Food Chemistry 131 (2012) 1531–1538

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Quantification of rotenone in seeds of different species of yam bean (*Pachyrhizus* sp.) by a SPE HPLC–UV method

E. Lautié [a,*], E. Rozet [b,1], P. Hubert [b], J. Quetin Leclercq [a]

[a] Laboratoire de Pharmacognosie, LDRI, Av. E. Mounier, 72 – Box 7203, Université Catholique de Louvain, 1200 Brussels, Belgium
[b] Laboratoire de Chimie Analytique, CIRM, Université de Liège, CHU B36 B-400 Liège, Belgium

ARTICLE INFO

Article history:
Received 20 August 2010
Received in revised form 25 May 2011
Accepted 24 September 2011
Available online 5 October 2011

Keywords:
Isoflavonoïd
Rotenone
Pachyrhizus seeds
HPLC
SPE
Method validation

ABSTRACT

This study describes the development of a validated method for the quantification of rotenone in yam bean. The milled seeds were submitted to a Soxhlet dichloromethane extraction which allowed extracting 90% of the seeds rotenone. Elimination of the lipids was obtained via solid phase extraction. Rotenone was eluted with dichloromethane/methanol and the solution dried under vacuum and solubilised directly in methanol before injection in HPLC. The whole process was realised as much as possible protected from light and at temperatures lower than 40 °C which allowed high recovery rates of spiked rotenone. Total error was used as criterion for the validation process and accuracy profiles drawn. The method allows the quantification of rotenone in yam bean seeds from 0.07% up to 1.25% (w/w). This method was applied to the quantification of rotenone in the seeds of several accessions of *Pachyrhizus erosus* and *Pachyrhizus ahipa*. The results range from 1.13 to 2.76 mg/g dry material.

1. Introduction

Due to the nutritional content of its roots and seeds as well as its agronomical yields and the resistance of the plant itself, there is a great deal of interest in widespreading the culture of the underutilized yam bean (*Pachyrhizus* sp.). Nowadays only the heavy storage roots are used as food with appreciable carbohydrates content (Noman, Hoque, Haque, Pervin, & Karim, 2007) while the seeds contain high protein and oil yields as well as several minerals and essential amino acids that could all be made available for human consumption and processing (Grüneberg, Goffman, & Velasco, 1999; Morales-Arellano, Chagolla-Lopez, Paredes-Lopez, & Barba de la Rosa, 2001; Santos, Cavalcanti, & Coelho, 1996). Nevertheless, in order to be introduced as a food crop, it is necessary to know the exact amount of potential toxic compounds, such as rotenone, of the seeds in the different species or accessions of *Pachyrhizus*.

This isoflavonoïd reported in the seeds was formerly used for its insecticidal activity especially on organic crops but it has been removed from the Annex I of Directive 91-414-CEE (European Commission, 2008) and its use is forbidden in the European Union since October 2009. Concerning its mechanisms of action, the acute toxicity of rotenone on insects is related to its capacity to block the NADH-ubiquinone oxidoreductase complex and to inhibit the mitochondrial respiratory chains. On mammals rotenone was proven to cause as well oxidative damage at the cellular level and dopaminergic neuronal loss implicated in the pathophysiology of Parkinson's disease. Subcutaneous chronic rotenone exposure of rats was set to cause dopaminergic degeneration (Sherer, Kim, Betarbet, & Greenamyre, 2003) but the detailed mechanism of action is still under investigation. Rat oral LD50 is 132–1500 mg/kg (WHO, ILO, & UNEP, 2009) and the simulated lethal oral dose for a 70 kg human is variously estimated between 10 and 200 g. Nevertheless, one fatal case has been reported in Thailand after ingestion of 100 g of seeds from *Pachyrhizus erosus* (Narongchai, Narongchai, & Thampituk, 2005) and another case of intoxication of five patients in Taiwan is described, one of them life-threatening (Hung et al., 2007).

It is thus important to release on the market seeds with known rotenone content, and develop a validated quantification method. Methods to assay rotenone are numerous in the literature with two main scopes:

(i) To find new sources, extraction methods and formulations of rotenone in stems and roots of species of *Derris, Lonchocarpus* and *Tephrosia* (Cabizza et al., 2004; D'Andrea et al., 2007; Pereira et al., 2000; Sae-Yun, Ovatlarnporn, Itharat, & Wiwattanapatapee, 2006).
(ii) To display trace analyses of this pesticide, sometimes simultaneously with other compounds, in matrices as diverse as river water (Di Donna, Mazzotti, Sindona, & Tagarelli, 2005), soils (Drozdzynski & Kowalska, 2009), olive and olive oil (Di Donna, Grassi, Mazzotti, Perri, & Sindona, 2004), fruits

* Corresponding author. Tel.: +32 2764 7234; fax: +32 2764 7293.
E-mail address: emmanuelle.lautie@uclouvain.be (E. Lautié).
[1] F.R.S.-FNRS Post-Doctoral Researcher (Belgium).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.09.125

and vegetables (Caboni, Sarais, Angioni, Garau, & Cabras, 2005; Taylor, Keenan, Reid, & Fernández, 2008) and human serum (Caboni et al., 2008).

Nevertheless, none of these methods was suitable for yam been seeds rotenone quantification as developed for the quantification of traces (rotenone used as a pesticide) or developed in matrices without high lipids contents.

Most publications in the trace analysis framework use very sensitive detectors as mass spectrometers but high performance liquid chromatography with UV detection is the accepted method as rotenone specific absorbance is high (Jimenez et al., 2000; Simeone et al., 2009).

The objective of this study was to develop and validate a simple and economical method to quantify rotenone in different samples of yam bean seeds of several *Pachyrhizus* accessions from different ecogeographical origins.

2. Experimental

2.1. Chemicals and plant material

Rotenone (⩾98%) was purchased from Enzo Life Science (Zandhoven, Belgium), methanol HPLC grade from Prolabo, VWR (Leuven, Belgium), acetonitrile HPLC grade from Fisher Scientific (Tournai, Belgium), and dichloromethane reagent grade from Sigma–Aldrich (Belgium).

Around 200 seeds of several accessions *P. erosus* (EC-533, EC-KEW and EC-041) and *Pachyrhizus ahipa* (AC-525, AC-102 FN, AC-102 FB and AC-524) were collected in Peru, milled and sent to Belgium in September 2009.

