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Research

Determination of Rotenone in Soy Milk

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Rotenone is a broad-spectrum pesticide and piscicide that inhibits the transport of electrons in NADH-Q-oxidoreductase (complex I), thereby inhibiting cellular respiration. For the past two decades, epidemiologists have suspected that exposure to rotenone increases the risk of developing Parkinsonism. Although restricted for food use in the United States, rotenone is still used in countries exporting produce. Research has determined rotenone's presence in produce as well as processed food products, such as tea and olive oil, raising the concern that it does not degrade before and during food processing. Furthermore, there are documented issues with the organic certification of imported produce into the United States, including soybeans. Organic and non-organic national brands of soy milk were purchased from a supermarket in the Greater Cleveland Area, and two samples were analyzed in triplicate for rotenone using high-performance liquid chromatography equipped with an ultraviolet light detector (HPLC-UV). A positive determination would indicate that rotenone is not degraded during the process that turns soybeans into milk. The samples were analyzed and compared to samples intentionally spiked with rotenone before they were filtered and dissolved in acetonitrile in preparation for the detection by HPLC- UV. Rotenone was not detected in these samples. However, a reliable method was developed for filtering soy milk and analyzing it for rotenone residues. The method detection limit allows for the determination of rotenone residues in soybeans beyond the maximal residual limit fixed by the European Union.

INTRODUCTION

Rotenone is an isoflavone found in the roots and stems of several plant species of the Fabaceae family (Figure I). It has been used as a natural piscicide and pesticide since the nineteenth century. Rotenone inhibits the transport of electrons in NADH-Q-oxidoreductase, thereby inhibiting cellular respiration (Singer and Ramsay, 1994). Its chemical isolation around the turn of the twentieth century from the roots of Derris elliptica and Derris chinensis led to widespread commercial and domestic applications (Ujváry, 2010).

Ujváry (2010) lists the many studies on rotenone, including those that investigate its action on insects, its metabolization in mammals and fish (Fukami et al., 1969), its carcinogenicity and cytotoxicity, as well as its implication in the development of Parkinson's Disease (PD). An early study linking rotenone and PD noted that the stereotaxic administration of rotenone to the brain of mammals resulted in damage to nerve cells in substantia nigra (Heikkila, 1985). A more recent study has determined that intravenous administration of rotenone to rats leads to impaired activity of mitochondrial complex I throughout the brain and to the development of Parkinsonian behavior (Betarbet, 2000; Sherer, 2003). Epidemiological studies also suggest a link between rotenone and PD (Chen and Ritz, 2018). In a statistical analysis of data related to an ongoing project on health in the agricultural industry, farmers who were known to have applied rotenone in fields between 1993 and 1997 were more prone to develop PD in comparison to those who had not. This "provided strong evidence of the association between rotenone use and PD in humans" (Tanner, 2011). Similarly, human populations living near cultivated fields also show a statistically higher rate of PD (Dorsey, 2020).

In the United States the agri-food industry has voluntarily banned rotenone since 2007 after the Environmental Protection Agency issued a data call-in of its studies on the inhalation neurotoxicity of the pesticide (EPA 2007). Federal regulations have outlawed its use for organic farming since 2018 (ECFR 2018). It is still employed, however, in many countries that export produce to the United States. While some marine biologists invoke Fukami's research on the metabolism of rotenone in the 1960s as evidence of the limited risks of ingesting rotenone (Robertson and Smith-Vainz, 2008), researcher who have conducted more recent studies

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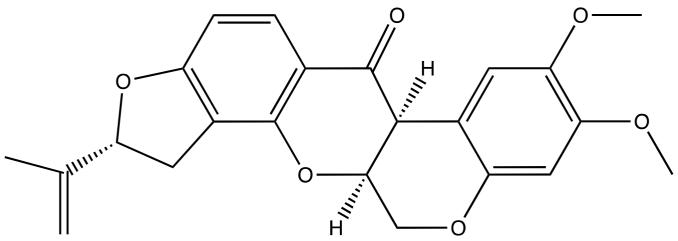

Figure 1. Chemical Structure of Rotenone. The rings in the structure provide the required conjugation for rotenone to be detected by the HPLC UV sensor.

on rotenone's metabolism have called for further assessment of "pesticide exposure from food" (Dorsey, 2020). While there is a link between gastric administration of rotenone and the progression of PD (Pan-Montoyo, 2010), more research is necessary on the ingestion of food containing rotenone and its presence in the gut. Researchers have developed protocols for analyzing the presence of rotenone in food (Choubbane, 2022; Kang and H, 2016; Moore, 2000), tools which will be useful for future epidemiological studies. In Italy, researchers found rotenone residues in olives and in olive oil at nearly three times the maximal residual limit fixed in Italy at the time, despite applying the pesticide and harvesting the olives according to official guidelines (Cabras, 2002) In China, researchers designing an experimental model to routinely test produce for rotenone determined, in the process, residues of the pesticide in tea products (Xu, 2010). These findings suggest that rotenone is not completely degraded during the transformation of produce into a product.

