

***Kakadu Plum Chemical Analysis: Cyanogens,
Alkaloids and Oxalate***

Rebecca Miller and Ian Woodrow

Plant Physiology Research Group
School of Botany
The University of Melbourne

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Summary

- None of the samples (including fruit tissue separated into seed and flesh) contained detectable levels of cyanogens. Sufficient tissue was used to detect levels down to $1 \mu\text{g g}^{-1}$ dry weight, which is harmless for human consumption.
- None of the samples contained detectable quantities of alkaloids. The method employed is the best and most commonly used one for alkaloid screening, but it must be noted that some alkaloids are not detected by this method. (There is no method or combination of methods that will detect all alkaloids.)
- Oxalate was clearly detectable in two of the three fruit samples. The mean concentration for the entire fruit was 16.6 mg g^{-1} dry weight. One fruit sample contained a mixture of organic acids that did not allow for clear quantification of oxalate using the HPLC method. The powdered samples were variable in oxalate content. One was similar to the fruit (about 10.5 mg g^{-1} dry weight), but the other two were much lower, with values estimated to be between 25 and $64 \mu\text{g g}^{-1}$ dry weight.

Methods

Sample preparation

Plum samples (DD26, MC01 and another batch named KP) were dissected into fruit and seed, snap frozen in liquid nitrogen, freeze dried and ground to a fine powder used for all analyses.

Cyanide concentration

The concentration of cyanide in kakadu plum tissue was measured by trapping cyanide liberated following hydrolysis of cyanogenic glycosides/compounds. Cyanogenic glycosides were hydrolysed by adding 1–2 mL buffer (0.1M citrate buffer – HCl pH 5.5) to different quantities of tissue (20, 50, 100 and 200 mg). Samples were analysed in triplicate, with and without the addition of non-specific enzymes – *Rhizopus* pectinase (which has non-specific glycosidase activity) and emulsin (cyanogenic β -glucosidase from almond) which cleaves a wide range of cyanogenic glycosides. Following incubation at 37°C for 24 hours. Cyanide trapped in a NaOH well was determined using a miniaturised cyanide assay adapted from Brinker and Seigler (1989) for use with a photometric microplate reader (Multiskan @ Ascent, with incubator, Labsystems, Helsinki, Finland). The absorbance was measured at 590nm with NaCN as the standard. In addition, 20 mg sample tissue was added to cyanogenic *Prunus* sp. tissue of known CN concentration, to investigate whether the KP tissue interfered with the glycosidase catalysed release of cyanide from *Prunus*.

Alkaloid detection (presence/absence)

Alkaloids were extracted and analysed by TLC using two protocols. Tissue was extracted (5 g) in 8 mL of acetic acid (10 %) in ethanol (v/v), or in 85% ethanol for 1 hour at RT, and centrifuged at $10\,000 \text{ g}$ for 20 minutes. The supernatant was collected, and filtered ($0.45 \mu\text{m}$), and concentrated *in vacuo*, resuspended in $500 \mu\text{L}$ extraction solvent and loaded ($30 \mu\text{L}$) on TLC silica gel G 60 plates. Two solvent systems were used – methanol:ammonium (200:3) and *n*-butanol:acetic acid:water (100:10:10). Plates were sprayed with Dragendorff's spray reagent (Sigma), a general reagent for the detection of alkaloids (e.g. Barbetti *et al.* 1987; Wagner & Bladt 1996; Harborne 1998; Vessel *et al.* 1999). Alkaloids appear as orange spots on a yellow background. Two positive controls (*Nicotiana tabacum* and *Alstonia scholaris*) were extracted and analysed. Dragendorff's reagent is the most common broad spectrum reagent used in the detection of alkaloids. However, it must be noted that it may not detect all alkaloids.

Oxalic acid concentration

Tissue (0.5 g) was extracted twice with 4 mL of 0.025 M HCl (as described by Tolrà *et al.* 1996). The extract was centrifuged at $10\,000 \text{ g}$ for 20 minutes, and the pooled supernatant was passed through a C₁₈ solid phase extraction cartridge (Phenomenex). After filtration through $0.45 \mu\text{m}$, an aliquot of the extract

(50 μL) was analysed by High Performance Liquid Chromatography (HPLC). Organic acids were separated using an HPX-86H Aminex column (Bio-Rad) with 0.025 M HCl as the mobile phase, at a flow rate of 0.5 mL min^{-1} . The retention time of an oxalic acid standard solution was used to identify the peak corresponding to oxalic acid, and peak integration of the standard was used to quantify the oxalic acid.