2.2. Extraction and clean-up procedure

The whole process was realised as much as possible protected from light. Regarding the extraction method, we tested the classical soxhlet as well as ultra sound extractions.

One gram dried powdered material was extracted in a soxhlet vessel of 70 mL with 200 mL dichloromethane (DCM). The appropriate time of extraction was evaluated as further described and is reported either in number of cycles of extraction (with a cycle varying from 5 to 6 min) or in the corresponding extraction time. At the end of the extraction, the solution was evaporated to dryness under reduced pressure at 35 °C and kept at −18 °C.

Successive ultrasound extractions were tested with the same ratio of powdered seeds/solvent as with the soxhlet extraction (1 g for 70 mL DCM). Between each of them the sample was filtered and re-extracted with fresh solvent. Finally they appeared laborious as well as not allowing a complete extraction after 4 times 30 min as compared to soxhlet (79% of the soxhlet value). Thus, the extraction method chosen for all analysis was the classical soxhlet extraction.

The resulting viscous crude extract of around 300 mg was reconstituted in 5.0 mL DCM and 0.5 mL was purified by solid phase extraction (SPE). Silica gel cartridges of 1 g were previously washed with methanol (MeOH) and conditioned in DCM. After application of the sample, the cartridge was eluted with 20 mL DCM and 14 mL DCM/MeOH (98:2, v/v). The DCM fraction as well as the first 4 mL of DCM/MeOH fraction was discarded, whereas the following 10 mL were collected and evaporated to dryness under reduced pressure at 35 °C and stored at −18 °C.

This fraction was then solubilised in 5.0 mL methanol on the day of the analysis, filtered with a 45 μm filter (Millipore, Brussels, Belgium) and directly injected into HPLC.

2.3. UV/MS HPLC analyses

The analysis were performed on a LaChrom Elite HPLC integrated system (Merck Hitachi, VWR, Leuven, Belgium) equipped with a L-2450 UV detector, a L-2300 oven, L-2130 autosampler and L-2130 pump all piloted by EZChrom software.

The chromatographic separation was performed on a RP-18e 250 mm × 4 mm LiChroCART® column (5 μm) equipped with a guard column. The column was eluted at a constant flow rate of 1 mL/min by a 45 min gradient of acetonitrile/water with the following steps: initial mobile phase 48:52 (v/v) during 26 min, then a 1 min gradient to 68:32 stabilized during 9 min and finally back to the initial conditions stable for another 9 min. Analysis were carried out at room temperature at a wavelength of 295 nm. The quantification was performed using the external calibration method.

The evaluation of the selectivity of the method was done checking the purity of the rotenone peak through a LC–MS system using the same separation conditions hyphenated to a LTQ-Orbitrap XL (Thermofisher Scientific, Belgium) from the UCL MASSMET platform that was used in ESI positive mode with the following conditions: a capillary temperature and voltage of respectively 275 °C and 30 V, the source voltage set to 5 kV and the tube lens voltage to 80 V. N_2 was used at sheath flow rate of 20 and auxillary flow rate of 10 (arbitrary units).

2.4. Evaluation of the extraction and the clean-up characteristics

To determine the extraction time, peak area of rotenone was determined after different numbers of cycles: 50, 60, 70, 80, 90. After each period, DCM was replaced.

The extraction yield (Ye) (the average amount of rotenone that can be extracted from the plant material) was then determined by the HPLC–UV method described for an 80 cycles extraction which correspond to 8 h extraction. The 8 h extracted residue was submitted to another 8 h extraction with DCM. The Ye was calculated as follows:

$$\text{Ye } (\%) = \frac{\text{RSP1}}{\text{RSP1} + \text{RSP2}} \times 100$$

where RSP1 and RSP2 are the peak areas of rotenone after the first 8 h extraction and the second 8 h extraction, respectively. Six extractions were used to compute the Ye ($n = 6$).

A recovery rate Rt, accounting for possible loss of the compound during the whole procedure (the soxhlet extraction and the SPE step) was also evaluated. It was calculated as follows from a plant material spiked with a known amount of rotenone (1 mg):

$$\text{Rt } (\%) = \frac{\text{RSP}_{\text{spext}} - \text{RSP}_{\text{ext}}}{\text{RSP}_{\text{sp}}} \times 100$$

Three repetitions of the samples EC-KEW and EC-533 were analysed.

$\text{RSP}_{\text{spext}}$ is the peak area of the spiked flour extract, RSP_{ext} is the peak area of the unspiked flour extract and RSP_{sp}, is the peak area of a solution of pure rotenone in methanol at the corresponding concentration.

2.5. Standard solutions

Purity-corrected fresh stock solution of rotenone was prepared the first day of each series in methanol at 250 μg/mL. Calibration standards in methanol at six concentration levels ($m = 6$) ranging from 12.5 to 75 μg/mL of rotenone were analysed three times ($n = 3$) for 3 series of experiments ($k = 3$). The stability of a methanol solution of rotenone at 0.5 mg/mL was assessed by monitoring the variation of the rotenone peak in the standard solution after

0.5, 1, 2, 3, 7, 14, 28 and 42 days (normalisation process calculation).

Another set of six calibration standards was prepared in the extract itself in order to compare the calibration in methanol and in the matrix. They were prepared by spiking an extract with the same amount of rotenone as for the methanol calibration standards. The signal from the rotenone initially present in the extract was subtracted to rotenone peaks obtained with these solutions. The number of matrix based calibration standards, series and repetitions per series were the same as for the methanolic ones ($m = 6$, $n = 3$ and $k = 3$). For each series the spiked extracts originated from a different extraction.

The validation standards were prepared in the same way as the matrix based calibration standards. The spiked extract was different from the extract used for the calibration standards in the matrix and was renewed as well for each series. Five concentrations were analysed ($m = 5$) and for each of the three series ($k = 3$), three independent samples ($n = 3$) from different SPE were analysed.

2.6. Validation of the method

The validation of the method was realised for three different series of experiments, each one lasting 3 days. In fact 60 injections were needed for each series. For each set of analysis of three replicates, the samples were taken out of the freezer 20 min before the first injection in order to limit the degradation of rotenone at room temperature. The same mobile phase was used all along one series.

