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Dithiocarbamate Fungicides and Ethylenethiourea Residue Levels in Tomato and Sweet Pepper from Markets: Kirinyaga and Nairobi Counties, Kenya

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Abstract:

Vegetables are a major source of essential nutrients such as minerals, proteins, energy and also a source of roughage. Some vegetables such as tomato and sweet pepper can be eaten raw or cooked. These vegetables are affected by pests and diseases that are controlled using pesticides among which are fungicides such as dithiocarbamates (DTCs) that are toxic to human and animals at high concentrations. DTCs are commonly used in different combinations in Kenya and one of their metabolites ethylenethiourea (ETU) a carcinogenic compound has a long residual time of between five to ten weeks. This study determined the residue levels of propineb, mancozeb and its metabolite ETU on tomato (*Lycopersicon esculentum* Mill) and sweet pepper (*Capsicum annum* L) during wet and dry seasons. The analyte was extracted from the vegetable using acetonitrile-dichloromethane-chloroform mixture and analyzed using HPLC together with method validation. The method of analysis had R^2 values (0.948-0.999) and recoveries ranging from 91.26-95.89 %. The levels of mancozeb in tomato were ranging from 2.56 ± 0.12 mg/kg in the wet season to below detection limit (BDL) in the dry season while in sweet pepper the levels ranged from 2.69 ± 0.57 mg/kg in wet season to 0.16 ± 0.00 mg/kg in dry season. Propineb levels ranged from 3.97 ± 0.50 mg/kg in wet season to BDL in dry season in tomato while in sweet pepper the range was 6.54 ± 0.72 mg/kg in wet season to BDL in dry season. The ETU levels in tomato samples were significantly higher in dry season than wet season and ranged from 27.94 ± 0.39 mg/kg to BDL in wet season while in sweet pepper the levels ranged from 8.88 ± 1.55 mg/kg in dry season to BDL in wet season. Two out of four markets had propineb residues in tomato above maximum residue limit (MRL) set by WHO/FAO and EU of 3 mg/kg while there were no levels of mancozeb above MRL. ETU mean residue levels exceeding the MRL of 0.05 mg/kg were noted in all vegetable samples. The results from this study indicates high residue levels of mancozeb, propineb and ethylenethiourea with significantly higher levels of dithiocarbamates in wet season and significantly higher levels of ETU in dry season.

Keywords: Dithiocarbamates, ethylenethiourea, residue, Vegetables, seasons, Kenya

1. Introduction

Vegetable production is one of the major branches of horticulture. Vegetables are considered as an asset providing income to the growers and providing a vital part of human diet. Vegetables such as tomato and sweet pepper are eaten raw or cooked. Tomato (*L. esculentum*) and Pepper (*Capsicum annum*) originated from South America. The vegetables are affected by various fungal diseases such as leaf spot (*Septoria lycopersici*), powdery mildew (*Leveillula taurica*), late blight (*Phytophthora infestans*), early blight (*Alternaria Solani*), Anthracnose or ripe fruit rot caused by (*Colletotrichum capsici*) to a lesser degree and mainly (*Colletotrichum leosporioides*) among others and hence are usually sprayed for protection or for curative purpose. Dithiocarbamates are commonly used fungicides to control such diseases. They are a group of organosulphur compounds that exist as strong complexes with various metal ions in a polymeric form and include ethylenebis-dithiocarbamates (EBDCs) such as mancozeb, and propylenebis-dithiocarbamates (PBDCs) like propineb. In contemporary agriculture over 30% of the produce does not reach the consumers market, and without use of chemical agents the loss would be doubled (Gupta and Thind, 2006) and yield could drop by as much as a third while food prices can increase by as much as 75% (Yadav, 2010). World health organization (WHO) and other bodies have set maximum residue levels on vegetables when consumed, however farmers may apply more than the recommended pesticide quantities especially during wet seasons while some may not even wait for Post-Harvest Interval (PHI) based on demand. During the wet seasons a lot of rain may wash off some fungicides affecting the levels while during dry seasons some fungicides may degrade due to high temperatures. Metabolites of fungicides such as dithiocarbamates which are used in vegetables have been shown to cause cancer and high toxic residues of the fungicides are injurious to the environment (Lopez *et al.*, 2012). Dithiocarbamates and the metabolites especially ethylenethiourea (ETU), have thyroid effect, are neurotoxic, mutagenic and teratogenic hence suspected to have carcinogenic effects on test animals. Some other studies

