Fast method for the simultaneous quantification of toxic polyphenols applied to the selection of genotypes of yam bean (Pachyrhizus sp.) seeds

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A B S T R A C T

The purpose of the research was to develop and validate a rapid quantification method able to screen many samples of yam bean seeds to determine the content of two toxic polyphenols, namely pachyrrhizine and rotenone.

The analytical procedure described is based on the use of an internal standard (dihydrorotenone) and is divided in three steps: microwave assisted extraction, purification by solid phase extraction and assay by ultra high performance liquid chromatography (UHPLC). Each step was included in the validation protocol and the accuracy profiles methodology was used to fully validate the method. The method was fully validated between 0.25 mg and 5 mg pachyrrhizin per gram of seeds and between 0.58 mg/g and 4 mg/g for rotenone.

More than one hundred samples from different accessions, locations of growth and harvest dates were screened. Pachyrrhizine concentrations ranged from 3.29 mg/g to lower than 0.25 mg/g while rotenone concentrations ranged from 3.53 mg/g to lower than 0.58 mg/g. This screening along with principal component analysis (PCA) and discriminant analysis (DA) analyses allowed the selection of the more interesting genotypes in terms of low concentrations of these two toxic polyphenols.

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1. Introduction

The yam bean (Pachyrhizus spp.) is a small genus comprising three cultivated species of neotropical origin in the subtribe Glycininae. Pachyrhizus erosus is most widely spread with production in Central America, Southeast Asia including China, Indonesia and the Philippines, and South Asia including Bangladesh and India [1]. Production on lower scales can be found in the Caribbean, French Guyana, Brazil and several Pacific islands while the crop is in the process of being introduced in Central and West Africa as well as in Madagascar [2,3]. Pachyrhizus tuberosus is locally found in the humid tropical lowlands of South America and the Caribbean, whereas Pachyrhizus ahipa is only reported in the Andean highlands within 2200 and 2800 m.a.s.l. of Bolivia and Argentina [4]. All three species are grown exclusively for their storage roots, which are in the case of P. erosus and P. ahipa usually consumed raw. The seeds of the yam bean are known to be toxic and only used to propagate the crop. Unfortunately, intoxications after seeds ingestion were reported from Thailand, Taiwan or China [5-8]. However, seed yields of P. erosus can be extremely attractive (> 4.5 t ha⁻¹) without strong yield declines under drought stress conditions [2].

Besides an interesting protein content and fatty acids composition [9], yam bean seeds are known to contain different isoflavonoids and coumarins [10,11], the most toxic of each family of compounds being rotenone and pachyrrhizine (Fig. 1). This last compound is also reported in several species of Neorautanenia [12,13] and belongs to the linear furocoumarin group. Other members of this group such as psoralens are known for their photosensitization abilities as their planar structure allows them to intercalate into DNA, inhibiting DNA replication [14]. Rotenone, an inhibitor of the mitochondrial respiratory chain, was formerly extracted from Derris, Loncocarpus and Tephrosia species when its use as an organic insecticide was still allowed in the European Union (EU) [15]. It is not a rare compound as it has been reported in other genera such as Millettia, Mundulea, Neorautanenia and...
Picidia [12,16–18]. The cytotoxicity of both compounds was evaluated on breast (MCF-7) and lung (A-549) cancer cell lines and results showed that rotenone is much more cytotoxic than pachyrrhizine [12]. In the literature the toxicity of yam bean seeds is usually related to the content of rotenone [5,6]; however, pachyrrhizine could also participate to the overall toxicity of yam bean seeds. It is hypothesized that it is possible to find genotypes in the Pachyrhizus spp complex with less or no toxic compounds in seed. This requires accurate quantification of the compounds able to play an important role in the toxicity of yam bean seeds and to screen different accessions in order to identify those containing less or no toxic compounds. Such genotypes could limit possible toxicities and if the absence of toxic compounds is proved, they could be released as varieties for human consumption. A simple and transferable method was developed previously for rotenone quantification [19] comprising a dichloromethane soxhlet extraction, a purification by solid phase extraction (SPE) and a quantification by high performance liquid chromatography-ultra-violet detection (HPLC-UV) with an external calibration. This method takes at least 10 h per sample (9 h extraction and drying and at least another hour for SPE and HPLC) so it was not fast enough for a screening program, so we decided to improve the extraction speed and its efficiency [20]. However, other improvements were needed to lower the analysis time using the faster ultra high performance liquid chromatography (UHPLC) technology and the use of an internal standard would allow minimizing the error all along the procedure in this multi-step analysis.