In these conditions the following criteria were tested: response function, linearity, precision, trueness, accuracy, limit of detection and quantification, quantification range and matrix effect. The dilution effect was assed as well first by comparing the peak areas during the prevalidation from a pure sample or a sample diluted three times and also by comparing the rotenone concentration calculated ($n = 3$, $k = 3$).

Total error was used as decision criterion for the validation process (Hubert et al., 2004; Hubert, Nguyen-Huu, Boulanger, Chapuzet, Chiap, et al., 2007; Hubert, Nguyen-Huu, Boulanger, Chapuzet, Cohen, et al., 2007; Rozet et al., 2007). The acceptance limits were set at $\lambda = \pm 20\%$ and the minimum probability to obtain future results within these limits was set at $\beta = 95\%$. Statistical analyses were performed using the e-noval V3.0 (Arlenda, Liège, Belgium) software.

3. Results and discussion

3.1. Extraction and clean-up procedure

The development of a method of quantification of rotenone in yam bean seeds began with the optimisation of the soxhlet extraction and the evaluation of a convenient extraction time allowing both an efficient extraction and a feasible process easy to reproduce for a large number of samples.

The evolution of the peak area of rotenone in the extract in function of the number of cycles (Fig. 1) shows that after 50 cycles a large majority of the rotenone present in the plant material has already been extracted and additional number of cycles did not increase dramatically the amount of rotenone extracted.

Nevertheless, the process of extraction seems long to complete and after 80 cycles (about 7 h 30 min extraction) rotenone is still present in the residue. This may be due to the composition of the plant tissue that slows the liberation of rotenone or due to the nature of the solvent that does not enter the cells as easily as other solvents such as methanol. Moreover it can be a reason why in this case 4 successive ultrasound extractions are not sufficient for a complete extraction.

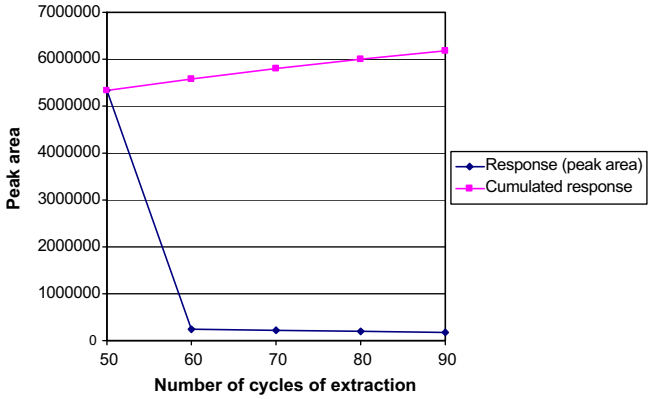

Fig. 1. Efficiency of the soxhlet extraction in function of the number of cycles (related to the extraction time).

As 8 h correspond to a working day, we decided that it was a good unit for extracting a large number of samples. These 8 h of extraction correspond to a number of cycles between 80 and 90 and comparing it with an extraction of 16 h considered as complete, it gave an extraction yield of 90.4% (standard deviation = 2.7%).

The recovery rate (Rt) was 100.1% (±12%) for the sample EC KEW and 108.8 (±14%) for EC 533. As the whole amount of rotenone is recovered at the end of the process, it could indicate that no degradation of the compound takes place in the soxhlet apparatus during the 8 h at 40 °C and that there is no loss during SPE. The important standard deviation obtained may be explained by the way this rate was calculated: a difference is calculated between two different soxhlet extractions on two different days and many factors can slightly vary between these 2 days.

The high values of Rt show that normal phase SPE on unmodified silica is suitable for rotenone extraction. When Jimenez et al. (2000) tested florisil cartridges, they faced strong interactions between rotenone and the stationary phase so they preferred reverse phase SPE (with octadecyl silica) for which Rt ranged from 78% to 98% with a 6% precision.

3.2. Validation of the method

The pre-validation step showed that (1) there was no dilution effect at the response level, which allows us to analyse the samples on a wider range of concentrations and (2) the matrix seemed to have a negligible effect, as observed by comparing the results from a standard addition method and from a calibration curve with rotenone in methanol. These findings from the prevalidation step had to be further validated, and for that reason the calibration standards were prepared in methanol as well as in the matrix.

3.2.1. Stability

Photosensitivity of rotenone was already described in Cheng, Yamamoto, and Casida (1972) reported its photodecomposition in oxygenated Methanol. More recently, studies focused more on "in vivo" conditions and the environmental factors that may have an effect on the degradation of this pesticide as for example the effect of fruit waxes (Angioni et al., 2004), soil components (Cavoski, Caboni, Sarais, Cabras, & Miano, 2007) or the incubating temperature of the soils (Cavoski, Caboni, Sarais, & Miano, 2008). Regarding the stability of rotenone solutions, a preliminary test confirmed an important degradation (more than 25%) of a 0.5 mg/mL methanol solution of a commercial standard kept at 70 °C during 3 h in the dark. This shows that rotenone can also be degradated at relatively high temperature. That is why we chose DCM, having a relatively

low boiling point, for the soxhlet extraction. Furthermore it was important for us to assess the stability of rotenone in (1) the standards stored and in (2) the solutions waiting for injections.

Jimenez et al. (2000) already reported degradation of standards at 4 °C and recommended a weekly preparation of the working solutions. For this reason each of our stock solutions was prepared each day of analysis. Regarding the stability study done on a rotenone solution at a concentration of 0.5 mg/mL, it showed that there was not more degradation during 42 days at −18 °C in the darkness than during 2 h at 25 °C ($p < 0.05$), which are the conditions of our experiment: each sample was kept at −18 °C, took out 20 min before the first of the 3 repeated injections to re-equilibrate at 25 °C. In these conditions, the degradation of a solution at 0.5 mg/mL was inferior to 0.32% (±0.03%, $n = 5$).

3.2.2. Selectivity

The selectivity of the analytical method was assed by investigating the purity of the rotenone peak with LC–MS (Fig. 2a–c). Mass spectra were analysed at three retention times corresponding to the beginning, the middle and the end of the peak of rotenone and were found to be very similar (Fig. 2d–f).

