from 13% to 67%). In contrast, no significant matrix effect (1.0%) was observed for PBD-Me in spinach matrix. Calibration curves in matrices were used for the quantification of targeted compounds.

Table 2: Slopes, determination coefficients, matrix effect, LOQ, and LOD in representative matrices (blueberries and spinach) and in acetonitrile.

Compounds	Matrix	Slope	R^2	Matrix effect (%)	LOD (mg/kg of CS_2)	LOQ (mg/kg of CS_2)
DMD-Me	ACN	109711	0.9993	/	/	/
	Spinach	158761	0.9951	45	0.08	0.27
	Blueberries	183152	0.9925	67	0.04	0.14
EBD-Me	ACN	2491	0.9995	/	/	/
	Spinach	2882	1.0000	16	0.38	1.27
	Blueberries	2880	0.9996	16	0.26	0.87
PBD-Me	ACN	75512	0.9968	/	/	/
	Spinach	76233	0.9997	1	0.01	0.03
	Blueberries	85113	0.9986	13	0.01	0.03

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In this paper, accuracy and precision were estimated using the recovery rate (Figure 6) in spinach and blueberry matrices on three different days (intraday and interday precision) [38]. Thus, in spinach, an average recovery rate of between 69% and 77%, with RSD = 3.0% – 8.1% for DMD-Me, was obtained. For EBD-Me, a recovery rate of 25% to 36% was obtained, with RSD = 4.4 – 3.4%. Lower recovery rates were obtained for PBD-Me (5.3% – 7.7%; RSD = 0.8 – 1.5%). Higher recovery values were obtained in blueberries (Figure 6). With recovery rates of 74% to 103% for DMD-Me, 55% to 74% for EBD-Me, and 7.8% to 8.5% for PBD-Me, $RSD_{(r)}$ values ranged from 0.7% to 12.5%. In addition, an analysis of variance (ANOVA) was performed to determine whether the mean recovery percentages obtained on each of the three days for each matrix were equivalent (Table SI-4). Thus, the ANOVA results showed that all mean recovery rates obtained on three different days for each Me-DTC in representative matrices were not significatively different ($p \geq 0.05$) except for the DMD-Me subgroup ($p = 0.02$). In addition, global recovery rates obtained on the three different days were also calculated for both matrices (Figure 6) with values ranging from 72% to 92% for DMD-Me in spinach and blueberries respectively and inter-day precision of 4.8% to 12%. For EBD-Me values ranged from 31% to 62% with an inter-day precision of 4.5% to 8.9% while it ranged from 6.3% to 8.2% for PBD-Me with an inter-day precision of 0.84% to 1.1%. The results presented above showed acceptable precision compliant with QC/QC requirements, but suitable accuracy only for DMD-Me (recovery 70% – 120%), and moderately acceptable accuracy for dimethyl EBD-Me [39]. The developed method achieved suitable performance in terms of intra and interday precision event if recovery rates remained low for propineb highlighting its lack of stability in these types of matrices. Even though a high recovery rate is usually recommended for analytical

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method, criteria such as consistency, accuracy, and precision also determine the relevance of the method. A low recovery rate like propineb may affect the sensitivity of the method. However, in terms of variability and LOD, results were in adequacy with required performance for analytical method and regulatory purposes since most of the RSD values did not exceed the limit for an analytical method according to Jenkins and al. and the US EPA [40,41].

The LOQ values (µg/kg CS_2) in berries and leafy vegetables ranged from 0.14 µg/kg to 0.27 µg/kg, 0.87 µg/kg to 1.27 µg/kg, and 0.03 µg/kg (for both types of matrices) for DMDs, EBDs, and PBD respectively (Table SI-5). The herein reported LOQ values are higher than the range reported by Kakitani et al. (LOQ < 0.007mg/kg for propineb, mancozeb and thiram) and Sayed et al. (LOQ: 0.05mg/kg for mancozeb) for similar methods [24,25]. The same conclusion can be made with previous work which reported LOQ = 0.03 – 2.69 mg/kg) [42].