This study had thus four objectives. First, to show if the fast extraction method developed for rotenone was able to extract pachyrrhizine. The second objective was to determine the SPE parameters allowing the simultaneous recovery of both analytes from the lipids of yam bean seeds. Then, the third objective was to develop an UHPLC method able to assay simultaneously rotenone and pachyrrhizine. And the fourth objective was to validate the overall procedure for each compound by using a validation protocol according to harmonization strategies proposed by the French society of pharmaceutical sciences and technics (SFSTP) commission [21–23].

2. Material and methods

2.1. Chemicals and plant material

Pachyrrhizine was purchased from Florida center for heterocyclic compounds (Gainesville, USA), rotenone from Enzo Life Science (Zandhoven, Belgium) and dihydrorotene [2-isopropyl-8,9-dimethoxy-1,12,12a,4h,6ah-chromeno[3,4-b] furan[2,3-h] from Sigma-Aldrich (Diegem, Belgium). Methanol (MeOH) HPLC grade from Prolabo, VWR (Leuven, Belgium), acetonitrile (ACN) HPLC grade from Fisher Scientific (Tournai, Belgium), tetrahydrofuran (THF) and ethyl acetate from VWR – GPR Rectapur (Leuven, Belgium) and dichloromethane (DCM) reagent grade from Sigma-Aldrich (Diegem, Belgium) were used as solvents. The catalyst palladium on calcium carbonate (extent of labeling: 5 wt% loading, poisoned with lead) was provided by Sigma-Aldrich (Diegem, Belgium).

In total 126 seeds samples from 35 different Pachyrhizus spp accessions were used comprising 5 accessions of P. erosus, 5 accessions of P. tuberosus, 18 accessions of P. ahipa and 7 hybrids of the type P. ahipa x P. tuberosus. The accessions are hold in trust at the International Potato Center (CIP) and were made available on request with permission of Peruvian authorities in the frame of a project funded by the Belgian Government, Federal Public Service Foreign Affairs, Directorate General for Development Cooperation – D2.2 (inclusive growth-agriculture). The plant material were grown in Peru in two eco-geographic regions, namely at CIP’s experimental stations in San Ramon (wet tropics) and La Molina (arid coastal pacific lowlands), in 2010 and 2012. The seeds harvests of each accession and environment were dried, milled and sent to UCL in Belgium.

2.2. Synthesis of dihydrorotene

We report here a fast and inexpensive method to synthesize dihydrorotene from commercial rotenone by catalytic hydrogenation of rotenone as proposed by O’Neil et al. [24]. Their protocol was modified as follows: 500 mg rotenone were solubilised in 15 mL ethyl acetate and 100 mg calcium carbonate poisoned with lead was added to the solution magnetically stirred. The reaction vessel was capped, degassed and filled with hydrogen gas. After 1 h, the reaction solution was filtered through celite and the solvent removed. The nature of the product was confirmed by 1H-NMR and its purity evaluated by HPLC-UV at 295 nm.

2.3. Standard solutions

Stock solutions of analytes and of internal standard (IS) were prepared for each series at 500 μg/mL and 400 μg/mL for pachyrrhizine and rotenone respectively and at 4 mg/mL for the IS, both in THF/MeOH (50:50, v/v). Calibration standards in MeOH/acetonitrile/water (50:25:25, v/v) were analyzed three times (n=3) for 3 series of experiments (k=3) at five concentration levels (m=5) ranging from 5 to 100 μg/mL for pachyrrhizine and from 4 to 80 μg/mL for rotenone. They were spiked with the internal standard (IS) to reach a final concentration of 40 μg/mL.