Results

Cyanogens

Sample	mass analysed	CYANIDE CONCENTRATION ² ($\mu\text{g/g}$ dry wt)		
		buffer only	buffer + emulsin (enzyme)	buffer + rhizopus pectinase
CD-2939	100 mg	0.0224	0.0123	-
CD-3280	100 mg	0.309	0.252	-
CD-3448	100 mg	0.123	0.377	-
MC-01 fruit	100 mg	0.012	0.121	-
MC-01 seed	100 mg	0.028	0.097	-
DD-26 fruit	100 mg	0.102	0.023	-
DD-26 seed	100 mg	0.000	0.000	-
KP ¹ fruit	50 mg	0.036	0.132	0.1590
KP fruit	100 mg	0.136	0.084	0.0517
KP fruit	200 mg	-	0.080	0.0912
KP seed	50 mg	0.3901	0.6320	0.6543

¹ The initial sample (KP; 3 masses) was analysed with buffer only, with the addition of emulsin (β -glucosidase from almond) and with rhizopus pectinase. Subsequent samples were analysed (100 mg) with buffer only, and with emulsin. Although it is rare, some tissues contain cyanogens but no enzymes to hydrolyse them. We therefore added the emulsin and pectinase mixtures to check for this possibility. It is extremely unlikely that there are cyanogens in the samples that cannot be detected by these protocols.

² Values less than 1 $\mu\text{g/g}$ would not significantly differ from noise in the assay. All values are means of 3 replicates, which gave consistent results. However, the results indicate that samples contain negligible cyanide.

Oxalic acid

Sample name	Oxalic acid concentration ($\mu\text{g/g}$ dry wt)
CD3448	10516.8
CD3280 ^a	25.5
CD2939 ^a	36.9 – 63.8
MC01 fruit	27563.3
MC01 seed	7678.1
KP fruit	19851.1
KP seed	11437.9
DD26 fruit ^b	-
DD26 seed ^b	-

^a For samples CD2939 & CD3280, there was no clear peak at 9.7 minutes corresponding to the elution time for oxalic acid (see chromatograms – Appendix 1). However, the chromatogram was not at the baseline, so while it is unlikely that there is oxalic acid present, we cannot rule out that there is a small amount. Hence, the area under the chromatogram between 9.6 - 10 minutes was used to determine an approximate oxalic acid concentration (effectively an upper limit).

^b We could not confidently determine the concentration of oxalic acid in the DD26 plum samples due to the complex nature of the mix in the sample. There were large overlapping peaks that interfered with the detection and quantification of oxalic acid at 9.7 minutes.

Alkaloids

TLC analysis showed that none of the samples contained detectable quantities of alkaloids. The method employed is the best and most commonly used one for alkaloid screening, but it must be noted that some alkaloids are not detected by this method. (There is not a method or combination of methods that will detect all alkaloids.) Two positive controls were used (tobacco and *Alsotonia*), which both showed clear alkaloid staining (Appendix 2).