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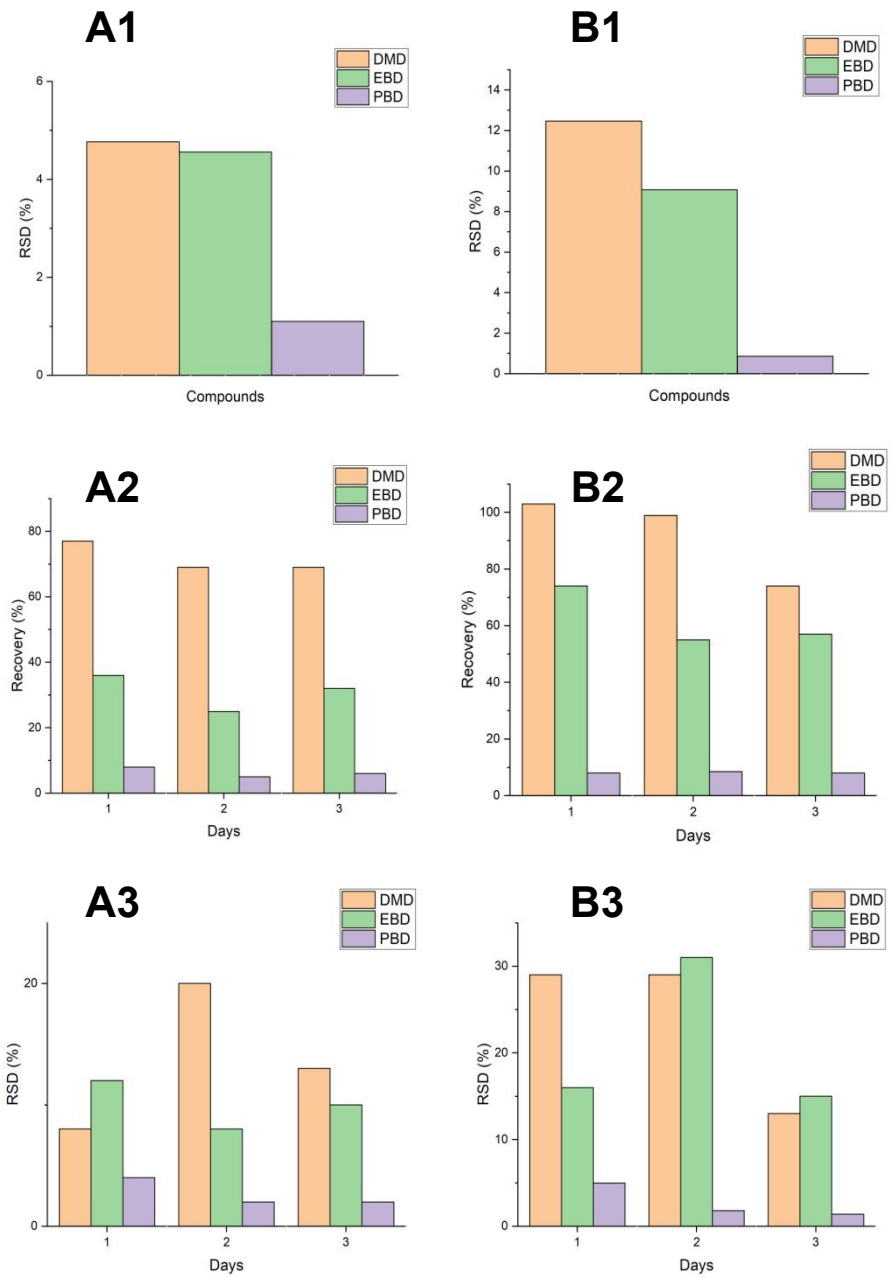

Figure 6: Accuracy and precision in spinach (A) and blueberries (B) matrices. A1 and B1 figures refers to the intermediate precision (n = 9). A2 and B2 figures reports recovery (% for 3 days) mean for 3 days while replicability (% RSD for 3 days) is reported in A3 and B3 figures.

3.4 Analysis of berries and leafy vegetable samples

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To ensure reliable results, fresh solutions must be prepared as many factors (such as aqueous media, temperature, and pH) promote degradation of dimethyl dithiocarbamate standards in solution. Thus calibration curves were prepared with fresh standard solutions and standards (≤ 1 month) were selected for determining the concentrations of DTCs in the samples. Calibration curves comprised nine to ten calibration levels ranging from 0.02 µg/kg to 1000 µg/kg (Figure SI-2). Calculated concentrations using these curves were subjected to ANOVA (Tables SI-6 to SI-11). Thus, 100% of the average concentration of DMDs and PBDs in the samples calculated with the two calibration curves in the corresponding matrices had no significant differences: $p \geq 0.05$. However, a significant difference ($p < 0.05$) between the average concentration of EBDs calculated using these curves was observed in 18% of the samples by comparing with curves established with methylated standards dating back approximately 1.5 years to observe degradation of the old DTC-Me standards (Figure SI-2). For the different matrices, differences of approximately 17% to 35% were observed between the slopes of fresh prepared calibration curves and older ones (Figure SI-2). Contaminant degradation led to a decrease in the observed instrumental response/slope.