The validation standards were reconstituted samples within the matrix containing known concentrations of the analyte of interest which are considered as true values by consensus. Validation standards were prepared by spiking the same amount of standards as for the calibration standards directly on the milled seeds of P. ahipa. They were dried under N2 flux to evaporate solvents from stock solutions and submitted to the microwave assisted extraction (MAE)–SPE-UHPLC protocol. The signals from the compounds initially present in the yam bean seeds were subtracted to the ones resulting from this spiking.

Five concentrations were analyzed (m=5) and for each of the three series (k=3), three independent samples (n=3) were analyzed.

The stability of the standards solutions was assessed during (1) the storage of the stock solutions at −18 °C and (2) during the analyses in the LC auto-sampler rack at 15 °C.
The stability at −18 °C of stock solutions of pachyrrhizine, rotenone and dihydrorotenone was evaluated through monitoring during 4 weeks the variation of the peak area. Weekly, diluted solutions were prepared from stock solutions and analyzed immediately.

The stability at 15 °C of solutions of pachyrrhizine, rotenone and dihydrorotenone in MeOH/acetonitrile/water (50:25:25, v/v/v) was evaluated during 20 h at respectively 50, 40 and 40 μg/mL.

2.4. Microwave assisted extraction and SPE clean-up procedure

20.0 mL of DCM/MeOH (50:50, v/v) and 1 mg IS solution were added to 0.5 g powdered seeds in a glass extraction vessel with a magnetic stir bar. The microwave extractor (MARS 5, CEM corporation, USA) conditions, 11 min at 55 °C, had been optimized in a previous work [20]. After extraction, the vessels were allowed to cool down until 38 °C before opening and centrifuged 5 min at 3000 g. 4.0 mL of the supernatant were taken and dried in a RapidVap equipment (Labconco) with the following conditions: first 15 min at 30 °C, 150 mbar and 40% vortex speed, then 40 min at 38 °C, 150 mbar and 40% vortex speed. The extracts were stored at −18 °C until analysis for which they were solubilised in 0.5 mL DCM and transferred quantitatively on the SiOH cartridge (6 mL/1000 mg Chromabond X, Filterservice, Belgium) previously washed with 15 mL of MeOH. The cartridges were then washed with DCM and eluted with DCM/MeOH. The washing and eluting volumes were optimized as well as the v/v ratio of the DCM/MeOH elution solution to obtain the best recovery of both compounds. The eluted volume was dried with the RapidVap (50 min at 32 °C, 150 mbar and 25% vortex speed) and stored at −18 °C until analysis.

2.5. UV/mass spectrometry (MS) UHPLC analyses

The analyses were performed on an Accela UHPLC system from Fisher Scientific (ThermoFisher Scientific, Bremen, Germany) consisting in a photo diode array (PDA) detector, an autosampler equipped with a column oven, a tray compartment cooler (set to 25 and 15 °C respectively) and a quaternary pump, all piloted by ChromQuest software. The chromatographic separation was performed on a Hypersil Gold aQ C18 column 1.9 μm, 100 mm × 2.1 mm ID (ThermoFisher Scientific, Alost, Belgium) equipped with a VanGuard™ UPLC HSS C18 pre-column 1.8 μm, 5 mm × 2.1 mm ID (Waters, Belfast, Ireland). 5 μL of samples were injected in the full loop injection mode. The column was eluted at constant flow rate of 500 μL/min with water and ACN. The initial conditions (56:44) were maintained during 9 min, and then the column was washed with 100% ACN during 5 min and stabilized to the initial conditions during 4 min. Analyses were carried out at wavelengths of 244 and 295 nm corresponding to the absorbance maxima of pachyrrhizine and rotenone respectively.