3.2.3. Response function and accuracy

Different regression models were tested such as linear, weighted or unweighted quadratic, with or without transformations. Total error (systematic + random error) being the main decision criterion for our validation process, the suitability of the different models was assessed by plotting the accuracy profiles and obtaining the narrower 95% β-expectation tolerance intervals included in the ±20% acceptance limits. For the calibration in methanol the simple linear regression gave relatively good results (Fig. 3a). However, results in terms of total error could be slightly improved using a more complicated model when calibrating in the matrix (Fig. 3b and c).

As the method has to be used routinely for several hundreds of samples, its convenience was as well an important parameter. Thus we looked for the simplest model obtained with the less calibrating points possible that would fit in the acceptance limits. Fig. 3d shows the accuracy profile obtained calibrating in methanol with the simplest model, i.e. a linear regression using the highest level fitted through the origin. In these conditions, the β-expectation tolerance interval ($\beta = 95\%$) was within the acceptance limits for each one of the five concentration levels of the validation standards. Validated models with calibration in methanol are used routinely even for assays in plant extracts matrices (Rafamantanana et al., 2009) as well as single-point calibration used in many pharmacopeia methods (European Pharmacopeia, 2010). This single-point calibration was chosen and proved to provide accurate results between 12.5 and 75 μg/mL.

In relation with the influence of the matrix, the prevalidation results showing a slight matrix effect were confirmed. The comparison of Fig. 3a and b obtained with the same model and the same number of calibrating points illustrates this matrix effect.

Moreover, the average recoveries obtained with the methanol based linear regression model (Fig. 3a) at each spiked levels from 12.5 to 75 μg/mL are respectively 83.9%, 94.6%, 97.3%, 99.4%, 102.3%. As they are generally inferior to 100%, it means that the presence of the matrix in the validation standards decreases the

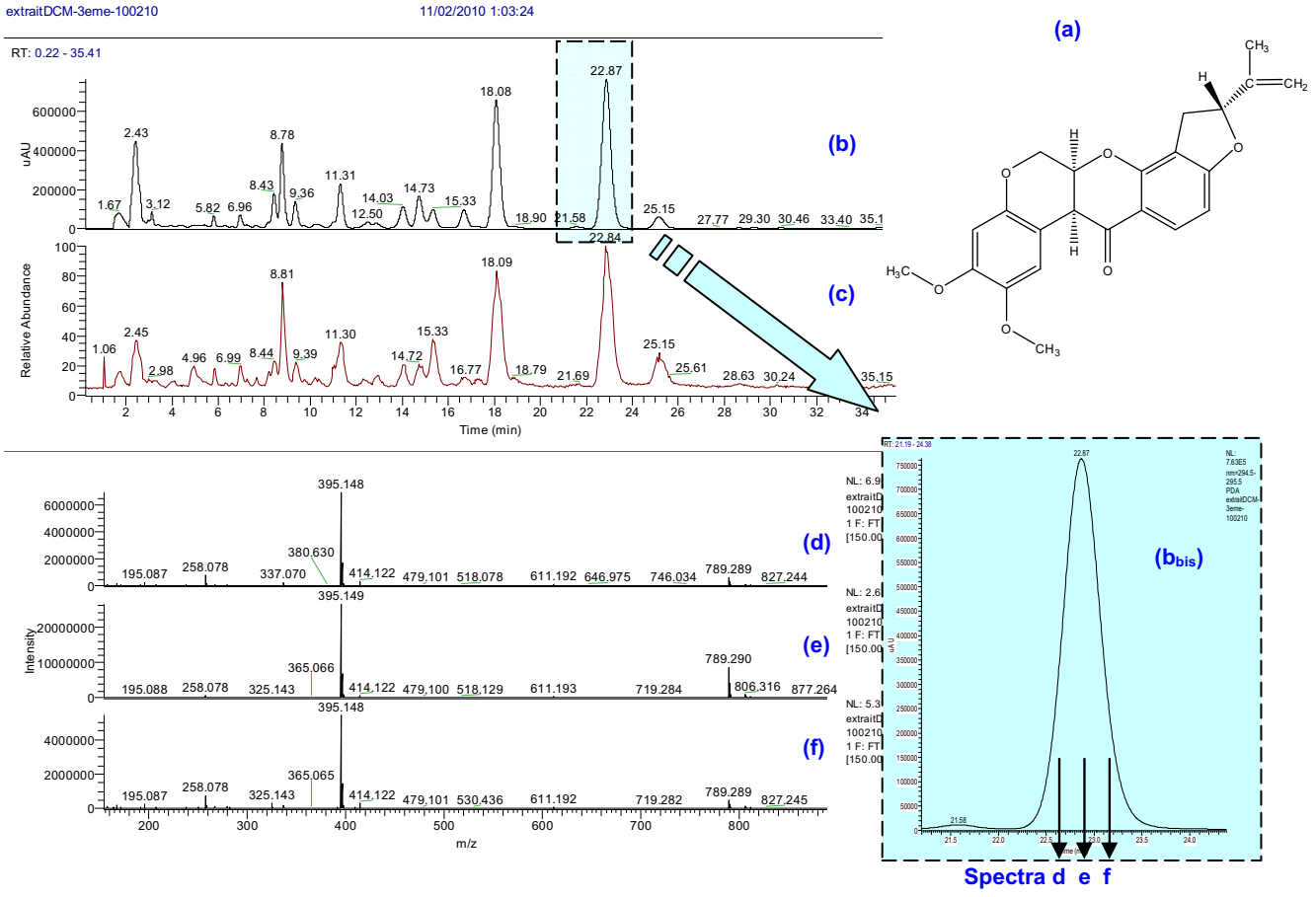

Fig. 2. (a) Structure of rotenone; (b) chromatogram of a yam bean seed extract with UV detection at 295 nm and (b_{bis}) enlargement of the rotenone peak; (c) same chromatogram with mass spectrometry detection (TIC). Mass spectra were taken at retention times corresponding to the beginning (d), the middle (e) and the end (f) of the rotenone peak.

