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Ethylenethiourea in fruits: Optimization and in-house validation of a method by liquid chromatography tandem mass spectrometry, occurrence and dietary exposure assessment

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ABSTRACT

Ethylenethiourea (ETU) is a toxicologically relevant degradation and/or biotransformation product of fungicides ethylenebisdithiocarbamates, that should be considered in a consumer risk assessment. The aims of this research were the validation of a method for analysis of ETU in fruits by liquid chromatography tandem mass spectrometry, the evaluation of ETU occurrence in fruits and ETU and dithiocarbamates dietary exposure assessment. The validated method was fitness for the purpose. Linearity was demonstrated between 1 and 25 ng/mL, without matrix effects. Recovery averages ranged from 75 to 110% for spiked samples at 1.0, 2.0 and 10.0 μg/kg, with relative standard deviation from 5 to 17%, indicating trueness and precision. The limits of detection and quantification were 0.5 and 1.0 μg/kg, respectively. Ninety samples of apple, papaya and strawberry were collected from different regions of São Paulo city and over all seasons of the year. ETU residues were found in 32 (35%) samples with levels ranging from 1.0 to 5.3 μg/kg. The exposure assessment to evaluate the health risk by eating fruit with ETU residues corresponded, respectively, for adults, teenager and children 0.05, 0.05 and 0.09% of the acceptable daily intakes (ADI) and 0.9, 1.1 and 1.6% of the oral dose for chronic non-carcinogenic effects. The contribution to the risk by consumption of food containing residues of dithiocarbamates, whereas the highest values found in the products monitored by the national Program in the period from 2001 to 2010 and the *per capita* food acquisition for urban population, represented 19.2% of the ADI (Mancozeb) for adult, 22.8% for teenager and 34.0% for children.

1. Introduction

Ethylenethiourea (ETU) is a toxic substance, the mainly degradation and/or biotransformation product of ethylenebis dithiocarbamate (EBDC) fungicides, such as mancozeb, maneb and zineb (USEPA, 2005; WHO, 1988).

The ethylenebisdithiocarbamates (EBDC) pesticides are organic salts of manganese, zinc or zinc and sodium, that have a wide range of approved uses on agricultural and horticultural crops in many countries (ANVISA 2013; EC 2010; USEPA, 2005). These pesticides belong to the dithiocarbamates class and are widely used as fungicides because of their efficacy against a broad spectrum of fungi and their associated plant diseases. EBDC fungicides are metabolized to ETU in the body and also degrade to ETU in the environment. Generally unstable in alkaline or acid, in the presence of oxygen, as well as in biological systems, the EBDC decompose rapidly in water. Their degradation to ETU can even occur during the storage (Lo & Ho, 1993) or manufacturing of processed foods (Geetanjali & Santosh, 2009; Kontou, Tsip, & Tzia, 2004) and in the field after the pesticide application (Araujo, 1998; Lemes, Barretto, Kussumi, & Colacioppo, 2005). Microorganisms form ETU from ethylene bistiouram disulfide (DIDT), a product of spontaneous decomposition of EBDC, and others metabolites such as ethyleneurea ethylene bisisothiocyanate shulphide and ethylene thiouram disulphide that can be found in soil (Mestres & Mestres, 1991; USEPA, 2005; WHO, 1988).

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Some EBDC (maneb and zineb) are suspected endocrine disrupter (EC, 2012a). Thyroid toxicity was manifested as alterations in hormones, increased weight, microscopic lesions and tumors. A rat subchronic toxicity study demonstrated microscopic neuropathology with associated clinical signs and loss of muscle mass (USEPA, 2005).

The ETU has the ability to induce tumor in rodent thyroid and in mouse liver (WHO, 1988) and has been classified as group 3 by the International Agency for Research on Cancer (IARC), with sufficient evidence of carcinogenicity studies in experimental animals and inadequate evidence in humans (IARC, 2001). It is suspected that ETU is an antithyroid compound (Mnif et al., 2011).

Since 2008, Brazil has taken the place of the world largest consumer of pesticides. More than 40% of all fungicides used in Brazil belong to the group of EBDC, being mancozeb consumption about 3000 ton in 2009, authorized for use on a variety of agricultural crops and most fruit cultures and others vegetables (ANVISA, 2013; Jardim & Caldas, 2012).

Most fruits and vegetables that are produced with EBDC are consumed *in natura* and also processed or industrialized, such as juices, purees, pulps and pasta. During these processes the conversion of EBDC to ETU can occur (Geetanjali & Santosh, 2009; Kontou et al., 2004; Lentza-Rizos, 1990).

Also, soil erosion and water runoff transfer the pesticide residues from treated to untreated areas and to rivers and lakes. Some researchers have shown the possibility of ETU presence in groundwater, rivers, drinking water, mainly in agricultural areas where the EBDC are used, due to the relative stability and high polarity of ETU (Geissen et al., 2010; Hogendoorn, Van Zoonen, & Brinkman, 1991; Ripollés, Sancho, López, & Hernández, 2012; Van Der Poll, Versluis-de Haan & De Wilde, 1993).