The SPE purified extract was solubilised in 5.0 mL methanol on the day of the analysis, filtered with a 0.22 μm PVDF (polyvinylidene fluoride) filter (Millipore, Brussels, Belgium) and directly injected into UHPLC.

Selectivity of the method was evaluated on the same system hyphenated with a LITQ-Orbitrap XL (ThermoFisher scientific, Belgium) from the UCL MASSMET platform that was used in positive mode electrospray ionization (ESI) 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 RF lens 1 voltage to 105 V. Nitrogen was used as the sheath gas and helium as auxiliary gas with flow rates of 20 and 10 arbitrary units respectively.

2.6. Validation and data analysis

Validation was done through the accuracy profiles procedure using total error as a decision criterion. This procedure allows simplifying the validation process and monitoring of the risk related to the use of the method [21,25,26].

The following criteria were tested for the validation procedure: response function, linearity, precision, trueness, accuracy, limits of detection and quantification and dosing range.

Several publications using complex bioanalytical procedures [27–32] report β-expected tolerance limits of ±30% in accordance with the recommendations of the American association of pharmaceutical scientists (AAPS) report [33]. Furthermore, we decided to set the acceptance limits to λ = ±30% on the upper part of the dosing range and to ±35% on the lower part of the dosing range where the error is usually higher as the food and drug administration (FDA) allows 5% more around the low limit of quantification (LLOQ). The minimum probability to obtain future results within these limits was set at β = 90% which is adequate for our method designed mainly for screening purposes.

Statistical analyses for the accuracy profiles were performed using the e-noval V3.0 (Arlenda, Liège, Belgium) software.

Principal component analysis (PCA) and quadratic discriminant analysis (DA), analysis of variance (ANOVA) and multiple ANOVA (MANOVA) were carried out using JMP v10 software (SAS institute, Cary, NC).

3. Results and discussion

3.1. Synthesis of dihydrorotenone

Unlike what is proposed by O’Neil et al. [24] we did not obtained satisfactory results with the catalyst they mentioned (palladium on carbon) but we had to use palladium on calcium carbonate poisoned with lead. In this case, the reaction was complete after only one hour and produced exclusively dihydroyotenone without any by-products. The 1H-NMR spectrum recorded at 300 MHz in CD3CN was identical to the one described in O’ Neil et al. and the purity of the synthesized dihydrorotenone (97%) was superior to the purity of the commercial product (93%) evaluated both by the normalization process on UHPLC at 295 nm.

3.2. Microwave assisted extraction

The MAE conditions were optimized for rotenone extraction in a previous article [20] and we confirm here that these conditions also allow the complete extraction of pachyrrhizine from milled yam bean seeds.

Three samples from P. ahipa (AC102 accession) milled seeds were submitted to the complete protocol with the optimized extraction time: 11 min [20], while three other were submitted to the same protocol but with 44 min extraction, time after which the extraction was considered as complete. Then the extraction recovery after 11 min extraction was calculated and compared with 44 min extraction. We obtained 99.42% (SD = 0.74) for rotenone and 98.68% (SD = 1.17) for pachyrrhizine. The quantification responses (ratio of the analyte’s peak area on the IS’s peak area) for 44 and 11 min extraction times were also compared for both compounds and they were statistically not different (α = 5%), so confirming that 44 min of extraction did not extract more pachyrrhizine nor more rotenone than 11 min extraction.

3.3. Solid phase extraction

SPE conditions were optimized from those described in Ref. [19] to allow the best recovery of both compounds and the shorter analysis time. In [19] the cartridges were washed by 20 mL of DCM previously to the elution of rotenone but we noticed that pachyrrhizine was already eluting between the 10th and the 15th
3.4. UV/MS UHPLC analyses

The choice of an internal standard calibration allows limiting run-to-run variation in extraction efficiency and chromatographic responses and is adequate especially in a 3-steps analytical method. Several compounds with chemical behavior similarities to the target analytes were tested but dihydrorotenone had the best analytical response. It showed two absorbance maxima at 241 and 297 nm, corresponding to the maxima of the analytes. The UHPLC-UV chromatograms at 295 (a) and 244 (b) nm of the yam bean seeds purified extract spiked with the IS are represented in Fig. 2: with retention times of 4.9 min, 6.1 min and 8.2 min for pachyrrhizine, rotenone and IS respectively we can see that the compounds are well separated with resolutions between each pair of compounds of interest superior to 1.5. Regarding the IS, its retention time is close to the analytes and furthermore it is a synthetical compound never found in plants.