References

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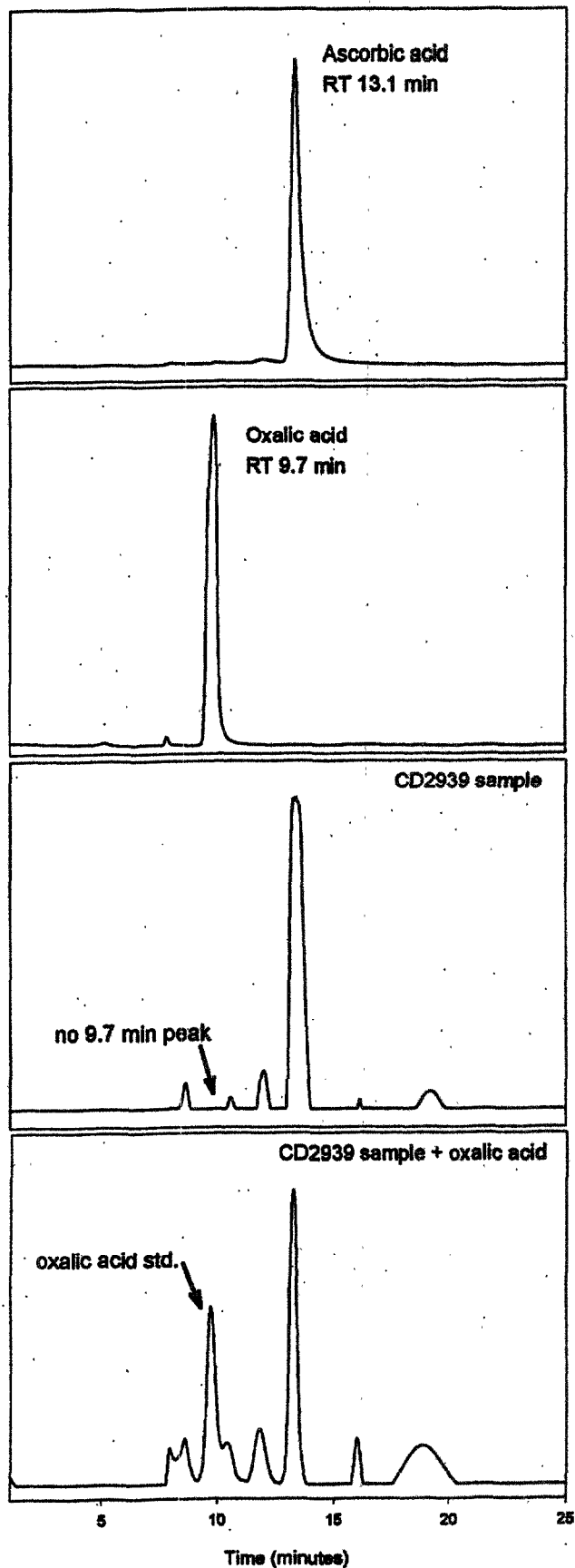
Department of Pharmacy and Pharmacology, University of Witwatersran, Johannesburg, South Africa.
Protocol: [Http://www.wits.ac.za/pharmacy/Pcognosyweb/Prac7.htm](http://www.wits.ac.za/pharmacy/Pcognosyweb/Prac7.htm)

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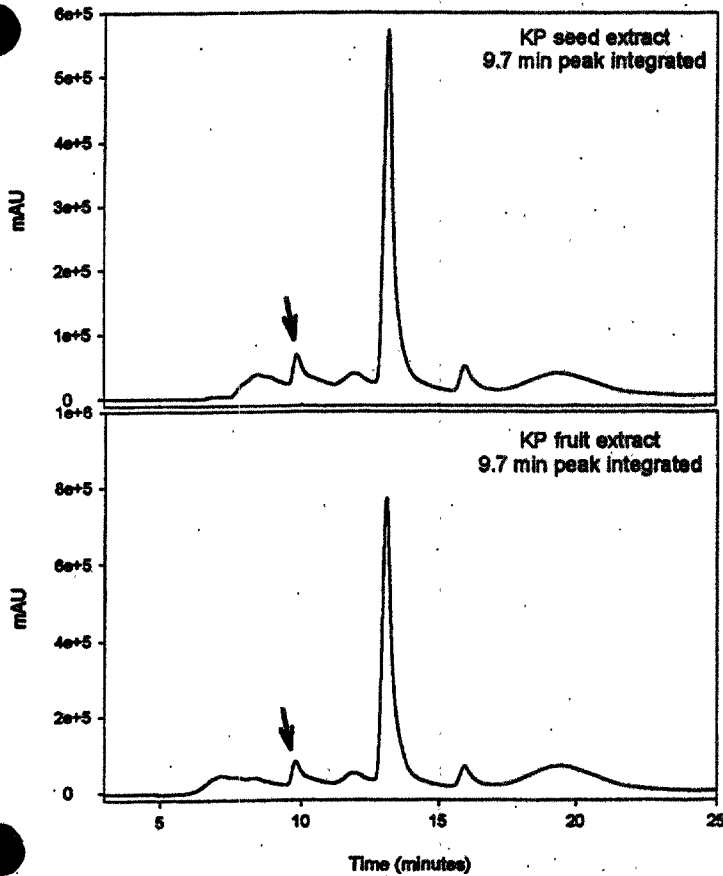
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APPENDIX 1: HPLC fractionation spectra.