have shown that ethylenethiourea (ETU) has a half-life of 1-7 days in the environment, a long half-life in the soil of between 5-10 weeks and can enter through placental barrier hence affecting the unborn by increasing tumor development (Shukla *et al.*, 2001). Mancozeb is a cholinesterase inhibitor which affects the nervous system and also causes spontaneous abortions in Rabbits at a dose of 80 mg/kg/day (Kroes *et al.*, 2004). In Kenya cancer is ranked third as a cause of death, which is 7% of total mortality yearly. Globally there are over 18.1 million new cases of cancer with over 9.6 million deaths translating to over 13% of total deaths (Freddie *et al.*, 2018). Some of the cancer cases may arise from diets containing carcinogenic compounds like ethylenethiourea. The main aim of the study was to investigate the levels of dithiocarbamates (propineb and mancozeb) and ethylenethiourea in tomato and sweet pepper vegetables obtained from different markets and seasons.

2. Materials and Methods

2.1. Sampling and Sample pre-treatment

Vegetable samples which were analyzed for ETU and dithiocarbamates (DTCs) were obtained in quantities of one kilogram per seller from six different sellers per market. They were pooled together and then one kilogram taken as a sample from the market. Three samples of each vegetable were studied from each of the four markets during dry and wet season. The markets were (Githurai and Wakulima); Nairobi County, within latitudes 1° 13'4, 65'S to 1° 17'15, 21'S and longitudes 36° 55'13,18°E to 36° 49'55,99°E respectively and (Kiamutugu and Kutus); Kirinyaga County, within the latitudes 0° 35'42, 91'S to 0° 28'3, 15'S and longitude 37° 12'30,33°E to 37° 23'21,84°E. Figure 1 shows some tomato and sweet pepper used in the study. Samples were collected in the morning hours then packed in polythene bags, transported the same day in a cold box and stored in a refrigerator (deep freezer) awaiting extraction.

Figure 1: Tomato from Kiamutugu Market and Sweet Pepper from Githurai Market

2.2. Cleaning of Glass Apparatus

Glass ware was soaked for 10 hours using fresh hydrochloric acid, then soaked in distilled and de-ionized water for 8 hours, rinsed with fresh distilled and de-ionized water then finally dried in an oven at temperatures of 100°C.

2.3. Chemicals and Reagents

All chemicals and reagents used in the study were analytical grade and above, they included;

- Analytical standard ethylenethiourea, mancozeb, propineb (purities; 98%,96.8% 103%) respectively from Pestanal
- Acetonitrile, chloroform, dichloromethane and methanol HPLC grade, from Chemoquip limited (Nairobi Kenya)
- sodium dodecyl sulfate, HPLC grade, Kobian Kenya limited (Nairobi Kenya)
- Water (distilled and deionized), HPLC grade.

2.4. Preparations of Standards and Mobile Phases

The stock solutions of standards of propineb and mancozeb were prepared by dissolving 10 mg in 10 ml acetonitrile while ETU was made by dissolving 12.5 mg in 50 ml of acetonitrile and stored in a freezer. The mobile phases were, 95% sodium dodecyl sulphate (SDS) + 5% Acetonitrile made by adding 50 cm³ of Acetonitrile to 950 cm³ of 0.1 molar sodium dodecyl sulphate, 0.1 Molar SDS made by dissolving 57.676 g of sodium dodecyl sulfate per 2 liters, methanol and acetonitrile. Methanol and acetonitrile required no preparation.

2.5. Extraction Procedure

The vegetables (tomatoes and sweet pepper) were finely chopped separately using a knife. A 1.0-gram sub-sample was placed in a 50 milliliters beaker containing a volume of 3 milliliters of a mixture of acetonitrile, dichloromethane and chloroform in the ratio of 1:1:1. Extraction was achieved by 2 minutes of mechanical shaking. The suspension obtained was filtered through a filter paper in a Buchner funnel and rinsed with 2 ml of extractant followed by addition of 2 milliliters of methanol to filtrate. The mixture was then evaporated to dryness under a gentle stream of nitrogen gas at room temperature. Residues obtained were dissolved in 500 µl of a solution of acetonitrile and water in the ratio of 1:1. The solution was filtered through a Millipore 0.45 µm nylon syringe into sample vials then stored in a freezer before HPLC analysis (Garcinuno *et al.*, 2004).