3.5. Validation

3.5.1. Analytes and IS stability

Rotenone is known to be a sensitive compound and care was recommended for the handling of stock solutions [19,34] while nothing was known on the stability of pachyrrhizine and dihydorotenone. After 4 weeks at –18 °C the peak responses of pachyrrhizine, rotenone and dihydorotenone were 99.4% (SD = 1.8), 99.4% (SD = 2.7) and 97.4% (SD = 0.4), respectively, of the initial value T0. As all values were included between 95% and 105% the stability of the stock solutions was confirmed in the prescribed conditions [30].

For the choice of the temperature of the LC autosampler rack, a compromise had to be found because low temperatures limit pachyrrhizine’s solubility and high temperatures promote rotenone’s degradation. At 15 °C, after 20 h, the peak responses of rotenone, pachyrrhizine and dihydorotenone were 98.5% (SD = 0.6), 97.8% (SD = 0.6) and 98.8% (SD = 0.6), respectively, of the initial value. It means that all compounds were stable during at least 20 h in the analyses conditions.

3.5.2. Method selectivity and matrix effect

The evaluation of the selectivity of the method was realised by checking the purity of pachyrrhizine, rotenone and IS peaks by UHPLC-MS. The mass spectra analyzed at three retention times corresponding to the beginning, the middle and the end of the peak were compared to search for any interfering compound in this retention time window. The three mass spectra from the same peak were found very similar in each case (data not shown). Selectivity was evaluated on five different matrices (2 P. erosus and 3 P. ahipa) spiked with the IS showing the absence of interfering compound for the three molecules of interest.

Secondly by injecting blank samples after the most concentrated calibration standard, we made sure that there was no carry over.

As recommended for bioanalytical methods, the matrix effect was assessed. A quantitative approach was used in order to evaluate if the presence of the sample matrix had an influence on the analytes quantification. Three P. ahipa samples (AC102 accession) were analyzed three times by two methods: the “standard addition method” taking into account the matrix and the “neat calibration method” where the calibration standards are prepared in MeOH/acetonitrile/water. The results obtained for pachyrrhizine were 22.1 ± 0.7 μg/mL (mean ± SD) and 21.5 ± 0.9 and for rotenone 38.3 ± 0.7 and 37.1 ± 1.9, respectively. In both cases, a paired student t-test (α = 5%) could not show any statistical difference between both results. We concluded that matrix did not influence the quantification and that matrix-based calibrators were not required (neat calibrators could be used).

3.5.3. Response function and accuracy profiles

The response function represents, within the dosing range, the relationship between the analytical response (Y i.e. analyte peak area/IS peak area) and the introduced concentration of the analyte X in μg/mL. The calibration model was selected using a software that allows fitting several regression models and comparing them. The respective accuracy profiles were plotted and we looked for

![Fig. 2. UHPLC-UV chromatograms at 295 nm (a) and at 244 (b) of the yam bean seeds extract spiked with the IS. 1: pachyrrhizine, 2: rotenone.](image-url)
the narrower 90% $\beta$-expectation tolerance intervals included in the acceptance limits. For rotenone, several models such as linear regression, linear regression after logarithmic transformation or quadratic regression gave similar results but the last one gave the better compromise between the simplicity of the model, the LLOQ (lower limit of quantification), LOD (limit of detection), relative expanded uncertainty and the higher precision index. We chose thus the quadratic model for rotenone calibration and the linear model for pachyrrhizine calibration for the same reasons. The validation results with those models are presented in absolute values in Table 1. The resulting graphical accuracy profiles are illustrated in Fig. 3 showing the total error (i.e. systematic + random error) resulting from the procedure. It is represented in relative values at each one of the concentration levels tested. From there, the 90% $\beta$-expectation tolerance interval (Fig. 3: blue dashed lines) can be calculated. The method is declared valid when this interval is included within the acceptance limits (Fig. 3: black dotted lines). For pachyrrhizine, the procedure was validated on the whole domain (5–100 mg/mL) while for rotenone the 90% $\beta$-expectation tolerance interval was not included in the acceptance limits at the first level of concentration. Thus the procedure was validated for rotenone from the LLOQ to 80 mg/ml.