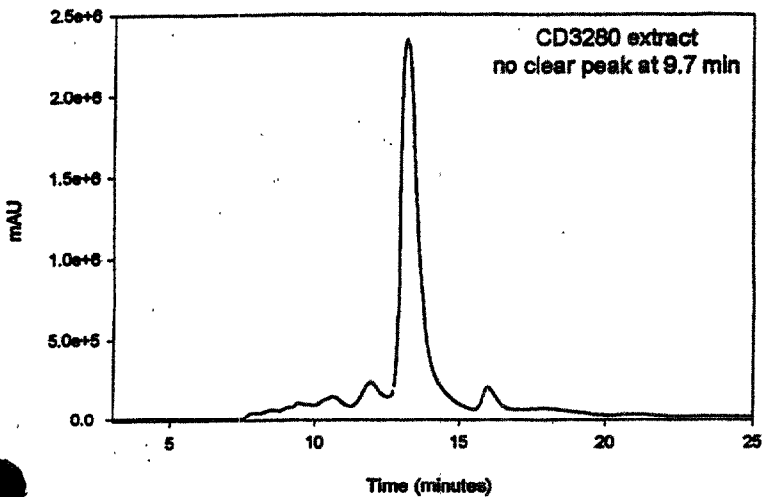


N.B. No oxalic acid was detected in the initial extractions of CD2939 and CD3280 samples. A second extraction from a larger mass of tissue was analysed, and oxalic acid concentration was approximated from the area under the chromatogram between 9.6 and 10 minutes.

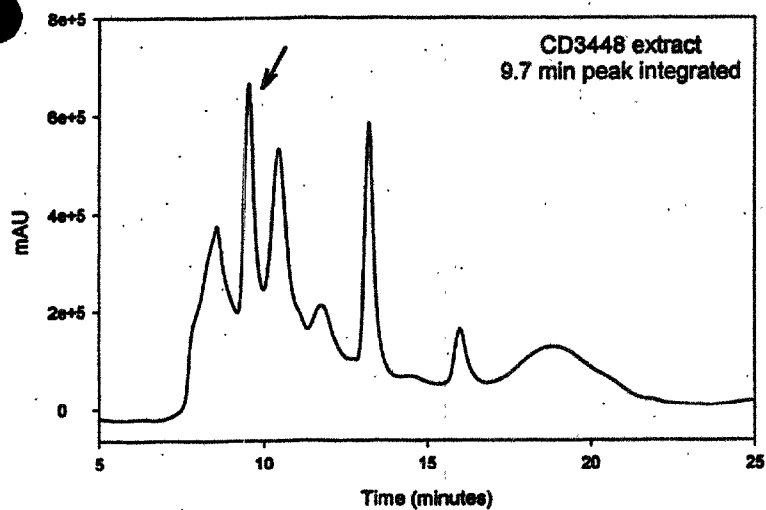
Example HPLC chromatogram (absorbance units v time) for KP seed and fruit extracts. The peak at 9.7 minutes corresponds to oxalic acid, and was integrated to determine the concentration relative to standard.



HPLC chromatogram for CD3280 extract illustrating the absence of a clear peak for oxalic acid at 9.7 minutes. The chromatogram is not at baseline, so the area under the chromatogram from 9.6 – 10 minutes was used to determine an approximate concentration or upper limit for oxalic acid. The profile for CD2939 was similar and the same process used to approximate oxalic acid concentration.



The 9.7 minute peak was integrated to determine the concentration of oxalic acid. CD3448 was extracted twice and the extract analysed multiple times to ensure repeatability.



APPENDIX 2. Alkaloid fractionation

1	Positive controls (tobacco, <i>Alsotonia</i>)
2	CD3448
3	CD2939
4	MC01 fruit
5	MC01 seed
6	DD26 fruit
7	CD3280
8	DD26 seed

Cyanogenic glycoside and oxalic acid content of edible plants

1. Cyanogenic glycosides

The concentrations of cyanide determined for Kakadu plum samples ranged from 0 – 0.632 $\mu\text{g CN/g dwt}$. The acute oral lethal dose of HCN for human beings is 0.5 - 3.5 mg/kg body weight for humans (see Jones 1998).

In a survey of cyanogenic species, samples yielding more than 10 $\mu\text{g CN/g fwt}$ were considered 'cyanogenic', samples yielding 2.5 – 10 $\mu\text{g/g fwt}$ were 'slightly cyanogenic' and any samples containing less than that were considered acyanogenic (Adsersen *et al.* 1988).