2.6. Sample Analysis Using HPLC-DAD

The HPLC analysis was carried out at Jomo Kenyatta University of Agriculture and Technology, Department of Food Science and Technology using the instrument Shimadzu model LC-20A which had a Quaternary pump (LC-20AD), PDA detector (SPD-M20A), system controller (CBM-20A), auto sampler (SIL-20A) and column oven (CTO-10ASVP). The machine was connected to a computer with a software Shimadzu LC solution. The HPLC column used was NUCLEODUR Batch 34500033, packed with C-18 5µm particles and length 250 mm×4.6 mm internal diameter, obtained from Macherey Nagel Germany and supplied by Chemo quip Kenya limited. The mobile phases used were, 95% sodium dodecyl sulfate (SDS) + 5% acetonitrile (pump A), 0.1 molar SDS (Pump B), methanol (Pump C) and acetonitrile (Pump D). Other conditions include a flow rate of 0.9 ml/min and an oven temperature of 40°C. Quantification was by multi-wavelength monitoring done with a band width of 4nm and absorbance spectra recorded from 200-400 nm. The detector end time was 10 minutes while the LC stop time was 15 minutes. The column separation conditions were such that; the 95% sodium dodecyl sulphate (SDS) + 5% acetonitrile connected to Pump A started at 100% for 2 minutes while the pumps B, C and D were at 0%. At 2.01 minutes Pumps B, C and D were run at 30% 0.1M SDS, 33% methanol and 37% ACN up to 6.00 minutes while pump A was at 0%. At 6.01 minutes Pump A was run at 100% up to 10.00 minutes while the other three pumps were at 0%. The detector end time was 10 minutes while the LC stop time was 15 minutes.

3. Results and Discussions

The results for the validation of the methods of analysis used and discussion of the results for the study are presented in the sections that follow.

3.1. Method Validation and Recovery Studies

The methods used in this study were validated by use of calibration curves for the different standards where regression equations, correlation coefficients and limits of detection were obtained. Recovery data, limit of blank and chromatogram separation data is also provided. The calibration curves for mancozeb, propineb and ethylenethiourea were obtained. The regression equations and correlation coefficients for propineb, mancozeb and ETU are shown in Table 1

Analyte	Reg. Equation	R ²	LOD	LOB	% Recovery
Ethylenethiourea	Y=21706X+9159	0.999	0.0100	0.0085	95.89
Mancozeb	Y=2971X+5187	0.948	0.0990	0.0080	91.26
Propineb	Y=2171.7X+297.79	0.996	0.6500	0.0900	95.26

Table 1: Calibration Data and % Recovery

The R² values (0.948-0.999) correlate with other studies on same compounds is such as (Garcinuno *et al.* (2004), Gul and Buket (2008) and in Jafari *et al.* (2010). The correlation coefficients make the study appropriate for use in analysis of residue levels of dithiocarbamate fungicides and the metabolite. The R² values indicate that the established calibration curves are linear over the respective range of concentrations, explaining that more than 94.8 % correlation between concentration and responses. Recovery studies were evaluated by recovery test according to Equation 1 (David and Terry, 2008).

$$\% \text{ Recovery} = \frac{C_F - C_U}{C_A} \times 100 \quad \text{Equation 1}$$

Where C_U represents the concentration in unfortified sample; C_A is the concentration of fortification (spiking solution); C_F is the concentration determined in fortified sample after extraction. The % recoveries together with limits of detection (LOD) and limits of blank (LOB) are shown in Table 1. The percentage recovery lies within the range 91.26 % to 95.26 %. The recovery studies compare favourably with other studies where 94.15 % recovery for maneb and 96.46 % for ETU was achieved (Gul and Buket 2008), which confirms the method fit for analysis of the above parameters. The limits of blank (LOB) and limits of detection (LOD) were calculated according to Equation (2) and (3), (David and Terry, 2008).