Furthermore, there are reports of longstanding difficulties in the certification of organic products imported from countries where rotenone is still used in non-organic farming. In 2017, the Washington Post reported examples of fraudulent organic certification of massive shipments of grains and legumes transiting to the United States from Ukraine via Turkey, including 36 million tons of soybeans treated with aluminum phosphide, of which 21 million tons had been distributed to consumers before the fraud was discovered. This shipment is part of a larger trend in the importation of soybeans, which has seen an increase of imports transiting via Turkey between 2014 and 2016, from 14,000 tons to 165,000 tons. Organic grain and legumes are not only imported from Ukraine but from over 100 countries, many of which have not banned rotenone for agricultural use (Whoriskey, 2017) .

Given the confirmed presence of rotenone in processed products like tea and olive oil, as well as the reported difficulties in the organic certification of non-domestic soybeans, the present experiment's goal was to develop a method for determining rotenone in another processed product, soy milk. A positive determination would indicate that rotenone is not degraded during the process that turns soybeans into milk. It could also suggest that irregularities in the organic certification of imported soybeans or the leaching of rotenone into organic fields could lead to the contamination of the supply of organic soy products. This research was approved by, and conducted under the supervision of, Dr. Anne O'Connor, principal supervisor of the REEL Lab in the Chemistry Department of Cleveland State University, Cleveland, Ohio.

MATERIALS AND METHODS

Experimental Design

National brands of organic and non-organic soy milk were purchased from a supermarket in the Greater Cleveland Area. As rotenone is not readily soluble in water but is soluble in lipids (Ujváry, 2010), it should be more likely to be found in the fatty acid content of soy milk compared to in its aqueous content. Given the high melting point of rotenone (176°C), the samples and spiked samples were first dried at 25°C in a dark oven over several days, as rotenone degrades upon irradiation. The dried fatty matter of the milk was then dissolved in acetonitrile, as rotenone is highly soluble in this solvent, and there is a long-held protocol to determine rotenone using acetonitrile in the organic phase of high-performance liquid chromatography with an ultraviolet detector (HPLC-UV) (Smith, 1994).

Reagents and Standards

Analytical grade rotenone was purchased from Millipore Sigma (201-501-9) (Sigma-Aldrich, Switzerland). HPLC-grade acetonitrile and Milli Q Water for HPLC were used in this experiment. Stock solutions of 2% rotenone were made in acetonitrile.

Preparation of Standard Solutions

Three sets of standards were serially diluted from a stock solution of 2% rotenone and acetonitrile, using analytical grade and HPLC-grade materials from Millipore Sigma. The standard solutions had known rotenone concentrations ranging from 0.0571 to 3.6512ppm. Solutions were freshly prepared for HPLC-UV analysis to avoid rotenone degradation and were stored in dark, sub-zero conditions.

Sample Preparation

Three 10mL samples were acquired for organic soy milk, non-organic soy milk, spiked, organic soy milk and spiked, non-organic soy milk were dried at 25°C in a dark oven for four days. The mass of the dried milk samples was determined by weighing beakers before and after dehydration. The samples were then dissolved in 10mL of acetonitrile, and dried particles were crushed with a

pestle. All samples were placed on a stirring plate for 20 minutes before dehydration and after dissolution in acetonitrile. Samples were separated by vacuum filtration, centrifugation and syringe filtration, and the supernatant was analyzed using data collected from the HPLC-UV device.

Liquid Chromatography

Three sets of standards, six samples and six spiked samples were analyzed for rotenone using a Shimadzu HPLC-UV device, or high-performance liquid chromatography device coupled with an ultraviolet light detector set at 290nm and 299nm wavelengths. The separation of rotenone was achieved in a C_{18} column (150mm X 4.6mm X 4μm, ThermoFisher Thermo Scientific Accucore XL). Mobile phases were 50:50 water (A) and acetonitrile (B).

EXPERIMENTAL SETUP

The serial dilutions of 2% stock of rotenone in acetonitrile (Table I) were analyzed by HPLC-UV to calculate their coefficient of determination (Figure II).

A linear equation was determined by plotting the HPLC areas over the known concentrations of the standards (Figure II). The method of detection and quantification limits were then calculated by using this linear equation.