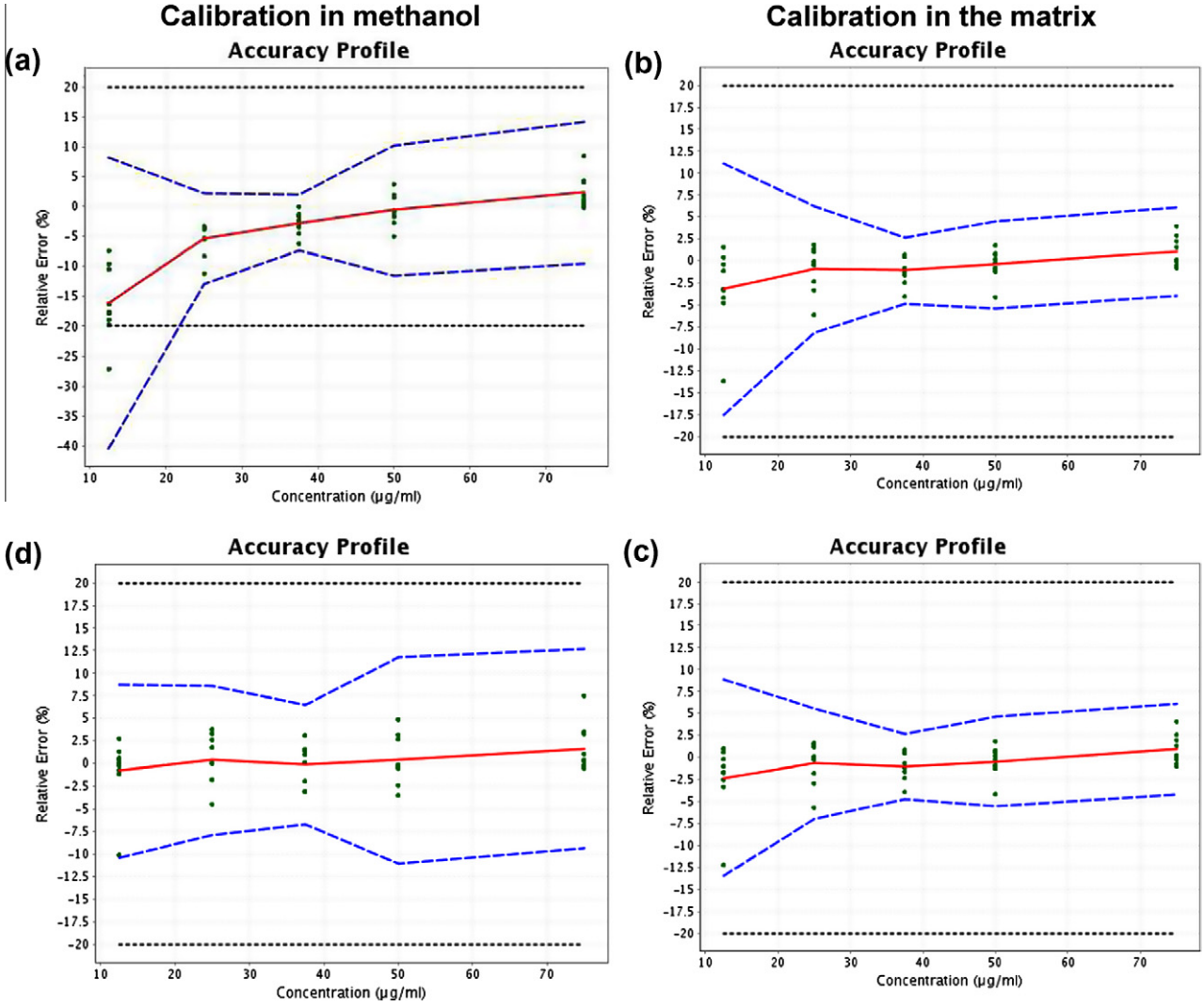

Fig. 3. Accuracy profiles in relative values for different calibration models using a 6 points linear model in methanol (a) and in the matrix (b), using a 6 points weighted (1/*X*) linear regression in the matrix (c) and a single point calibration in methanol (d). (The continuous line represents the relative bias, the dashed lines the 95% β-expectation tolerance limits and the dotted lines the ±20% acceptance limits.)

detector response compared to the rotenone response in methanol for the calibration standards. This effect is similar to the one called by Pizzutti et al. (2007) a "negative effect" of the matrix for apolar pesticides in soya extracts.

Furthermore, as the matrix effect seems to be related to the concentration level, it can explain why in methanol the single point calibration model using the highest level fitted through the origin is the one showing the best results. In fact, the matrix shows a greater influence at lower concentrations of rotenone added (the concentration of the matrix being always the same). As a consequence, the linear model constructed on 6 points (Fig. 3a) involving calibrating points at low spiked concentration was not as good as the linear model constructed on one point (75 µg/mL) and forced to the origin (Fig. 3d).

The values of accuracy are summarised in Table 1 for each of the validation standards concentrations for this last model.

3.2.4. Trueness, precision and linearity

Values obtained are summarised in Table 1.

Trueness explained by the relative bias is inferior to 1% except at the highest concentration level (1.67%) which shows the very good trueness of the method. The precision is evaluated intra-day (repeatability) and inter-day (intermediate precision) through relative standard deviations (RSD). The repeatability is better for higher concentrations (the RSDs decrease as concentrations increase), whereas it is more homogeneous for intermediate precision (between 2% and 4% as illustrated in Table 1).

The linearity is the ability, in a definite range, to obtain results directly proportional to the concentration. The concentrations estimated by the calibration can be plotted as a function of the introduced concentrations, a regression line is constructed and compared to the identity line $y = x$. The parameters of the equation of the regression line are presented in Table 1 and the slope value close to 1 demonstrates that the method is linear. The linearity of the model is demonstrated as well because the absolute 95% β-expectation tolerance limits are within the absolute acceptance limits (Rozet et al., 2007).

3.2.5. Dilution effect

Our aim was to explore the possibility of assaying rotenone in highly concentrated samples so we compared the results measured from the extract and the results estimated from the extract diluted three times. Equivalency was first determined at the average level and then at the individual result level.

First of all, the relative difference of the averages of the results and its confidence interval at 95% were calculated and gave

Table 1
Validation results obtained for the quantification method of rotenone in extracts of yam bean seeds.

Validation criteria		Concentration levels (μg/mL)				
		12.5	25	37.5	50	75
Response function		Linear regression forced at the origin One point methanol calibration (75 μg/mL)				
Trueness (relative bias)		−0.79%	0.38%	−0.09%	0.38%	1.67%
Precision	Repeatability (RSD)	3.43%	2.25%	1.73%	1.42%	1.43%
	Intermediate precision (RSD)	3.74%	2.90%	2.28%	3.00%	2.93%
Accuracy (95% relative β-expectation lower and upper tolerance limits in %)		−10.60, 9.02	−8.44, 9.21	−7.05, 6.87	−14.25, 15.02	−12.61, 15.95
Linearity		Slope: 1.0203 Intercept: −0.5325 R^2: 0.998				

respectively 1.9% and [−8.2%, 12.0%]. This interval was within the ±20% acceptance limits indicating that on average samples diluted three times can be considered as equivalent to the undiluted extracts.