$$\text{LOB} = \text{mean blank} + 1.645 \text{SD blank} \quad \text{Equation 2}$$

$$\text{LOD} = \text{LOB} + 1.645 (\text{SD low concentration sample}) \quad \text{Equation 3}$$

The limits of blank range from 0.09 mg/kg for propineb, 0.0085 mg/kg for ethylenethiourea (ETU) and 0.008 mg/kg for mancozeb, while the limits of detection ranged from 0.01 mg/kg for ETU to 0.099 mg/kg for mancozeb. A study done on dithiocarbamates and the metabolites obtained the limit of detection (LOD) of 0.01 mg/kg for ETU and 0.1 mg/kg for maneb (Blasco *et al.*, 2004). In another study done in Iran, the LOD of 0.1 mg/kg for mancozeb and propineb was attained, (Jafari *et al.*, 2012). The values show that the method is robust for the analysis of the fungicides and the metabolite in vegetables studied, since results are consistent with other studies. Simultaneous analysis and peak identification was done whereby the retention time for ethylenethiourea (ETU) was 3.2±0.2 minutes while that of propineb was 3.9 ±0.2 minutes and that of mancozeb was 8.7±0.2. The retention times achieved in this study were as a result of the experimental settings, equipments and procedures for this study with similarities to other studies where; using a C18 column the retention time of ETU was 3.3 minutes, maneb was 4.1 minutes while that of mancozeb was 9.1 minutes (Gul and Buket, 2008). Quantification was done by multiplying the amount obtained from the graph (using the regression equations for standard) with a dilution factor of 0.5 as shown in Equation 4.

$$Q(\text{mg/kg}) = \left(\frac{P-C}{M} \right) \times 0.5$$

Equation 4

Where **Q** (X in equation) is the levels in mg/kg, **P** is the peak area (Y) from the table; **C** is the Y intercept while **M** is the gradient of the regression equation.

3.2. Residue Levels in Vegetables during Dry and Wet Seasons

Tomato and sweet pepper samples from different markets during wet and dry seasons were analyzed for propineb, mancozeb and ETU. The results are discussed in the following sections.

3.2.1. Residue Levels of Propineb

The results showing mean residue levels of propineb are presented on Table 2.

Market	Tomato (mg/kg) n=3		Sweet Pepper (mg/kg) n=3	
	wet	dry	wet	dry
Wakulima	3.15±0.08 ^{Cd}	BDL	BDL	BDL
Githurai	0.83±0.05 ^{Ab}	1.91±0.08 ^C	BDL	2.91±0.06 ^{Ac}
Kutus	3.97±0.50 ^{dE}	BDL	0.99±0.13 ^{aB}	0.87±0.04 ^{Bb}
Kiamutugu	1.48±0.87 ^{Bc}	BDL	6.54±0.72 ^{bD}	1.09 ^{cC}
P-values	< 0.001	< 0.001	< 0.001	< 0.001
LSD	0.04	1.00	0.03	1.00

Table 2: Mean Levels of Propineb in Vegetables during Dry and Wet Seasons

From Table 2, propineb residue in tomato ranged from 0.83±0.05 to 3.97±0.50 mg/kg during the wet season with tomato samples from Kutus market having the highest level and Githurai market having the least. During the dry season the tomatoes from three markets had values below detection limit (BDL), however tomatoes from one market (Githurai) had propineb mean residues of 1.91±0.08 mg/kg during the wet season. Mean values followed by the same small letter within the same column do not differ significantly from one another while those followed by the same capital letter in the same row do not differ significantly. Tomatoes from different markets showed significant difference in the residue levels of propineb. The results also show that tomato samples obtained during the dry season gave the highest number of results below detection limit. This can be explained from common agricultural practices, whereby frequency of spray is increased in wet season but decreased in dry season. The propineb residue on sweet pepper ranged from BDL to 6.54±0.72 mg/kg during the wet season with Kiamutugu market pepper samples having the highest levels at a mean of 6.54±0.72 mg/kg. During the dry season propineb residues on sweet pepper ranged from BDL to 2.91±0.06 mg/kg with the sweet pepper samples from Githurai market having the highest level. The residue on sweet pepper vegetable differed significantly on comparing Kutus market and Kiamutugu market during the wet and dry seasons and also Githurai and Kutus during dry season but did not differ significantly when the dry and wet seasons were compared in Kutus market. The lack of any significant difference can be explained by low usage of the fungicide (Momanyi *et al.*, 2019). The levels of propineb on tomato samples from Wakulima and Kutus markets (3.15±0.08 and 3.97±0.5 mg/kg, respectively) and sweet pepper samples from Kiamutugu market (6.54±0.72 mg/kg), were above the maximum residue levels (MRL) set by FAO/WHO of 3mg/kg (FAO/WHO, 2004). The results on residue levels of propineb from this study correlates with similar studies such as that of green houses and non-green houses tomatoes in Iran, which found residues in one greenhouse tomato sample above the set MRL at 3.2 mg/kg (Jafari *et al.*, 2012). Another study in Congo had tomato samples with residues of mancozeb at 4.65 mg/kg which is above the MRL of European Union and FAO/WHO (Mpiana *et al.*, 2014).