In the Brazilian legislation, maximum residues levels (MRL) of dithiocarbamates are established for CS_2 (mg/kg), depending on the use of the pesticide, although there is not defined an MRL for ETU (ANVISA, 2013; Brasil, 2003, pp. 48–50).

The consumption of fruits brings numerous health benefits once they are rich in vitamins and minerals, among other components (IBGE, 1985). On the other hand, food is an important source of consumer exposure to chemicals and the monitoring of pesticides residues in food constitutes one of the most significant topics to estimate the risk to human health (WHO, 2003).

Brazil ranks as the third world producer of fruits, behind China and India, and as the fifteenth main exporter. In 2009, 41 million ton of fruits were produced in Brazil, in two million hectares, 65% for the domestic market and 35% for exportation (IBRAF, 2012).

In 2010, the Brazilian apple production was 1.3 million ton, corresponding to the ninth country in the world ranking (FAO, 2012). About 10–20% of apple production in the country is exported to various markets, mainly to Europe.

According to FAO (2012), world production of papaya represents 10% of the total tropical fruits, around 8 million ton, of which 39% are from Latin America and Caribbean. In 2010, the second main producing country was Brazil, with a production of 1.9 million ton of papaya (FAO, 2012).

The national production of strawberries, according to the Brazilian Fruit Institute, is approximately 105,000 ton/year (IBRAF, 2012), the state of Minas Gerais contributed with 40,000 ton/year and São Paulo with 29,000 tons/year (EMATER, 2011).

Residues of dithiocarbamates have been found in strawberry, apple and papaya samples in monitoring studies carried out in Brazil (ANVISA, 2008, 2009, 2010, 2011; Caldas, Miranda, Conceição, & Souza, 2004; São Paulo, 2005). However, considering the ETU residues, there are only few researches concerning Brazilian fruits (Araújo, 1998; Lemes et al., 2005).

Several publications related to the analysis of ETU in food by liquid and gas chromatography (Aprea et al., 1997; Blasco, Font & Pico, 2004; Diserens, 1991; Dubey, Heberer & Stan, 1997; Ozhan & Alperturga, 2008), but few of them were conducted in Brazil, as described by Araújo (1998) and Lemes et al. (2005).

The aims of this work were the optimization and validation of an analytical method for determining residues of ETU in apple, papaya and strawberry by liquid chromatograpy tandem mass spectrometry (LC–MS-MS), the study of the occurrence of ETU residues in samples collected in São Paulo city (SP, Brazil) and the estimation of the risk to the consumers health due to ETU and dithiocarbamates intake.

2. Material and methods

2.1. Sampling

For the occurrence study, a total of 90 samples were analyzed, including apple, papaya and strawberry, 30 of each one. The samples were purchased in supermarkets in São Paulo city (SP-Brazil), during the period of one year. A stratified sampling plan was performed, according the Codex Alimentarius (1999) requirements. The samples were collected randomly to represent the seasons (spring, summer, autumn and winter) and the consumption in five regions of São Paulo city (north, south, east, west and center).

Considering the validation experiments, apple, papaya and strawberry samples were obtained from a certified organic production, representing the blank samples.

The fruits of each sample were cut by quartering method, mixed and a representative portion of them were chopped, homogenized, reduced to 200 g and transferred to labeled amber glass jars with lids. The samples were prepared on the same day that they were collected and kept frozen at −20 °C to −18 °C until the moment of the analysis.

2.2. Standard, solvents and reagents

The certified standard of ETU, produced by Riedel-de Haën with 99.9% purity, was purchased from Sigma–Aldrich Co. Ltd. (Dorset, UK).

The HPLC grade solvents and analytical grade reagents were dichloromethane, methanol, acetonitrile, sodium hydroxide and ammonium acetate supplied by Merck (Darmsdadt, Germany) and Mallinckrodt Baker (North Caroline, USA). Water was purified (<18 MΩ resistivity) through a Milli Q Purification System (Millipore, USA).

2.3. Instrumentation

Analyses were performed using an HPLC Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, degasser system, autosampler with temperature control and column oven thermostated. The HPLC was coupled via an electrospray interface to a triple quadrupole MS–MS API 5000™ (Applied Biosystems/MDS Sciex, Concord, Canada).

The data acquisition and quantitation were carried out by the Analyst software version 1.4.1.

2.4. Method

The extraction and purification procedures were based on those described by Diserens (1991) and Lemes et al. (2005), with optimization of LC-MS/MS conditions.