However, at the individual results level, the 95% β-expectation tolerance interval for the results estimated from the diluted extracts gave [−26.2%, 30.0%]. This means that the prediction interval lays outside the ±20% limits around the average value of the undiluted extract results.

In summary, we can conclude that in average the difference between this two kind of samples (diluted and undiluted) is acceptable, whereas the variability of individual results increases when diluting. As a consequence if there is a need to dilute very concentrated samples for example, the assay should be repeated several times.

3.2.6. Limits of detection and of quantification

The limit of detection (LOD) estimated from the standard curve residual standard-deviation and slope gave 0.46 μg/mL while it is 0.14 μg/mL when estimated with the signal/noise method from the European Pharmacopeia. The detection of this compound is very good as rotenone solubilised in methanol at a concentration of 0.1 μg/mL is still detected experimentally.

The lower and upper limits of quantification (LLOQ and ULOQ) that have been validated are respectively 12.5 and 75 μg/mL. They are the smallest and highest quantities of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy. Thus the method allows the quantification of rotenone from 0.07% to 0.42% (w/w) in yam bean seeds or from 0.07% up to 1.25% rotenone when diluting the more concentrated extracts.

3.3. Measurement of uncertainty

Method validation demonstrates the reliability of the results generated by the method. It is however insufficient to allow the correct interpretation and comparison of the results. To achieve this, measurement uncertainty has to be evaluated. Feinberg, Boulanger, Dewé, and Hubert (2004) demonstrated that the β-expectation tolerance interval used in the accuracy profile validation methodology is directly related to the uncertainty of the measurements. This allows obtaining estimates of the measurement uncertainty of the rotenone method without additional experiments as long as the method validation experimental design includes the major sources of uncertainty that will be involved during the routine application of the method. Estimates of measurement uncertainty were therefore obtained at each of the concentration levels of the validation standards and are given in Table 2. The expanded uncertainty is obtained by applying a coverage factor of $k = 2$ (Eurachem/Citac, 2000; European Co-operation for Accreditation, 2004). This corresponds to a 95% confidence interval around the results where the measure may lie. Table 2 indicates that the relative expanded uncertainty of rotenone is at most 8% over the concentration range validated. This maximum value is obtained for the 12.50 μg/mL concentration level. This means that with a confidence level of 95% the measure and is situated at maximum ±8% around the measured result. Table 2 also shows that the relative uncertainty is relatively constant over the valid concentration range fluctuating from 5% to 8%. Therefore, in order to obtain the uncertainty of the routine results, the relative uncertainty that will be used to provide a first estimation of the measurement uncertainty of future routine results will be of 8%.

3.4. Application to the determination of the quantity of rotenone in several samples of yam bean seeds

The amount of rotenone in the milled seeds was calculated using the following formula:

$$\text{Rotenone (in mg/g dry powdered yam bean seeds)} = \frac{C \times D_f}{m \times \text{Ye} \times \text{Rt} \times 1000} \quad (1)$$

with C being the concentration of rotenone in μg/mL calculated from the regression model, D_f being a dilution factor (50), m the exact mass in g of the initial dry plant material, Ye the extraction yield

Table 2
Measurement uncertainty estimations of rotenone results at each concentration level investigated during the method validation using the selected regression model. The expanded uncertainty was computed using a coverage factor of 2.

Concentration level (μg/mL)	Uncertainty of the bias (μg/mL)	Uncertainty (μg/mL)	Expanded uncertainty (μg/mL)	Relative expanded uncertainty (%)
12.5	0.1791	0.5011	1.002	8.0
25.0	0.3243	0.7948	1.590	6.4
37.5	0.3886	0.9402	1.880	5.0
50.0	0.7995	1.701	3.402	6.8
75.0	1.165	2.489	4.978	6.6

Table 3
Results of rotenone determination in different yam bean seeds samples.

Yam bean species	Samples	Rotenone in mg/g dry powdered yam bean seeds	Standard deviation on $n = 3$	Measurement of uncertainty $u(x)$
P. erosus	EC-533	2.76	0.164	0.662
	EC-KEW	1.13	0.014	0.271
	EC-041	2.50	0.029	0.600
P. ahipa	AC-525	2.14	0.008	0.513
	AC-102 FN	2.06	0.013	0.494
	AC-102 FB	2.25	0.078	0.540
	AC-524	2.31	0.080	0.554
	209003	2.21	0.060	0.530
	209004	2.06	0.054	0.494
	209006	2.30	0.079	0.552
	209007	2.21	0.012	0.530
	209021	1.97	0.035	0.473
	209022	1.45	0.018	0.348
	209023	2.29	0.031	0.550
	209024	2.50	0.059	0.600
	209025	2.39	0.044	0.574
	209026	1.82	0.022	0.437

after the 8 h of extraction and Rt the total recovery of rotenone (the factor 1000 is due to conversion from μg to mg).

The measurement uncertainty values in Table 2 are not those of the final rotenone amount in the milled seeds of yam bean following Eq. (1). The relative uncertainty $\left(\frac{u(R)}{R}\right)$ of the final results is then obtained by:

$$\frac{u(R)}{R} = \frac{u(C)}{C} + \frac{u(\mathrm{Ye})}{\mathrm{Ye}} + \frac{u(\mathrm{Rt})}{\mathrm{Rt}} \quad (2)$$

In other words, the relative uncertainty of the rotenone amount in the milled seeds is the sum of the relative uncertainty of the concentration of rotenone obtained by the analytical method (C) with the expanded uncertainty of the extraction yield after the 8 h of extraction (Ye) and with the expanded uncertainty of the total recovery of rotenone (Rt).