3.2.2. Residue Levels of Mancozeb

The mean residue levels of the fungicide mancozeb in tomato and sweet pepper obtained from markets during different seasons are shown on Table 3.

Market	Tomato (mg/kg) n=3		Sweet Pepper (mg/kg) n=3	
	wet	dry	wet	dry
Wakulima	BDL	0.25±0.09 ^{aA}	BDL	0.16±0.00 ^{Aa}
Githurai	1.29±0.04 ^{aB}	1.06±0.07 ^{bC}	BDL	2.21±0.86 ^{Bc}
Kutus	2.10±0.03 ^{bD}	1.60±0.19 ^{cB}	1.49±0.08 ^{aB}	2.52±0.77 ^{bC}
Kiamutugu	2.56±0.12 ^c	BDL	2.69±0.57 ^{bC}	2.13±0.27 ^{Bc}
P-values	< 0.001	< 0.001	< 0.001	0.004
LSD	0.01	0.02	0.02	0.03

Table 3: Mean Levels of Mancozeb in Vegetables during Wet and Dry Seasons

From Table 3, the mean residues of mancozeb in tomato ranged from below detection limit (BDL) to a mean of 2.56±0.12 mg/kg during the wet season and from 0.25±0.09 to 1.60± 0.19 mg/kg during dry season. Mean values followed by the same small letter within the same column do not differ significantly from one another while mean values followed by the same capital letter within same row do not differ significantly. The residues during the wet season were significantly

higher than those obtained during the dry season in Kutus market while in Kiamutugu market the wet season had high residues at 2.56 ± 0.12 mg/kg compared to BDL in the dry season. Within seasons different markets showed significant differences of mancozeb residue levels on tomato. The mean levels of mancozeb on sweet pepper ranged from BDL in two markets to a mean of 2.69 ± 0.57 mg/kg during the wet season. The sweet pepper samples obtained during the dry season had mancozeb residue values ranging from 0.16 ± 0.00 to 2.52 ± 0.77 mg/kg. Different markets had significant difference in the levels of mancozeb on sweet pepper within a season. The residues were significantly higher during the dry season than during the wet season in Kutus market and other markets with the exception of Kiamutugu. The significance can be attributed to wash off during wet season.

The results from the study indicated there were no mancozeb residues in tomato and sweet pepper which were above the MRL set by FAO/WHO of 3mg/kg. This may be attributed to cleaning or polishing to remove surface dirt in the markets before selling and also the high decomposition rate of mancozeb in the environment (Olaniyi *et al.*, 2010). The study findings have some uniqueness and also compare favorably with those obtained from other studies, such as findings done in Turkey Mehmet *et al.* (2014) and Brazil Caldas *et al.* (2004).

3.2.3. Mean Levels of Ethylenethiourea

The mean residue levels of the metabolite ethylenethiourea in both vegetables obtained from various markets are shown on Table 4.