The cyanide content in cassava ranges from 240 – 890 mg CN/kg fwt in the tubers (Jones 1998). The threshold cyanide concentration, above which cassava needs to be detoxified is reported as 100 mg/kg fwt (Hegarty *et al.* 2001). Lima beans contain 100-4000 mg/kg fwt, sorghum grain contains 6 mg/kg fwt, and bamboo shoots contain as much as 8000 mg/kg. (Jones 1998). Macadamia nuts (*M. integrifolia*, *M. tetraphylla*) contain 0.12-0.15 $\mu\text{mol CN/g fwt}$ (3.2 – 3.9 $\mu\text{g/g fwt}$) (Dahler *et al.* 1995).

Table1 : Cyanide concentrations in some Australian bushfoods.

Nb. the threshold of detection for HCN was 0.1 mg CN/100g fwt (Hegarty *et al.* 2001), based on an indicator paper test. Using 0.5 as the conversion factor, (assuming 50% water content), this threshold equates to 20 $\mu\text{g/g dwt}$. The technique used to determine CN concentration in Kakadu plum samples is more sensitive.

Species	HCN (mg/100g fwt)
Lemon myrtle	0.52
Illawarra plum	0.29
Cinnamon myrtle	0.22
Kakadu Plum	<0.1

Source: Hegarty, M.P., Hegarty, E.E., Wills, R.B.H. (2001) Food Safety of Australian Bushfoods. Rural Industries Research and Development Corporation Publication No 01/28

2. Oxalic acid

The concentrations of oxalic acid determined for Kakadu plum samples ranged from 25.5 – 27563 $\mu\text{g/g dwt}$. Oxalic acid concentrations in fresh material have been reported for common food plants (Table 2), and some Australian bushfoods (Table 3). To enable comparison, conservatively, assuming 50% water content in samples, multiply dwt concentrations by 0.5.

Table 2: Levels of oxalic acid in common food plants

Species	Plant part	Quantity of oxalic acid (ppm)
<i>Fagopyrum esculentum</i> (buckwheat)	Leaf	111,000
<i>Averrhoa carambola</i> (star fruit)	Fruit	50,000 – 95,800
<i>Piper nigrum</i> (pepper)	Fruit	34,000
<i>Papaver somniferum</i> (Poppyseed)	Seed	16,200
<i>Rheum rhubarbarum</i> (rhubarb)	Leaf	13,360
<i>Spinacia oleraceae</i> (spinach)	Leaf	6,580
<i>Musa x paradisa</i> (banana)	Fruit	5,240
<i>Theobroma cacao</i> (cacao)	Seed	5,000
<i>Zingiber officinale</i> (ginger)	Rhizome	5,000
<i>Prunus dulcis</i> (almond)	Seed	4,073
<i>Anacardium occidentale</i> (cashew)	Seed	3,184
<i>Capsicum annum</i> (green pepper)	Fruit	1,171
<i>Ipomoea batatas</i> (sweet potato)	Root	1,000
<i>Glycine max</i> (soybean)	Seed	770
<i>Avena sativa</i> (oats)	Plant	400
<i>Cucurbita pepo</i> (pumpkin)	Juice	400
<i>Phaseolus vulgaris</i> (green/string bean)	Fruit	312
<i>Mangifera indica</i> (mango)	Fruit	300
<i>Solanum melongena</i> (eggplant)	Fruit	291
<i>Lycopersicon esculentum</i> (tomato)	Fruit	263
<i>Ribes nigrum</i> (black currant)	Fruit	200
<i>Manihot esculenta</i> (cassava)	Root	171

Source: Phytochemical database, USDA, ARS, NGRL, Beltsville Agricultural Research Center, Maryland, U.S.A. Web address: <http://www.ars-grin.gov/cgi-bin/duke/highchem.pl>

Table 3: concentration of oxalic acid in some Australian bushfoods

Species	Oxalic acid concentration (g/100g)
Kakadu plum	0.24
lemon myrtle	0.68 – 1.52
aniseed myrtle	0.5 – 1.49
wild rosella	0.11
bush tomato	0.10
mountain pepper	0.15

Source: Hegarty, M.P., Hegarty, E.E., Wills, R.B.H. (2001) Food Safety of Australian Bushfoods. Rural Industries Research and Development Corporation Publication No 01/28

For comparison, and method verification, silver beet, rhubarb and spinach were also analysed, and found to contain 0.53, 0.52, 0.53 g/100g, respectively, values similar to those reported by Wills (1997). Only lemon myrtle and aniseed myrtle contained oxalic acid in levels higher than those in mainstream fruit and vegetables (Hegarty et al. 2001 cite Wills 1997).

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