The amount of rotenone was then determined for samples of yam bean seeds of two different species *P. erosus* and *P. ahipa* and different accessions for each one. In the chromatograms, similar to those obtained in Fig. 2b, no interfering compounds appeared in the retention time window of interest for the quantification thus further illustrating the good selectivity of the method. The results given in Table 3 range between 1.13 and 2.76 mg/g of milled seeds which corresponds to amounts around 0.2% (w/w) for the majority of them. Magalhaes, Sales, Magalhaes, and Valio (1992) evaluated the seed content of several rotenoids from *Pachyrhizus tuberosus* and also found rotenone contents in the range of 1–2 mg/g dried seeds. The differences do not seem to be related with the species but more with the accession. For example, the highest and the lowest values of rotenone were obtained from accessions from the same species *P. erosus* (respectively EC-533 and EC-KEW). Indeed, this last accession is of special interest as it shows half the rotenone content compared to the others (all p-values <0.0001).

4. Conclusions

The method developed was found to be selective, linear, accurate, true and precise from 12.5 to 75 μg/mL which allowed the quantification of rotenone from 0.07% to 0.42% (w/w) in yam bean seeds or from 0.07% up to 1.25% rotenone taking into account the 3-fold dilution. The methodology described was shown to be convenient for routine analysis and is meant to be used as a selection tool of less toxic seeds between a large panel of accessions of seeds of *Pachyrhizus*.

A precise quantification of rotenone allowing a comparison between seeds of species of *P. erosus* and *P. ahipa* was realised for the first time. Results showed that already one accession (EC-KEW) has a lower rotenone content.

Recent data on toxicological endpoints for dietary exposure to rotenone is scarce. However, the US Environmental Protection Agency uses a "no observed adverse effect level" (NOAEL) of 15 mg/kg/day for acute and 0.375 mg/kg/day for chronic dietary exposure scenario. Applying a uncertainty factor of 1000 (which takes into account the inter-and intra-species variability as well as the potential neurotoxicity) and considering daily intakes of 50 g yam bean for 50 kg adults, the endpoints regarding a potential food crop would be around 0.015 and 0.004 mg/g yam bean seeds for acute and chronic dietary exposures respectively. Then the content of the accession EC-KEW is still too high to make it a possible food crop for the moment and the selection of less toxic seeds between a large panel is thus important. Breeding or agronomical alternatives can be found as well as solutions coming from the process. In fact, during the analytical procedure, we observed the degradation of rotenone with temperature. There can be of interest for further research to analyse the cooking process itself: indeed a grain selected for its very low content of rotenone could yet become totally safe after cooking it under drastic conditions.

Acknowledgements

We wish to thank Dr. Grüneberg from the CIP Peru for the plant material, Marie-Christine Fayt and Jean Paul Vanhelleputte for their wise technical advices and Gabrielle Chataigné for her kind assistance during the LC–MS analyse. Financial support from the Belgian Federal Public Service (FPS) Foreign Affairs for the "Yam Bean Central & West Africa" project, the National Fund for Scientific Research (FNRS) (FRFC 2.4555.08), the Special Fund for Research (FSR) and the faculty of medicine of UCL is also gratefully acknowledged. A research grant from the Belgium National Fund for Scientific Research (FRS-FNRS) to E. Rozet is gratefully acknowledged.

References

Angioni, A., Cabizza, M., Cabras, M., Melis, M., Tuberoso, C., & Cabras, P. (2004). Effect of the epicuticular waxes of fruit and vegetables on the photodegradation of rotenone. *Journal of Agricultural and Food Chemistry, 52*(11), 3451–3455.

Cabizza, M., Angioni, A., Melis, M., Cabras, M., Tuberoso, C. V., & Cabras, P. (2004). Rotenone and rotenoids in cubè resins, formulations, and residues on olives. *Journal of Agricultural and Food Chemistry, 52*(2), 288–293.

Caboni, P., Sarais, G., Angioni, A., Garau, V. L., & Cabras, P. (2005). Fast and versatile multiresidue method for the analysis of botanical insecticides on fruits and vegetables by HPLC/DAD/MS. *Journal of Agricultural and Food Chemistry, 53*(22), 8644–8649.

Caboni, P., Sarais, G., Vargiu, S., De Luca, M. A., Garau, V. L., Ibba, A., et al. (2008). LC–MS–MS determination of rotenone, deguelin, and rotenolone in human serum. *Chromatographia, 68*(9–10), 739–745.

Cavoski, I., Caboni, P., Sarais, G., Cabras, P., & Miano, T. (2007). Photodegradation of rotenone in soils under environmental conditions. *Journal of Agricultural and Food Chemistry, 55*(17), 7069–7074.

Cavoski, I., Caboni, P., Sarais, G., & Miano, T. (2008). Degradation and persistence of rotenone in soils and influence of temperature variations. *Journal of Agricultural and Food Chemistry, 56*(17), 8066–8073.

Cheng, H.-M., Yamamoto, I., & Casida, J. E. (1972). Rotenone photodecomposition. *Journal of Agricultural and Food Chemistry, 20*(4), 850–856.

D'Andrea, A., Aliboni, A., De Santis, A., Mariani, S., Gorgoglione, D., & Ritieni, A. (2007). SFE of *Derris elliptica* (Wallich) benth roots: Influence of process parameters on yield and purity of rotenone. *The Journal of Supercritical Fluids, 42*(3), 330–333.

Di Donna, L., Grassi, G., Mazzotti, F., Perri, E., & Sindona, G. (2004). High-throughput assay of rotenone in olive oil using atmospheric pressure chemical ionization tandem mass spectrometry. *Journal of Mass Spectrometry, 39*(12), 1437–1440.

Di Donna, L., Mazzotti, F., Sindona, G., & Tagarelli, A. (2005). Assay of rotenone in river water by high-throughput tandem mass spectrometry and multiple-reaction monitoring methodology. *Rapid Communications in Mass Spectrometry, 19*(12), 1575–1577.

Drozdzynski, D., & Kowalska, J. (2009). Rapid analysis of organic farming insecticides in soil and produce using ultra-performance liquid chromatography/tandem mass spectrometry. *Analytical and Bioanalytical Chemistry, 394*(8), 2241–2247.

Eurachem/Citac. (2000). *Eurachem/Citac guide "Quantifying the uncertainty in analytical measurement"* (2nd ed.). <http://www.eurachem.org>.