To demonstrate method performance, leafy vegetables and berries were analyzed. Results are presented in tables 3 and 4 according to the brand. Details on the origin of the samples are available in additional material (Table SI-1). Concentrations are expressed in mg/kg of CS_2 as the maximum residue limits (MRLs), and no corrections (Table 3 – 4) were made based on the recovery rates obtained (Figure 6). Thus, out of a total of 51 samples, EBD and DMD were detected in almost every sample (94%) and 20% of them contained traces only (traces < LOQ). PBDs were detected in 99% of the samples and 43% of these

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contained traces. Residues of EBDs and DMDs in leafy vegetables and berries were detected at concentrations ranging from 1.2 to 70 µg/kg, and propineb residues at concentrations ranging from 0.032 to 0.23 µg/kg. These results are consistant with those reported in the literature, for example by Dong et al. who conducted routine monitoring of EBDs and PBDs in fruit and vegetable samples purchased at local markets in the city of Chongqing [26]. Indeed, reported levels for EBDs ranged from 7.3 to 16.5 µg/kg, comparable to values obtained in this study while PBDs concentrations ranged from 6.6 to 11.3 µg/kg. These concentrations are significantly higher than those obtained in our study. Our results were also in the same range than reported concentrations by Crnogorac et al. who determined DTCs fungicide residues in fruits and vegetables at concentrations ranging from 9 to 185 µg/kg [43]. This range encompasses most of our results.

None of the calculated concentrations for EBDs and DMDs exceeded the MRLs established by the European Union. Indeed, these concentrations were 1000 times lower than the established MRLs [34]. Some of the analyzed samples presented levels of PBD that exceeded the permitted limits of 0.00003 mg/kg of CS_2). To this end, given that the Food and Agriculture Organization of the United Nations has banned plant protection products containing propineb since June 22, 2019 the samples analyzed should no longer contain it [44]. To the best of our knowledge, there is no official Health Canada document mentioning that propineb is also banned in Canada. However, the presence of DTCs in most of the analyzed crops is not surprising given that previous reports from 2020 – 2022 of the European Food Safety Authority (EFSA) classified these pesticides as the most frequently detected in crops [34,45].

Table 3: Results of dithiocarbamates analysis in leafy vegetables. Concentrations are expressed in µg/kg of CS_2 (European Food Safety Authority). Traces = < LOQ; NF = Not Found

Categories	Vegetables	State	Brands	(DMD + EBD) (µg/kg)[a]	MRL (mg/kg of CS_2)	PBD (µg/kg)[a]	MRL (µg/kg of CS_2)
Lettuce	Iceberg lettuce	Fresh	Oak Canyon Farms	Traces	0.1	Traces	0.03
			Naturels Rewards	NF	0.1	Traces	0.03
			Happy Green	NF	0.1	Traces	0.03
	Curly lettuce		Folia (GreenHouses)	Traces	0.1	Traces	0.03
			Good Leaf (no pesticides)	Traces	0.1	Traces	0.03
	Curly green lettuce		Marché C&T	6.3 ± 0.8	0.1	0.053 ± 0.003	0.03
	Rocket		Attitude	3.4 ± 0.4	14.0	NF	0.03
	Romaine lettuce hearts		Dole	Traces	0.1	Traces	0.03
			Ocean Mist Farms	Traces	0.1	0.034 ± 0.002	0.03
			Happy Green	Traces	0.1	Traces	0.03
			Tanimura & Antle	1.8 ± 0.2	0.1	Traces	0.03
	A-choy lettuce		Marché C&T	2.2 ± 0.2	0.1	Traces	0.03
Spinach	Spinach	Fresh	Les marques Métro	1.5 ± 0.1	0.1	Traces	0.03
			Harvest Fresh	Traces	0.1	0.039 ± 0.002	0.03
			Queen Victoria	Traces	0.1	Traces	0.03
			Frisco's	NF	0.1	0.077 ± 0.004	0.03
	Spinach	Frozen chopped	Selection	1.7 ± 0.2	0.1	Traces	0.03
	Young spinach	Fresh	Organics	2.4 ± 0.2	0.1	0.041 ± 0.002	0.03
	Chopped spinach	precooked	President's choice	1.2 ± 0.1	0.1	0.075 ± 0.004	0.03
	Chinese spinach	Frosen	J.L Trading	2.4 ± 0.3	0.1	0.033 ± 0.002	0.03
	Spinach	Frozen chopped	Artic Gardens	Traces	0.1	Traces	0.03
Other	Cress	Fresh	B&W (non-OGM)	1.8 ± 0.2	30.0	0.078 ± 0.004	0.03
Cabbage	kale	Chopped	Arte	6.7 ± 0.7	0.1	0.055 ± 0.003	0.03
	Taiwanese bok choy lettuce	Fresh	Marché C&T	4.3 ± 0.5	0.1	0.047 ± 0.003	0.03