ETU residues were extracted from 20 g of the homogenized samples with 100 mL of methanol, under stirring for 30 min. The

extract was filtered under vacuum and re-extracted with 50 mL of methanol. The filtrate was concentrated and the pH of the extract was adjusted to 8.5 with a solution of 10% of sodium hydroxide (final volume of 10 mL). An aliquot of 1 mL of the extract was transferred into an *Extrelut* column (Merck KGaA Darmsdadt, Germany). After 20 min, residues of ETU were eluted with 20 mL of dichloromethane and collected into a flask containing 0.5 mL of purified water. The eluate was concentrated at 45 °C, under partial vacuum, and completed to 1.0 mL with purified water. This solution was filtered through a PTFE membrane of 0.45 μm (Millipore, USA) into a glass autosampler vial for LC/MS/MS analysis.

This extract can be used for the analysis of other polar pesticides. Considering the use of this extract only for the determination of ETU by LC/MS/MS, 2 g of the homogenized samples can be extracted with 10 mL of methanol and re-extracted with 5 mL of methanol. In this case, the purification must be performed with entire extract (1 mL).

2.5. Validation

The parameters studied in the method validation were selectivity, linearity, working range, detection limit, quantification limit, recovery and precision. These parameters were considered to verify the fitness for purpose of the method (EC 2012b; Thompson, Ellison & Wood, 2002). The significance levels in hypothesis tests was $\alpha = 0.05$.

2.5.1. Linearity

The evaluation of linearity was conducted according the procedures proposed by Souza and Junqueira (2005).

Solvent calibration curves were prepared at seven concentration levels, being 1.0, 2.0, 5.0, 10.0, 15.0, 20.0 and 25.0 ng mL^{-1} corresponding to 0.5, 1.0, 2.0, 5.0, 7.5, 10.0 and 12.5 μg/kg of sample, in three independent replicates of each level. The solutions were prepared and analyzed randomly.

The ordinary least squares method was applied to the experimental data to estimate the regression parameters. Outliers were investigated by Jacknife standardized residuals test. Violations of the assumptions related to the simple linear regression were evaluated: normality (Ryan–Joiner test), homoscedasticity (Brown–Forsythe test) and independence of regression residuals (Durbin–Watson). F tests were applied to check the significance of the regression and deviation from linearity. Matrix effects were investigated by the comparison of the slopes obtained for the solvent curve with that achieved for the matrix-matched curves by *t* test.

2.5.2. Selectivity

Blank samples (obtained from organic production) were analyzed in six independent replicates to check interferences from the matrix. Other interferences, such as that due to solvents, glassware and reagents, were investigated by the analysis of solvent blank, also in six replicates (EC 2012b; Thompson et al., 2002).

2.5.3. Limits of detection (LD) and quantification (LQ)

The theoretical limits of detection and quantification were established as the mean plus three and six times the standard deviation of the results obtained for the blank samples (EC, 2012b).

2.5.4. Accuracy

Considering the unavailability of certified reference material for this scope, trueness was assessed by spiking/recovery experiments. Three concentration levels were studied (1.0, 2.0 and 10.0 μg/kg) in six replicates. For each level, recovery values were considered acceptable when between 70 and 120% (EC, 2012b).

Precision, under repeatability conditions, was evaluated by relative standard deviations (RSD) obtained in spiking/recovery experiments, at each studied level. The acceptability criterion for the RSD was ≤20% (EC, 2012b).

2.6. Risk assessment

2.6.1. Risk assessment estimated by results of this study for ETU occurrence

The Maximum Daily Intake (MDI) was calculated assuming: i) that the consumed fruits had the highest values found in this study for ETU occurrence; and ii) the average for fruit consumption *per capita* for the city of São Paulo, according to the Brazilian Institute of Geography and Statistics (IBGE, 2012a).

The Brazilian Household Budget Survey (POF) is a household survey conducted by representative sampling of the Brazilian population, which is carried out by the IBGE. The analysis of food consumption of the Brazilian population involved interviews in 59,548 permanent households sampled in Brazil from May 2008 to May 2009. In São Paulo city, the residents with 10 or more years of 3623 households were interviewed in relation to the quantities of products "food" purchased for consumption. The annual *per capita* amount of each product was estimated by the ratio between the total amount of acquired food and the resident population (IBGE, 2012a).

For risk estimates were considered the median body weight of 69 kg for adult (20–75 years), 58 kg for teenager (13–19 years) and 39 kg for children (10–12 years), obtained from the concomitant study conducted by IBGE in the period May 2008–May 2009 related to the population anthropometric measurements (IBGE, 2012b). The risk characterization was estimated for age group (adults, teenagers and children) comparing the MDI with the acceptable daily intake (ADI) of 2 μg/kg bw/day for the general population, according to FAO/WHO (1994) and the oral dose for chronic non-carcinogenic effect of 0.08 μg/kg bw/day, including sensitive subgroups of the population, established by the United States Environmental Protection Agency (USEPA, 1998).