European Commission. (2008). DÉCISION DE LA COMMISSION du 10 avril 2008 concernant la non-inscription de la roténone, de l'extrait d'Equisetum et de l'hydrochlorure de quinine à l'annexe I de la directive 91/414/CEE du Conseil et le retrait des autorisations de produits phytopharmaceutiques contenant ces substances. *Journal officiel de l'Union européenne 18.4.2008*, L 108/30–32.

European Co-operation for Accreditation. (2004). *EA guidelines on the expression of uncertainty in quantitative testing*, EA-4/16. <http://www.european-accreditation.org>.

European Pharmacopeia. (2010). *European Pharmacopeia* (7th ed.). <http://www.edqm.eu>.

Feinberg, M., Boulanger, B., Dewé, W., & Hubert, Ph. (2004). New advances in method validation and measurement uncertainty aimed at improving the quality of chemical data. *Analytical and Bioanalytical Chemistry, 380*, 502–514.

Grüneberg, W. J., Goffman, F. D., & Velasco, L. (1999). Characterization of yam bean (*Pachyrhizus* spp.) seeds as potential sources of high palmitic acid oil. *Journal of the American Oil Chemist Society, 76*(11), 1309–1312.

Hubert, Ph., Nguyen-Huu, J.-J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., et al. (2004). Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal—Part I. *Journal of Pharmaceutical and Biomedical analysis, 36*, 579–586.

Hubert, Ph., Nguyen-Huu, J.-J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., et al. (2007). Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal—Part I. *Journal of Pharmaceutical and Biomedical analysis, 45*, 70–81.

Hubert, Ph., Nguyen-Huu, J.-J., Boulanger, B., Chapuzet, E., Cohen, N., Compagnon, P.-A., et al. (2007). Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal—Part III. *Journal of Pharmaceutical and Biomedical analysis, 45*, 82–96.

Hung, Y.-M., Hung, S.-Y., Olson, K. R., Chou, K.-J., Lin, S.-L., Chung, H.-M., et al. (2007). Yam bean seed poisoning mimicking cyanide intoxication. *Internal Medicine Journal, 37*(2), 130–132.

Jimenez, J. J., Bernal, J. L., del Nozal, M. J., Novo, M., Higes, M., & Llorente, J. (2000). Determination of rotenone residues in raw honey by solid-phase extraction and high-performance liquid chromatography. *Journal of Chromatography A, 871*, 67–73.

Magalhaes, A. F., Sales, B. H. L. N., Magalhaes, E. G., & Valio, I. F. M. (1992). Flavonoids and 3-phenylcoumarins from the seeds of *Pachyrhizus tuberosus*. *Phytochemistry, 31*(5), 1831–1832.

Morales-Arellano, G. Y., Chagolla-Lopez, A., Paredes-Lopez, O., & Barba de la Rosa, A. P. (2001). Characterization of yam bean (*Pachyrhizus erosus*) proteins. *Journal of Agricultural and Food Chemistry, 49*(3), 1512–1516.

Narongchai, P., Narongchai, S., & Thampituk, S. (2005). The first fatal case of yam bean and rotenone toxicity in Thailand. *Journal of the Medical Association of Thailand, 88*(7), 984–987.

Noman, A. S. M., Hoque, M. A., Haque, M. M., Pervin, F., & Karim, M. R. (2007). Nutritional and anti-nutritional components in *Pachyrhizus erosus* L. tuber. *Food Chemistry, 102*, 1112–1118.

Pereira, A. S., Afonso Serrano, M. A., Aquino Neto, F. R., Cunha Pinto, A., Furtado Texeira, D., & Gilbert, B. (2000). Analysis and quantitation of rotenoids and flavonoids in Derris (*Lonchocarpus urucu*) by high-temperature high-resolution gas chromatography. *Journal of Chromatographic Science, 38*(4), 174–180.

Pizzutti, I. R., de Kok, A., Zanella, R., Adaime, M. B., Hiemstra, M., Wickert, C., et al. (2007). Method validation for the analysis of 169 pesticides in soya grain, without clean up, by liquid chromatography–tandem mass spectrometry using positive and negative electrospray ionization. *Journal of Chromatography A, 1142*(2), 123–136.

Rafamantanana, M. H., Rozet, E., Raoelison, G. E., Cheuk, K., Ratsimamanga, S. U., Hubert, P., et al. (2009). An improved HPLC–UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (APIACEAE). *Journal of Chromatography B: Analytical Technologies in the Biomedical Life Sciences, 877*(23), 2396–2402.

Rozet, E., Ceccato, A., Hubert, C., Ziemons, E., Oprean, R., Rudaz, S., et al. (2007). An analysis of recent pharmaceutical regulatory documents on analytical method validation. *Journal of Chromatography A, 1158*, 111–125.

Sae-Yun, A., Ovatlarnporn, C., Itharat, A., & Wiwattanapatapee, R. (2006). Extraction of rotenone from *Derris elliptica* and *Derris malaccensis* by pressurized liquid extraction compared with maceration. *Journal of Chromatography A, 1125*(2), 172–176.

Santos, A. C. O., Cavalcanti, M. S. M., & Coelho, L. C. B. B. (1996). Chemical composition and nutritional potential of yam bean seeds (*Pachyrhizus erosus* L. Urban). *Plant Foods for Human Nutrition, 49*, 35–41.

Sherer, T. B., Kim, J.-H., Betarbet, R., & Greenamyre, J. T. (2003). Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and [alpha]-synuclein aggregation. *Experimental Neurology, 179*(1), 9–16.

Simeone, V., Baser, N., Perrelli, D., Cesari, G., El Bilali, H., & Natale, P. (2009). Residues of rotenone, azadirachtin, pyrethrins and copper used to control *Bactrocera oleae* (Gmel.) in organic olives and oil. *Food Additives and Contaminants Part A: Chemistry, Analysis, Control, Exposure and Risk assessment, 26*(4), 475–481.

Taylor, M. J., Keenan, G. A., Reid, K. B., & Fernández, D. U. (2008). The utility of ultra-performance liquid chromatography/electrospray ionisation time-of-flight mass spectrometry for multi-residue determination of pesticides in strawberry. *Rapid Communications in Mass Spectrometry, 22*(17), 2731–2746.

World Health Organization, International Labour Organization, & United Nations Environment Programme (2009). *The WHO recommended classification of pesticides by hazard WHO 2009*. ISBN: 92-4-154663-8. <http://www.who.int/ipcs/publications/pesticides_hazard/en/>.