Market	Tomato (mg/kg) n=3		Sweet Pepper (mg/kg) n=3	
	Wet Season	Dry Season	Wet Season	Dry Season
Wakulima	BDL	21.99 ± 5.19^{bc}	BDL	8.88 ± 1.55^{bd}
Githurai	8.52 ± 1.25^{bb}	9.30 ± 1.82^{ab}	BDL	0.65 ± 0.16^{aA}
Kutus	9.36 ± 0.78^{bb}	27.99 ± 0.67^{bd}	0.56 ± 0.24^{ab}	1.63 ± 0.03^{bc}
Kiamutugu	1.81 ± 0.21^{aA}	BDL	7.23 ± 1.24^{bc}	1.53 ± 0.04^{bd}
P-values	< 0.001	0.001	< 0.001	< 0.001
LSD	0.02	0.03	0.39	0.16

Table 4: Mean Levels of Ethylenethiourea in Vegetables during Different Seasons

From Table 4 the residue levels of ethylenethiourea (ETU) in vegetables ranged from below detection limit (BDL) to 27.99 ± 0.67 mg/kg. The tomato samples had residue levels ranging from below detection limit (BDL) to 9.36 ± 0.78 mg/kg in the wet season and BDL to 27.99 ± 0.67 mg/kg in the dry season with tomato from Kiamutugu having lowest positive value at 1.81 ± 0.21 mg/kg while Kutus had the highest mean at 9.36 ± 0.78 mg/kg in the wet season. In the dry season the lowest positive value was at 9.30 ± 1.82 mg/kg from samples obtained at Githurai market. Mean values followed by the same small letter within the same column do not differ significantly from one another while mean values followed by the same capital letter within same row do not differ significantly. The residue levels of ethylenethiourea (ETU) in tomato obtained during the dry season were significantly higher than those found during the wet season in Kutus market and Wakulima market with no significant difference in Githurai. The high mean residues in dry season is attributed to environmental decomposition of dithiocarbamates and also accumulation of ETU from spraying due to the ETU long lifetime in the environment among other reasons, while the low levels during wet season is attributable mainly to the high-water solubility of ETU at 1000 mg/l at 20 °C (Pallavi and Ajay., 2013). Mean residue levels from different markets differed significantly during dry season while during the wet season, only Kiamutugu and Kutus markets did differ significantly.

Sweet pepper had ETU residues above detection limit in only two markets from Kirinyaga County during the wet season with quantities of 0.56 ± 0.24 mg/kg at Kutus market and 7.23 ± 1.24 mg/kg at Kiamutugu market which differed significantly. During the dry season the mean residues of ETU in sweet pepper were 0.65 ± 0.16 mg/kg from Githurai market to 8.88 ± 1.55 mg/kg in Wakulima market. The residues from Wakulima market differed significantly from the ones from three markets while those from other markets had values which did not differ significantly from each other. The residues of ETU on sweet pepper did differ significantly between the different seasons in Kiamutugu market and Wakulima while in Kutus no significant difference. The results show there are high residue levels of ETU and correlate with some studies such as Gul and Buket (2008) where residue levels of ETU were noted in two tomato juices samples obtained from Istanbul Turkey at 0.08 and 0.11 mg/kg respectively. The mean residues in the vegetables were above the maximum residue limit (MRL) of 0.5 mg/kg set by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) (FAO/WHO., 2013). The chromatograms of 2 samples showing all study compounds in vegetables used are shown on Figure 3 and 4.

Peak Table

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.117	436399	22457	7.935	5.037
2	3.756	4039	16583	0.073	3.720
3	6.454	3888442	315414	70.704	70.749
4	7.138	1043299	76626	18.970	17.188
5	7.856	114817	7552	2.088	1.694
6	8.709	12606	7187	0.229	1.612
Total		5499602	445819	100.	100.

Figure 2: A Chromatogram of Tomato Sample from Githurai Market

The chromatogram shows levels of ethylenethiourea (ETU), Propineb and mancozeb at peaks 1, 2 and 6 respectively.

Figure 3: A Chromatogram of Sweet Pepper Sample from Kiamutugu Market

The chromatogram shows levels of ethylenethiourea (ETU), Propineb and mancozeb at peaks 4, 5 and 13 respectively.

4. Conclusion

The results from this study indicate that tomato analyzed from the Kenyan markets had some residue levels of pesticides exceeding the recommended MRL. The residue levels of mancozeb and propineb were higher during wet season than during dry season while ETU residue levels were higher during dry season than during wet season; which can be due to thermal degradation of mancozeb and other EBDCs which yield ETU as a metabolite or as an impurity. The Farmers, vendors and consumers should wash the vegetables properly while regulatory bodies should uphold regular surveillance on residue levels at all levels in the consumer chain.

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