2.6.2. Estimated risk due intake of EBDC residues by results of Brazilian Health Surveillance Agency/Pesticide Residues in Food Monitoring Program (PARA), 2001–2010

The MDI was calculated assuming: i) that the consumed food had the highest values of the dithiocarbamates in CS_2 detected by the Brazilian Health Surveillance Agency (ANVISA) in the Pesticide Residues Food Monitoring Program (PARA), in the period from 2001 to 2010 (ANVISA, 2008, 2009, 2010, 2011); and ii) the average for each food consumption *per capita* for the city of São Paulo, according to the Brazilian Institute of Geography and Statistics (IBGE, 2012a).

For risk estimates were considered the median body weight of 69 kg for adult (20–75 years), 58 kg for teenager (13–19 years) and 39 kg for children (10–12 years), obtained from the concomitant study conducted by IBGE in the period May 2008 to May 2009 related to the population anthropometric measurements (IBGE, 2012b). The risk characterization was estimated for age group (adults, teenagers and children) comparing the MDI with the ADI of 0.03 mg/kg bw/day for mancozeb for general population established by FAO/WHO (1994), and adopted in the Brazil and European Union (ANVISA 2013; EC, 2010).

3. Results and discussion

Tandem mass spectrometry with electrospray ionization, have been demonstrated to be useful in the determination of different pesticides classes (Chung & Lam, 2012; Sannino, Bolzoni, & Bandini,

2004). In this study, ETU was determined by LC-MS/MS with electrospray ionization (ESI), due to the high sensitivity and unequivocal identification of this technique (Martins Junior, Lebre, Wang, Pires, & Bustillos, 2011).

3.1. Optimized LC/MS/MS conditions

The LC separation was carried out on a *Symmetry* C-18 (Waters Technologies, USA) reversed-phase column (100 mm × 2.1 mm i.d. x 3.5 μm particle size). The column compartiment was maintained at 25 °C and the sample injection volume was 20 μL. The isocratic elution was composed by 30% of the mobile phase A (5.0 mmol L^{-1} of ammonium acetate) and 70% of the mobile phase B (methanol:water, 95:5, v/v, with 5.0 mmol L^{-1} of ammonium acetate), in a flow rate of 700 μL min^{-1}. The dead volume (Rt0) was less than 1.0 min and the retention time obtained for the optimized analytical conditions was 1.59 min.

The mass spectrometer was operated with ESI probe in positive ion mode. The MS/MS spectrum was obtained by ramping the collision energy from 5 to 130 eV.

ETU quantitative analysis was performed using Multiple Reaction Monitoring (MRM) mode and three *m/z* transitions were selected, including the *m/z* 103.1 → 44.0, *m/z* 103.1 → 86.0 and *m/z* 103.1 → 60.0, being the first transition for quantification and the others two for confirmation purposes (Fig. 1).

A dwell time of 300 ms was used to monitor ETU transitions with a pause time of 5 ms between of them. The first quadrupole (Q1) and the third quadrupole (Q3) were operated at unit resolution (0.7 ± 0.1 *m/z*) and the entrance potential (EP) was 10 V. The electrospray capilar voltage was maintained at 5.5 kV. Ultrapure air was used as drying and nebulizing gas, both at 50 psi. Nitrogen ultrapure was used curtain gas at 10 psi and in the collision cell at 6 arbitrary units. The optimized declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) are shown in Table 1.

3.2. Validation

For the linearity assessment, after the deletion of four outliers, the assumptions of normality, homoscedasticity and independence of the regression residuals were confirmed, with Ryan–Joiner coefficient of 0.975 ($p > 0.10$), tLevene statistic of −1.043 ($p > 0.05$) and Durbin–Watson statistic of 2.056 ($p > 0.10$), respectively. These results indicated the adequate estimation of the regression parameters by ordinary least square method without the need of weighting. The regression was significant ($p < 0.001$) while the lack-of-fit was not significant ($p > 0.05$), indicating linearity in the studied range from 1.0 to 25.0 ng mL^{-1} (transition quantification: *m/z* 103.1 → 44.0). The calibration function was $y = (4743.68 \pm 26.52)x + (1462.69 \pm 345.80)$, with determination coefficient R^2 of 0.9995, after the outlier deletion.

No matrix effects were detected, once no significant differences were observed between the slopes achieved for the solvent and matrix-matched curves ($p > 0.05$). Selectivity was also demonstrated, considering that no false positive results were obtained for blank samples and blank of solvents.

The estimated limits of detection and quantification for the method were 0.5 and 1.0 μg/kg, respectively.

The LC–MS/MS method for determining ETU residues showed adequate mean recovery and precision, under repeatability conditions, between 1.0 and 10.0 μg/kg, with mean recoveries and RSD in accordance with the acceptable limits established by European Commission (EC, 2012b). For apple, the mean recovery values ranged from 84 to 100%, with RSD between 13 and 17%. Considering the papaya spiked samples, the mean recovery and RSD were from 92 to 110% and from 5 to 15%, respectively. For strawberry samples the mean recovery varied from 75 to 87%, with RSD between 6 and 17%.