3.5.4. Trueness, precision and linearity

Trueness and precision are not considered here as decisional criteria but they allow evaluating respectively the systematic error (i.e. the relative bias) and random error produced by the analytical procedure. All values obtained for the two analytes are summarized in Table 1.

Table 1 Validation results for the two analytes.

<table>
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<th>Pachyrrhizine</th>
<th>Rotenone</th>
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<td>Quadratic ($Y = a' + b'X + c'X^2$)</td>
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<td>Mean introduced concentration</td>
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* Expressed in mg/mL.
* Expressed in RSD%.

Fig. 3. Accuracy profiles for pachyrrhizine (a) and for rotenone (b). The plain line is the relative bias, the dashed lines are the 90% $\beta$-expectation tolerance limits and the dotted lines represent the acceptance limits. The dots represent the relative error of the back-calculated concentrations and are plotted with respect to their targeted concentration. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
For pachyrrhizine the relative bias is below 10% while for rotenone it is below 8% except at one concentration level close to the LLOQ for which the relative bias is higher. This result is mainly due to one point (n=9) and the total error is still in the acceptance limits. The precision is expressed in term of repeatability (intra-day precision) and intermediate precision (inter-day). For pachyrrhizine it ranges between 5% and 10% for the repeatability and is around 10% for the intermediate precision. Regarding the precision of the procedure for rotenone, both repeatability and intermediate precision do not exceed 7% on the validated dosing range.

For the estimation of the linearity, the back-calculated concentrations were plotted against the introduced concentrations and a regression line was fitted and compared to the identity line \( y=x \). The parameters of the equation of the regression line for pachyrrhizine and rotenone are presented in Table 1: the values of the slopes close to 1 demonstrate that the procedure is linear.

### 3.5.5. Limits of detection and quantification

The LOD was estimated from the standard curve residual standard-deviation and slope. It gave values of the same order for pachyrrhizine and rotenone, respectively 1.2 and 1.5 \( \mu g/mL \) which correspond to 60 and 80 \( \mu g \) compound/g dried seeds. These LODs were verified experimentally: both compounds are still detectable at even lower concentrations.

The lower and upper limits of quantification validated were 5 and 100 \( \mu g/mL \) for pachyrrhizine which means that the given procedure allows assaying samples with the targeted substances concentrations between 0.25 and 5 mg pachyrrhizine/g dried seeds. For rotenone the validated lower and upper limits of quantification were respectively 11.6 and 80 \( \mu g/mL \) which correspond to 0.58 and 4 mg rotenone/g dried seeds.

### 3.5.6. Risk assessment and uncertainty

During routine analysis, many results are produced and assessing the risk or the probability for a measurement to be outside the acceptance limits is necessary.

For each of the two compounds, this probability linked with future measurements was evaluated using the accuracy profiles and the results obtained for each concentration levels are presented in Table 2. Globally, on the validated dosing range, there is less than 6% risk for the quantification of two compounds to fall outside the acceptance limits while a risk of 33% is tolerated for routine analysis according to the 4-6-15 rule of the FDA guidance (at least 4 upon 6 assays must be within \( \pm 20\% \) of their nominal values so1/3rd can fall outside the accepted limits).