Table 1. Concentration of Rotenone in Standard Solutions (ppm)

1	2	3	4	5	6	7
0.0571	0.1141	0.2282	0.4564	0.9128	1.8256	3.6512

First, the concentrations (x) of seven HPLC-UV readings of the area (y) of the standard with the lowest concentration (0.0571 ppm) were calculated. Second, the average of these concentrations was calculated and subtracted from each individual value. Third, these latter values were squared, averaged and divided by the number of samples minus one (n-1) before taking their square root (Figure III, Tables II & III). Having thereby obtained the standard deviation of the HPLC readings of the most diluted standard, this value was then multiplied by 3.143, which corresponds to the student's t-value for a 2% dilution, to obtain the method detection limit (Table III) (Csuros, 1997).

The determination of rotenone in the organic soy milk was carried out on the HPLC-UV device in triplicate (samples 1 to 3), as was its determination in non-organic soy milk (samples 4 to 6) (Table IV). The detection of rotenone in the spiked samples was carried out in the same fashion (Table V). The percent recovery of the rotenone in the spiked samples was calculated by dividing the amount of rotenone recovered over the theoretical amount of rotenone in (or the amount pipetted into) these samples (Table VI).

Table 2. Standard Deviation of Most Diluted Standard (0.0571 ppm)

Trial	HPLC-UV Area	Concentration (ppm)	Deviation	Squared Deviation
1	1655	4.26e-2	-7.38e-3	5.45e-5
2	1676	4.35e-2	-6.45e-3	4.17e-5
3	1523	3.67e-2	-1.32e-2	1.75e-4
4	1733	4.60e-2	-3.94e-3	1.55e-5
5	1448	3.34e-2	-1.65e-2	2.73e-4
6	1408	3.17e-2	-1.83e-2	3.35e-4
7	1698	4.45e-2	-5.48e-3	3.01e-5
Average		**3.98e-2**		**1.32e-4**

Table 3. Method Detection Limit, Limit of Detection, and Limit of Quantification

Variance	Standard Variance	MDL	LOD	LOQ
2.20e-5	4.69e-3	1.47e-2	4.42e-2	4.69e-2

Table 4. Concentration of Rotenone in Samples

Soy Milk Samples	HPLC-UV Area	Rotenone (ppm)	Concentration of Rotenone (mg/g)
1*	0	0	0
2*	0	0	0
3*	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0

* Organic Soy Milk

Table 5. Concentration of Rotenone in Spiked Samples

Spiked Soy Milk Samples	HPLC-UV Area	Rotenone (ppm)	Concentration of Rotenone (mg/g)
1*	50082	2.18	2.37e-2
2*	48770	2.12	2.28e-2
3*	49272	2.15	2.31e-2
4	49864	2.17	2.62e-2
5	139009	6.11	7.45e-2
6	58018	2.53	3.17e-2

*Organic Soy Milk

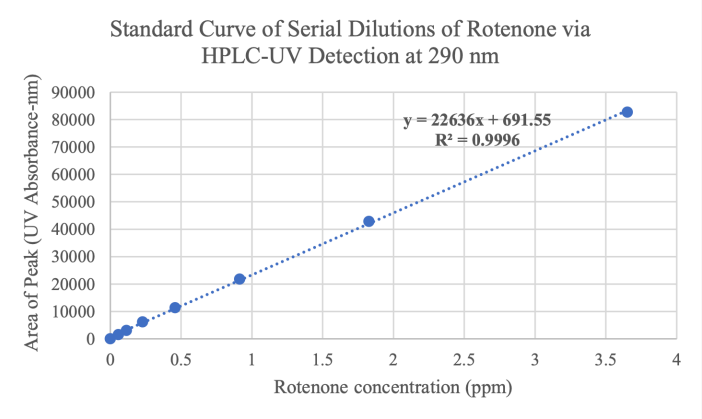

Figure 2. Plot of Peak Area vs Concentration (ppm) of Rotenone. Curve of Serial Dilutions via HPLC-UV. A 1217.08 ppm stock solution of rotenone was prepared by dissolving 23.8 mg of rotenone in 250 mL of acetonitrile. Standard solutions rangingfrom 0.0571 to 3.6512 ppm of rotenone were prepared and ran on the HPLC-UV. The coefficient of determination (R2) for the standards was 0.9996 indicating a linear relationship between the UV absorbance peak areas at 290 nm and rotenone concentrations. The equation generated from this standard curve was used to calculate the rotenone concentration in the samples.

$$s = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}}$$

Figure 3. Standard Deviation Equation. The mean of the measurements was found by calculating the square of each data point's distance to the mean, summing them, and dividing the sum by number of measurements, before taking the square root of this result.

RESULTS

Rotenone was not detected in all samples of soy milk in all three trials (Table IV).

The recovery rates of spiked samples were, for the most part, satisfactory (Table VI). The abnormal levels of recovery in samples 5 and 6 (Table VI) suggest a human error occurred during the spiking of sample 5, and that the high levels of rotenone were perhaps not completely flushed from the column when sample 6 was determined using the HPLC device.