Table 1
Optimized conditions for analysis of ethylenethiourea in tandem mass spectrometry with electrospray ionization (ESI-MS/MS) by Multiple Reaction Monitoring (MRM).

Transition (*m/z*)	DP[a] (V)	CE[b] (eV)	CXP[c] (V)
103.1 → 44.0	41	33	18
103.1 → 86.0	41	29	32
103.1 → 60.0	41	45	22

[a] DP: desolvation potential.
[b] CE: collision cell energy.
[c] CXP: collision cell exit potential.

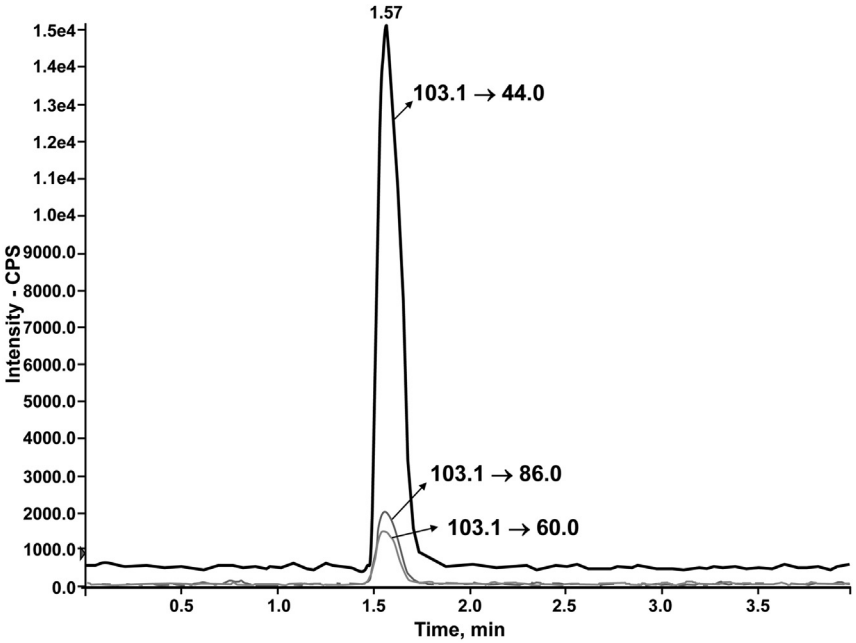

Fig. 1. Ethylenethiourea standard by Multiple Reaction Monitoring.

The results achieved in this study agreed with that obtained by other researchers. The analytical performance of the method proposed by Bonnechère, Hanot & Van Loco (2011) for ETU detection in food was evaluated with spiked celery samples at 50 and 300 μg/kg. The recoveries were between 65 and 90%, with RSD below 10%. Ripollés et al. (2012) validated a method for ETU determination by LC/MS/MS in drinking, ground and surface water and obtained mean recoveries ranging from 75 to 114%, with RSD less than 2%, for spiked samples at 0.1 and 1.0 μg/kg.

3.3. ETU residues in fruits commercialized in São Paulo city, Brazil

Residues of ETU were detected in 32 (35%) of the 90 analyzed samples, being 10 (33%) apple samples ranging from 1.0 to 3.7 μg/kg; 20 (67%) papaya samples from 1.0 to 5.3 μg/kg and 2 (7%) of strawberry samples from 1.0 to 1.4 μg/kg of ETU (Table 2).

In the FDA Total Diet Study (TSD), conducted in 2008, for apples and other fruits, except berries, the maximum level and mean levels of ETU were 3.0 and 1.0 μg/kg, respectively. In 2004 and 2006, ETU levels ranged, respectively, from 1.0 to 13 μg/kg and from 3.0 to 17 μg/kg for infant and toddler foods (FDA, 2013).

ETU was found in 61 of 600 samples analyzed by the First Hong Kong Total Diet Study, with levels ranging from 2 to 5 μg/kg, and in six samples ETU was detected in levels over than 100 μg/kg (Chung & Lam, 2012).

Papaya samples presented upper frequency (67%) and highest ETU level, followed by apple and strawberry samples (Table 2). The levels of dithiocarbamates residues that were found for same crops by the ANVISA Program, from several Brazilian States, showed the same profile. Furthermore, dithiocarbamates were detected in all cultures evaluated by PARA confirming the widespread use of these pesticides in Brazil (Table 3).

Considering the strawberry samples, the lower frequency of detection and levels of ETU also reflected the change in the Brazilian legislation, related to the prohibition of maneb and the authorization of metam sodium, with the MRL of 0.2 mg/kg (ANVISA 2013).