In order to interpret and compare the results, their uncertainty has to be evaluated and the 95% confidence interval around the results where the true value may be has to be defined. It has been shown [35] that uncertainty can be related to precision and trueness from the data collected previously to establish the accuracy profiles. The estimation of uncertainty measurements for each concentration level is shown in Table 2 where a coverage factor of \( k=2 \) has been applied for the relative expanded uncertainty [36,37]. For pachyrrhizine, the relative expanded uncertainty is maximum 25% which is included in the acceptance limits and for rotenone, less than 15% which is one of the best performances already reported for this compound on a whole procedure.

#### 3.5.7. Application to the screening of genotypes

Once fully validated, this fast analytical method was implemented in routine. As batches of 12 samples can be extracted and purified (SPE) together, the total analysis time ranges between 30 and 35 min per sample and we were able to screen more than one hundred Pachyrhizus samples. They were belonging to different germplasm accessions/genotypes and to three cultivated species all harvested from two different eco-locations in Peru during two different years.

The highest values of pachyrrhizine and rotenone from the 126 samples assayed were 3.29 mg/g and 3.53 mg/g dried seeds, respectively. The values for pachyrrhizine are relatively low with 75% under 1.5 mg/g while only 25% of them were under 1.50 mg/g for rotenone. Mean values were 1.13 and 1.94 mg/g, respectively, but the dispersion of results is more important.

For two samples, the targeted substances reached such a low level that it was under the LLOQ: accession 209038 of \( P. ahipa \) contained less than 0.58 mg rotenone/g and accession 209044, an interspecific hybrid of two \( P. ahipa \) and \( P. tuberosus \) species, contained a little less than 0.25 mg pachyrrhizine/g. They both might be considered as accession with “low toxic seeds”.

The principal component analysis (PCA) allows studying in more details the data trends. As seen in Fig. 4a and b, the axes allowing the best separation of the samples are linked to the sum of pachyrrhizine and rotenone concentrations as well as to the difference of their concentrations. The first component PC1 explains up to 74.1% of the total variance while the second component PC2 explains 25.9% of the total variance. It is interesting to notice that as PC1 is related to the sum of the two analytes, the global toxicity of the sample is represented by the X-coordinate in Fig. 4a. The small angle between the vectors representation of pachyrrhizine and rotenone concentrations in Fig. 4b shows that they are strongly positively correlated. Biologically, it means that there are good odds that candidates with low rotenone levels would contain low amounts in pachyrrhizine too. It can be noticed that the samples with low content in the two toxic compounds correspond to accessions 209017, 209040 and 209044 (\( P. erosus \) and hybrids). As biosynthetic pathways of both compounds are interrelated it is interesting to observe that a low content in one of the compound would not be biologically compensated by an increase in the content of the other.

For \( P. tuberosus \) samples, rotenone contents are intermediate (between 1.56 and 2.92 mg/g) for all of them while for pachyrrhizine, two groups of samples can clearly be distinguished: T1, i.e.

### Table 2

Risk and uncertainty for the quantification of pachyrrhizine and rotenone in yam bean seeds.

<table>
<thead>
<tr>
<th>Concentration levels(^a)</th>
<th>Pachyrrhizine</th>
<th>Roonen</th>
<th>Rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk(^b) (%)</td>
<td>Uncertainty(^c)</td>
<td>Relative expanded uncertainty(^d) (%)</td>
</tr>
<tr>
<td>1</td>
<td>5.7</td>
<td>0.56</td>
<td>22.5</td>
</tr>
<tr>
<td>2</td>
<td>4.9</td>
<td>1.96</td>
<td>24.5</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>6.27</td>
<td>25.1</td>
</tr>
<tr>
<td>4</td>
<td>2.8</td>
<td>10.20</td>
<td>20.4</td>
</tr>
</tbody>
</table>

\(^a\) Corresponding to 5; 16; 50 and 100 \( \mu g/mL \) for pachyrrhizine and 4; 12.8; 40 and 80 \( \mu g/mL \) for rotenone;  
\(^b\) risk of having measurements falling outside of the acceptance limits;  
\(^c\) expressed in \( \mu g/mL \);  
\(^d\) computed using a coverage factor of 2.
accessions 209014, 209015 and 209020, with very low levels (< 0.5 mg/g) and T2, i.e. accessions 209010 and 209011 with levels over 1 mg/g.