Table 6. Recovery of Rotenone in Spiked Samples

Spiked Soymilk Samples	Rotenone in Spiked Sample (ppm)	Theoretical Rotenone Value in Spike (ppm)	Percent Recovery (%)
1*	2.18	2.43	89.79
2*	2.12	2.43	87.41
3*	2.15	2.43	88.32
4	2.17	2.43	89.40
5	6.11	2.43	251.46
6	2.53	2.43	104.22

* Organic Soy Milk

The method detection limit developed in this experiment (1.47 x 10^{-2}ppm) falls slightly short of the mark for reportable results (Table III). According to the World Health Organization's International Programme on Chemical Safety, the acceptable daily intake of rotenone, or the proposed no-adverse-response level to rotenone, is 1.4 x 10^{-2} mg/L or 1.4 x 10^{-5} mg/g (Rahde, 1990). However, the method detection limit does meet the requirements for detecting rotenone in soybeans beyond the maximal residual limit fixed in the European Union (0.02ppm), while falling slightly short of the limit fixed in Japan (0.01ppm).

During this experiment, degradation of rotenone was observed when samples were not kept in the freezer. Based on the precision of the serial dilutions of the standards, however, rotenone did not degrade during the manipulation of materials in the laboratory.

DISCUSSION

A positive determination for rotenone in the soy milk samples analyzed in this experiment would indicate that it does not degrade during the processing of soybeans into soy milk. In the case of organic soy milk, a positive determination would also raise questions about organic certification and whether rotenone is leaching into the water tables of fields designated for organic farming. Rotenone was not determined in all samples of all three trials (Table IV). These results suggest that rotenone was not used as a pesticide or, if it was, it had either degraded before or during its transformation into soy milk.

To our knowledge, there is no existing research on rotenone in soy milk. Other studies have tested fresh produce for residues of rotenone (Choubbane, 2022; Kang and H, 2016; Moore, 2000; Xu, 2010). While rotenone is known for its rapid photodegradation, Xu et al. also found rotenone residues at 0.012 and 0.016mg/kg in tea, which is slightly below the European Union's maximal residual limit (0.02 ppm) but above Japan's fixed limit (0.01ppm) (Xu, 2010). Another study on olives determined residual levels of rotenone at 0.11mg/kg, 12 days after the last application

of the pesticide in an olive grove, which was nearly three times higher than Italy's fixed maximum residual limit of 0.04 mg/kg at the time (Cabras, 2002). Furthermore, when olives from this grove were pressed, about half of the rotenone residues found its way into the olive oil. While these studies were not conducted on soybeans and soy milk, the results of this research project on olives and olive oil point to a limitation in the interpretation of the present project's results. The experimental design of Cabras (2002) and colleagues included known amounts of applied rotenone and measurements of rotenone on olives and in olive oil from products harvested at different intervals after pesticide application. Further research on rotenone residues in soybeans and soy milk would benefit from a similar experimental design; namely, comparing the residue levels of soybeans knowingly treated with the pesticide to levels found in their transformed state (soy milk). More data on the application of rotenone, its degradation and the level of residues on the soybeans and in the soy milk would provide a clearer picture of how far rotenone degrades before harvesting and during processing, or if processing does not fully degrade the pesticide.

Nevertheless, given the widespread use of the organic pesticide in countries exporting produce and processed foods, developing methods for the filtering and analysis of processed products, like soy milk, is useful in helping to determine how people might be exposed to rotenone through their food supply. The methods employed in this experiment to test soy milk for rotenone residues show promise. Despite outlying results

in the recovery rate of spiked sample mentioned above (Table VI), the overall recovery rate suggests the filtration method is effective: the drying of the soy milk, the dissolution of the dried fatty contents in acetonitrile and the different methods of filtration would not have degraded rotenone had it been already present in the samples tested. While the method detection limit developed in this experiment (0.014ppm) falls slightly short of the daily acceptable intake (Table III), it does meet the requirements for detecting the maximal residual limit fixed in Japan and the European Union. Future trials using the same method should experiment with further serial dilutions and different ultraviolet-detection wavelengths on the HPLC device to obtain more contrasting results of the peak areas. If rotenone were positively determined at slightly lower levels, the results would therefore be reportable in more countries and useful from a public health standpoint.

A larger concern informing this experiment was how exposure to rotenone in the food supply might be linked with the development of Parkinsonism. As research continues to investigate the correlations between food exposure to rotenone, its metabolism in the digestive system and the development of Parkinson's Disease (PD), the availability of reliable methods for detecting pesticides in food could be useful in calculating potential exposure to risk factors. While the scope of the present experiment cannot contribute to a discussion of what qualifies as an environmental risk factor related to PD, the methods employed can be used to gather data that would eventually quantify exposure to rotenone through soy milk.

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