In general, apple and papaya samples acquired in summer (57%) and autumn (41%) had the highest percentage ETU residues than those obtained in winter (23%) and spring (30%). In summer the percentages were higher (Fig. 2). During the rainy season, with a greater chance of spreading disease, especially fungal, pesticides are applied more frequently. Additionally, the matrices collected in winter showed the lowest occurrence of residues of ETU, confirming the temperature effect (Geetanjali & Santosh, 2009).

Considering the regions, the frequency of ETU residues were: east (48%), west (37%), center (31%), south (30%) and north (28%) (Fig. 3). Except for the east region, the similarity is an indication that the fruits are uniformly distributed in a fairly.

However, when observing each matrix separately (Fig. 3), there is an indication that the frequency of ETU detection in the different regions is depending on the analyzed matrix. The highest frequency of ETU residues for apple was in the eastern region (57%), for papaya samples was in the northern region (80%) and for strawberry samples in the center (17%). No ETU residues were found in samples of apple collected in the north and also in strawberry acquired in the north, south and east. These differences should be attributed to some factors such as different sources of fruits, transport and storage conditions, and shelf life.

Table 2
Ethylenethiourea residues in apples, strawberries and papaya samples.

Fruit	> LOQ[a] N[b] (%)	Median (μg/kg)	Mean (μg/kg)	SD[c]	RSD[d] (%)	Geometric mean (μg/kg)	Range (μg/kg)
Apple	10 (33%)	0.5	1.0	0.9	90	0.8	1.0–3.7
Papaya	20 (67%)	1.6	1.3	1.2	92	1.2	1.0–5.3
Strawberry	2 (7%)	0.5	0.5	0.2	40	0.5	1.0–1.4
Total	**32 (35%)**	**0.5**	**0.8**	**1.0**	**125**	**0.8**	**1.0–5.3**

[a] LOQ: limit of quantification.
[b] N: number of samples.
[c] SD: standard deviation.
[d] RSD: relative standard deviation.

3.4. Risk assessment of exposure to ETU and dithiocarbamates (CS2) by food intake

Estimated intake of ETU residues for São Paulo population, considering *per capita* annual consumption and the highest level found in this study for apple, papaya and strawberry represented, respectively, for adults, teenager and children 0.05, 0.05 and 0.09% of the ADI established by FAO/WHO (1994) and 0.9, 1.1 and 1.6% of the chronic oral dose including non-carcinogenic effect for sensitive population subgroups described by the USEPA (1998) (Table 3).

The MDI of ETU obtained in this study were below that the ADI established by FAO/WHO (1994). However, the population exposure to ETU by the consumption has the contribution of several food products and other components, such as the residues of ETU formed before the ingestion, due to industrialization process or in the field; the remnants of the application of EBDC in crops; the ETU formed by processing or by preparation and cooking of food with EBDC residues; and/or the ETU metabolized in the body after ingestion.

In this study, the risk of exposure to dithiocarbamates residues was also estimated, considering the results published by ANVISA Program from 2001 to 2010 (ANVISA, 2008; 2009; 2010; 2011). Residues of dithiocarbamates were found in 752 (68.1%) of apple, 580 (55.2%) of papaya, 212 (19.9%) of strawberry and in other 15 food products, including 622 (50.4%) of tomato, 323 (32.1%) of lettuce, 36 (27.9%) of cabbage, 74 (23.8%) of pepper, 29 (17.6%) of grape, 107 (17.1%) of carrot, 36 (12.8%) of cucumber, 36 (11.4%) of beet, 90 (7.2%) of orange, 57 (6.6%) of banana, 14 (5.2%) of pineapple, 7 (4.3%) of beans, 18 (1.9%) of potato, 3 (1.9%) of mango and 2 (1.2%) of rice samples, with levels ranging from 0.01 to 7.60 mg/kg of dithiocarbamate in CS_2 (Table 4).

The contribution to the risk by consumption of food containing residues of dithiocarbamates, whereas the highest values found in the products (lettuce, pineapple, rice, bananas, potatoes, beets, carrots, cabbage, beans, oranges, apples, papaya, mango, strawberry, cucumber, pepper, tomato, grape) monitored by PARA and the *per capita* food acquisition for urban population represented 19.2% of the ADI (mancozeb) for adult, 22.8% for teenager and 34.0% for children. However only 18 of the 41 crops for which dithiocarbamates are authorized in Brazil were analyzed by PARA in the period between 2001 and 2010 (Table 4).

It is important to highlight that the Brazilian legislation establishes MRL for dithiocarbamates in CS_2 and the data available from Brazilian monitoring programs are reported in CS_2. Considering that ETU is formed only from EBDC, the results of dithiocarbamates in CS_2 could not be used for the estimation of the EBDC levels, because any dithiocarbamate residues results in CS_2 by the analytical methods. However, almost all of the authorized dithiocarbamates for use on fruits and other vegetables in Brazil belong to the class of EBDC, being mancozeb the main compound (ANVISA, 2013). Then, it was possible to estimate the risk of exposure by intake through the results reported by the Brazilian monitoring programs in recent years, considering that the CS_2 was derived from the use of mancozeb (authorized for most fruits and vegetables in Brazil), that is, as an indicative of the exposure to EBDC (Table 4).