Table 4: Results of dithiocarbamates analysis in berries. Concentrations are expressed in µg/kg of CS_2 (European Food Safety Authority). Traces = < LOQ; NF = Not Found

Berries	State	Brands	(DMD + EBD) (µg/kg)[a]	MRL (mg/kg of CS_2)	PBD ± SD (µg/kg)[a]	MRL (µg/kg of CS_2)
Blackberries	Fresh	North Bay Produce	1.9 ± 0.2	0.1	Traces	0.03
		Wish Farms	2.1 ± 0.2	0.1	0.035 ± 0.001	0.03
		President's choice	1.8 ± 0.2	0.1	Traces	0.03
		Driscoll's	2.3 ± 0.3	0.1	Traces	0.03
		Mariland Farms	1.5 ± 0.2	0.1	Traces	0.03
		Berry-Fresh	1.4 ± 0.2	0.1	0.042 ± 0.002	0.03
	Frozen	President's choice	0.30 ± 0.01	0.1	Traces	0.03
Blueberries	Fresh	Shajara	1.1 ± 0.1	2.0	0.033 ± 0.001	0.03
		Naturip Farms	2.6 ± 0.3	2.0	Traces	0.03
		Local farm	15 ± 2	2.0	Traces	0.03
		President's choice	70 ± 10	2.0	0.13 ± 0.001	0.03
		Clear Springs	8 ± 1	2.0	Traces	0.03
		Camposol	Traces	2.0	Traces	0.03
		Bleu et Bon (no pesticides)	1.9 ± 0.2	2.0	0.034 ± 0.001	0.03
	Frozen	No name	2.3 ± 0.3	2.0	0.036 ± 0.002	0.03
		President's choice	1.6 ± 0.2	2.0	0.047 ± 0.002	0.03
		Compliments	2.1 ± 0.2	2.0	0.042 ± 0.002	0.03
		Irrésistible	2.4 ± 0.3	2.0	0.037 ± 0.002	0.03
Raspberries	Fresh	Berry Valley	1.2 ± 0.1	0.1	0.060 ± 0.003	0.03
		Driscoll's	1.2 ± 0.1	0.1	0.23 ± 0.01	0.03
Strawberries	Fresh	Naturipe Farms	31 ± 4	0.1	0.04 ± 0.02	0.03
		Gem-Pack Berries	1.5 ± 0.1	0.1	0.032 ± 0.001	0.03
	Frozen	President's choice	1.9 ± 0.2	0.1	0.071 ± 0.003	0.03
		No name	1.3 ± 0.1	0.1	0.036 ± 0.001	0.03
		Naturalia	2.0 ± 0.2	0.1	0.035 ± 0.002	0.03
Mixed berries	Fresh	Snowcrest	2.1 ± 0.2	2.1	0.041 ± 0.002	0.03
		Selection	4.5 ± 0.6	2.1	0.032 ± 0.001	0.03