In order to confirm the existence of groups based on genotypes (species or accession) and to obtain classification rules, discriminant analysis (DA) was carried out. Two discriminant components were obtained and the distribution of the samples in the plane generated by them is shown in Fig. 5. The area under the Receiver Operator Characteristic (ROC) curves varies from 0.89 (for \( P. \) erosus) to 1.00 (for T1 accessions of \( P. \) tuberosus) showing the good adequation of the classification rule. Finally, Fig. 5 allows differentiating genotypes with respect to their toxicity. From the second canonical axis, it can be seen that the various genotypes do not differ much in rotenone concentrations: genotypes T1, T2 and E have their centroid (mean) very close, genotype A has the smallest projection of its centroid on the second canonical axis and for H it is in between. Post-hoc Tukeys test after a significant \( p \)-value < 0.0001 ANOVA for rotenone concentration that detected significant differences for all genotypes except between genotypes A and E. To assess the impact of the genotypes simultaneously on rotenone and pachyrrhizine concentrations, and hence on the global toxicity a multiple ANOVA (MANOVA) was performed. A significant effect \( (p \)-value < 0.0001) of the genotypes was detected and a contrast showed that genotypes T1 has significantly lower concentration of both compounds than all the other genotypes. In fact the global concentration in toxic compounds of the genotypes follows the order: \( P. \) tuberosus T2 accessions > \( P. \) erosus (E) accessions > \( P. \) ahipa (A) accessions > \( P. \) tuberosus \( \times \) \( P. \) ahipa (H) accessions > \( P. \) tuberosus (T1) accessions. Another main difference between the two groups with lower content in toxic compounds is their dispersion: T1 is quite homogeneous while the hybrid group (H) is more scattered, which is logical as the H samples can originate from hybridization of \( P. \) tuberosus T1 and T2 accessions \( \times \) \( P. \) ahipa accessions and thus have a higher genetical heterogeneity. Moreover, it should be noted that hybrids (H) were sampled on two different locations while \( P. \) tuberosus T2 was sampled in just one, which may partially explain the heterogeneity in the results obtained for hybrids (H).

These results show that in yam bean seeds the genotype has an influence on the content of toxic compounds and that there is genetic variation for the content of toxic compounds in yam bean seed so that screening and selection of genotypes makes sense to find genotypes of yam bean seeds with less toxic compounds. Nevertheless the influence of the environment and the genotype by
environment interactions on the production of toxic compounds by the plant should be tested too as these parameters can influence as well the toxicity of yam bean seed.

4. Conclusions

To our knowledge, it is the first time that a quantification method is described for pachyrhizine. It also allows assaying rotenone simultaneously and more than 2-fold faster than the methods previously published for rotenone thanks to the use of UHPLC. We have shown in this study that the conditions for the fast MAE optimized for rotenone allowed as well the complete extraction of pachyrhizine. We also developed SPE parameters to obtain a good recovery of both pachyrhizine and rotenone which was validated in a 3-steps analytical method. This faster analytical procedure has also quite low LOD for the two compounds: 60 μg/g for pachyrhizine and 80 μg/g for rotenone in yam bean seeds while the dosediging interval ranges from 0.25 mg/g to 5 mg/g for pachyrhizine and from 0.58 mg/g to 4 mg/g for rotenone. DA analyses showed that there are good chances to low content for at least one toxic compound. Furthermore PCA and validated at different locations. Several samples originating from the plant should be tested too as these parameters can in heterogeneous with respect to pachyrhizine and rotenone contents in yam bean seeds. Screening for low or no toxicity in yam bean seeds merits further research and in the next steps the effects of genotypes, environments, and genotype by environment interactions on pachyrhizine and rotenone contents in yam bean seeds should be evaluated

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