Considering that the risk to human health do not only includes the exposure by dietary but also by drinking water and

Table 3
Risk assessment estimated of exposure to ethylenethiourea (ETU) by food intake.

Fruit	Mean value (μg/kg)	Highest value (μg/kg)	Food consumption[a] (g/person/day)	Estimated risk (mean) (% ADI[b]) (% of DRF[c])			Estimated risk (highest value) (% ADI[b]) (% of DRF[c])		
				Age group[d] (years)					
				Adult 20–75	Teenager 13–19	Child 10–12	Adult 20–75	Teenager 13–19	Child 10–12
Apple	1.0	3.7	5.504	**0.01**	**0.01**	**0.01**	**0.02**	**0.02**	**0.03**
				0.09	0.11	0.18	0.36	0.43	0.65
Papaya	1.3	5.3	5.827	**0.01**	**0.01**	**0.01**	**0.03**	**0.03**	**0.05**
				0.14	0.16	0.24	0.56	0.66	0.99
Strawberry	0.5	1.4	0.367	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.01**
				0.00	0.00	0.00	0.01	0.01	0.01
		Total		**0.02**	**0.02**	**0.02**	**0.05**	**0.05**	**0.09**
				0.23	0.27	0.42	0.93	**1.10**	1.65

[a] Average of fruit consumption *per capita* for São Paulo city, according to IBGE (2012a).
[b] ADI: Acceptable Daily Intake of 2 μg/kg bw/day (FAO/WHO, 1994).
[c] DRF: oral dose for chronic non-carcinogenic effect of 0.08 μg/kg bw/day (USEPA, 1998).
[d] For risk estimates were considered the median body weight of 69 kg for adult (20–75 years), 58 kg for teenager (13–19 years) and 39 kg for child (10–12 years) (IBGE, 2012b). Results in "bold": estimated risk in % of ADI and results in "normal letter": estimated risk in % of Drf.

environmental sources, so the real risk of exposure to ETU is the sum of all these sources. The USEPA has considered the carcinogenic risk of 1.0 μg L^{-1} for ETU in water (USEPA, 1998). In the Brazilian legislation, a limit for dithiocarbamates or ETU residues in water intended for human consumption is not established.

Despite of the improvements, in recent years, related to the information on pesticide residues in food, through monitoring programs, there are still a lack of data concerning to the food consumption for important risk subgroups, such as vegetarians, infants, children and pregnants. For example, it should be

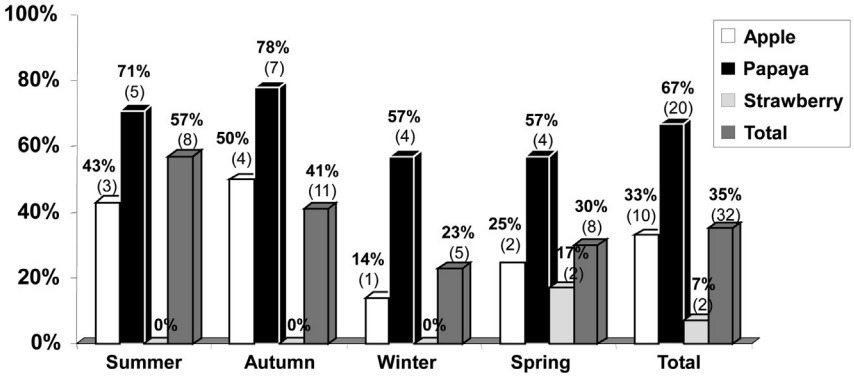

Fig. 2. Frequency of ethiylenethiourea residues in fruit samples collected in São Paulo city (SP, Brazil), in different seasons.

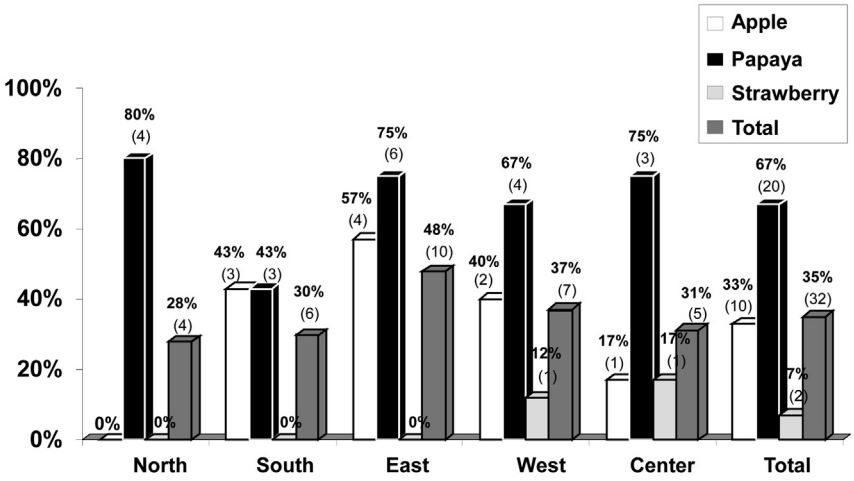

Fig. 3. Frequency of ethiylenethiourea residues in fruit samples collected in different regions of São Paulo city (SP, Brazil).