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It is also important to analyze the results according to the different categories of plants studied. Indeed, among the vegetables, lettuce, spinach, and cabbage were analyzed. Several types of berries, such as blackberries, raspberries, strawberries, and blueberries, were evaluated. With concentrations ranging from trace (67%) to 6.3 µg/kg for the sum of DMD + EBD and from trace (83%) to 0.034 µg/kg for PBD respectively, salad samples were the least contaminated with DTCs. Indeed, spinach samples had concentrations ranging from trace (43%) to 2.4 µg/kg for the sum of DMD + EBD and from trace (57%) to 0.077 µg/kg respectively. With concentrations ranging from 4.3 µg/kg to 6.7 µg/kg for the sum of DMD + EBD and from 0.047 µg/kg to 0.055 µg/kg for PBD respectively, cabbage samples are proportionally those which are most contaminated with DTCs. Regarding berries, most samples had comparable contamination levels. Highest concentrations were found in blueberry and raspberry samples with levels around 70 µg/kg and 31 µg/kg respectively for the sum of DMD + EBD. PBD levels were lower in blackberry samples with 71% having trace levels up to 0.042 µg/kg and in blueberry samples with levels ranging from trace (37%) to 0.13 µg/kg. Raspberry and strawberry samples consistently had levels above the LOQ with values ranging from 0.060 µg/kg to 0.23 µg/kg and from 0.032 µg/kg to 0.071 µg/kg respectively. It should be noted that the sample size for each of the categories studied differs and the percentages and variety of concentrations expressed must take this disparity into account. Finally, no significant difference was reported between the fresh and frozen samples analyzed in this study.

3.5 Exposure and risk assessment

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The varying levels of DTCs found in the samples in this study raise questions about human exposure and the risk associated with the consumption of such products. Based on average daily consumption in Canada, we assessed the amount of DTCs ingested per day [46]. Compared to the acceptable daily intake and acute intake values given by the EU, it was possible to assess the risk posed by the consumption of berries and leafy vegetables. Regarding PBD values, no sample analyzed represented a risk for human consumption for both ADI and ArfD of 0.007 and 0.1 mg/kg bw/day respectively. Regarding the combined DMD + EBD values, the mean value found of 0.0164 mg/kg bw/day in blueberry samples exceeded that of ADI (0.006 mg/kg bw/day) without exceeding the ArfD threshold (0.08 mg/kg bw/day). The same observation could be made for strawberry samples since the mean value determined was 0.0109 mg/kg bw/day (Table 5). Given the small sample size of blueberries and strawberries, the calculated ADI values should be interpreted with caution. A larger sample size would allow for a more precise refinement of this value and more accurate conclusions.

Table 5: Average values for daily exposure to DTCs. Results are expressed in mg/kg body weight/day.

Category	Mean value (kg/bw)	Mean DMD + EBD (µg/kg)	Mean PBD (µg/kg)	Average daily intake (mg/kg bw/day)	Average daily intake (mg/kg bw/day)
Blueberries	1.16	10.7	0.05	0.0124	0.0001
Cabbage	0.83	5.50	0.05	0.0046	0.0000
Spinach	0.62	1.84	0.05	0.0011	0.0000
Strawberries	1.45	7.54	0.15	0.0109	0.0002

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Raspberries	0.86	1.20	0.04	0.0010	0.0000
Lettuce	0.66	3.40	0.04	0.0022	0.0000
Acceptable Daily Intake (ADI)				0.006	0.007
Acute reference Dose (ArfD)				0.08	0.10

2. Conclusion

A sensitive and robust method for the analysis of DTCs was developed in this work. This method was satisfactorily validated using representative matrices of blueberries for small fruits and spinach for leafy vegetables, and the analysis was performed using UHPLC/MS-MS. It was applied to 51 samples of different berries and leafy vegetables purchased in the metropolitan Montreal area. As a result, 94% of the samples contained DMDs and EBDs, while PBD were detected in 99% of the samples. None of the DMDs and EBDs concentrations in the samples analyzed exceeded the MRLs established by the EU (which were 50 to 4,000 times higher than the MRLs). However, 56% of samples containing propineb exceeded the MRLs. The presence of propineb in these samples, even in trace amounts, provides evidence of its usage, despite the ban decreed by the Food and Agriculture Organization of the United Nations. There does not seem to be any prohibition by Canadian authorities. Furthermore, the results obtained provide an overview of the concentration of DTCs ingested by Montreal consumers. In addition, given these results, it can be said that the proposed methylation method, coupled with UHPLC/MS-MS, is suitable for monitoring DTCs in pigment-rich plant-based matrices. Based on these various comparisons, daily exposure and risk assessment, we can conclude that leafy vegetables

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and berries consumed by residents of the Montreal area contain low levels of DTC residues. However, a more in-depth study, i.e., one conducted over the long term, is needed to confirm this conclusion.

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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Data availability statement

The data supporting this article have been included as part of the Supplementary Information.