Table 4
Risk assessment estimated of exposure to dithiocarbamates in CS_2 by food intake.

Food	Frequency residues[a] *N*[b] (%)	Highest value[a] (mg/kg) CS_2	Food consumption[c] (g/person/day)	Estimated daily intake expressed in mancozeb[d] (μg/kg bw/day)	Estimated risk (highest value expressed in mancozeb) (% of ADI[e])		
					Age group[f] (years)		
					Adult (20–75)	Teenager (13–19)	Child (10–12)
Apple	752 (68.1%)	3.04	6.430	0.577	1.67	1.98	2.96
Papaya	580 (55.2%)	3.97	6.320	0.742	2.15	2.56	3.80
Strawberry	212 (19.9%)	2.15	0.468	0.028	0.09	0.11	0.14
			Subtotal 1	**1.347**	**3.91**	**4.65**	**6.90**
Banana	57 (6.6%)	2.97	21.769	1.912	5.54	6.59	9.80
Bean	7 (4.3%)	0.13	22.147	0.083	0.25	0.30	0.44
Beet	36 (11.4%)	0.48	1.358	0.019	0.05	0.05	0.09
Cabbage	36 (27.9%)	6.85	0.860	0.174	0.50	0.59	0.89
Carrot	107 (17.1%)	2.16	4.556	0.291	0.83	0.99	1.49
Cucumber	36 (12.8%)	0.46	1.326	0.018	0.05	0.05	0.09
Grape	29 (17.6%)	1.14	2.279	0.076	0.21	0.25	0.39
Lettuce	323 (32.1%)	7.60	2.449	0.550	1.58	1.88	2.82
Mango	3 (1.9%)	0.19	2.679	0.142	0.05	0.05	0.07
Orange	90 (7.2%)	1.66	16.016	0.781	2.27	2.70	4.03
Pineapple	14 (5.2%)	0.33	4.276	0.041	0.11	0.12	0.21
Pepper	74 (23.8%)	2.31	1.665	0.106	0.32	0.39	0.57
Potato	18 (1.9%)	0.43	16.008	0.202	0.58	0.69	1.05
Rice	2 (1.2%)	0.08	65.830	0.154	0.44	0.53	0.80
Tomato	622 (50.4%)	2.17	13.931	0.852	2.49	2.95	4.40
			Subtotal 2	**5.401**	**15.28**	**18.13**	**27.14**
			TOTAL	**6.748**	**19.19**	**22.78**	**34.04**

[a] Computed by the highest values of the dithiocarbamates in CS_2 obtained in the Brazilian Health Surveillance Agency (ANVISA) Pesticide Residues Food Monitoring Program (PARA) (ANVISA, 2008, 2009, 2010, 2011).
[b] *N*: number of samples.
[c] Average of food consumption *per capita* for São Paulo city, according to IBGE (2012a).
[d] Considering that all CS_2 was derived from the use of mancozeb.
[e] ADI: Acceptable Daily Intake of 0.03 mg/kg bw/day for mancozeb, according FAO/WHO (1994).
[f] For risk estimates were considered the median body weight of 69 kg for adult (20–75 years), 58 kg for teenager (13–19 years) and 39 kg for child (10–12 years) (IBGE, 2012b).

considered that infants consume fresh fruits and vegetables cooked and processed from the first months of life and the risk to health from exposure to ETU or other toxic substance by food will be higher when compared with other age groups. Furthermore, infants have reduced metabolic capacity to the elimination of toxic compounds and they are more susceptible to exposure than adults because they have lower body weight and active developmental processes (Au 2002), therefore the knowledge on food consumption data for the different age groups are very important.

4. Conclusions

The studied method for determining residues of ETU by LC–MS/MS was fitness for the purpose of the ETU monitoring in fruits.

Dietary exposure to ETU and EBDC is a health concern due to the toxicological effects of these compounds. This work presents the determination of residue levels of ETU in apples, papaya and strawberry, and represents an important contribution to estimate the risk to the health for consumer population.

In addition, continuous monitoring programs are needed to know the chronic exposure to pesticide residues and to subsidy the regulators in order to ensure the quality of food products and to improve the population health.

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