RIKILT
WAGENINGEN UR

Straightforward method to determine intact cyanogenic glucosides in almonds and flaxseeds by LC-MSMS

Patricia López, Elena de Vries and Hans Mol

Background

Cyanogenic glucosides (CNGs) are products of secondary metabolism of plants, which naturally occur in certain food crops, such as almonds, cassava, apple(seeds), linseeds and derived products. After consumption, when the plant tissue is disrupted, the CNGs are enzymatic hydrolysed thus causing the release of cyanide (HCN), which in high doses may cause health effects. Maximum limits for HCN have been set in EU Directive No 2002/32/EC for different feed materials and in EU Regulation No 1334/2008/EC for different food commodities.
A standardized method for control of CNGs (CEN 16160:2012) is based on the generation of HCN and its measurement by HPLC-Fluorescence. The method is quite laborious, time-consuming and entails all the risks associated to the manipulation of HCN. Besides, it lacks specificity.

Objectives

- Development and validation of a fast, straightforward method for the determination of the intact CNGs in food products by LC-MS/MS.
- Application of the method(s) for the analysis of almonds and flaxseed products and comparison of the results with CEN 16160:2012.

Results: Optimization of LC-MSMS conditions

The LC-MS/MS conditions to analyze the CNGs of interest (Figure 1) were optimized.

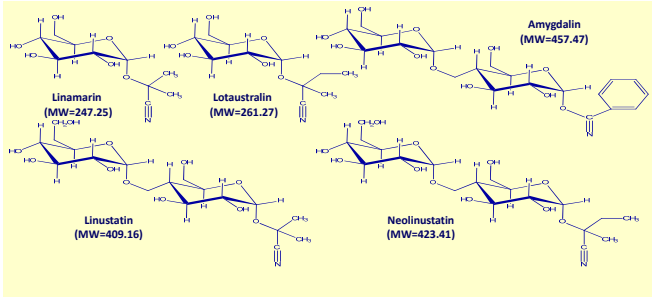

Figure 1. Cyanogenic glucosides (CNGs) under study.

The fragmentation pattern for the CNGs and the internal standard (phenyl-β-D-glucopyranoside) was optimized in ESI positive and negative mode by direct infusion in a Waters Micromass Quattro Ultima MS of the individual standard solutions at 10 μg/kg. ESI+ was observed to be more sensitive.

Table 1. MRM parameters in ESI+ mode and retention time under optimum conditions.

CNGs	RT (min)	Fragment	Cone (V)	CE (V)
Linamarin	4.22	265.3 > 248.2 265.3 > 179.9 265.3 > 163.1 265.3 > 144.9	20	10 10 10 15
Lotaustralin	5.54	279.3 > 179.9 279.3 > 163.1	20	10 10
Linustatin	4.22	427.1 > 410.4 427.1 > 325.2 427.1 > 163.1	20	10 10 15
Neolinustatin	5.11	441.2 > 424.2 441.2 > 162.9	20	10 15
Amygdalin	6.63	475.2 > 458.1 475.2 > 325.1 475.2 > 162.9	20	10 10 15
IS	6.05	274.2 > 180.1 274.2 > 162.9	20	5 10

- Precursor ion for all CNGs: ammonium adduct; common transition:loss of the ammonium.
- $[M+NH4]^+ > 325 \rightarrow$ diglucoside fragment of the CNG.
- $[M+NH4]^+ > 179 \rightarrow$ glucose fragment of the CNG.
- $[M+NH4]^+ > 163 \rightarrow$ glucose fragment with loss of O.

Mobile phases:
A: water + 0.1% formic acid + 5mM ammonium formiate
B: methanol/water (95:5, v/v) + 0.1% formic acid + 5mM ammonium formiate

Column X-Bridge C_{18} 150 x 3 mm (5μm) T=35 °C
Flow 0.4 mL/min

Results: optimization of the extraction

The extraction of CNGs from almonds and flaxseed was independently optimized. Half gram of sample was in a two-step procedure extracted with different solvents in triplicate (Figure 2 and 3). Temperature, organic solvents and/or acid conditions were used to denaturize enzymes.

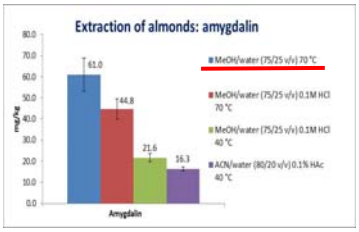

Figure 2. Optimization of the extraction of amygdalin from almonds.

- **Almonds**: Extraction of amygdalin: MeOH/water (75/25) at 70 °C (figure 2).
- **Flaxseed** (figure 3)
  - (Neo)linustatin: no significant differences.
  - Linamarin: ACN/water (80/20) 1%HAc at 40 °C.

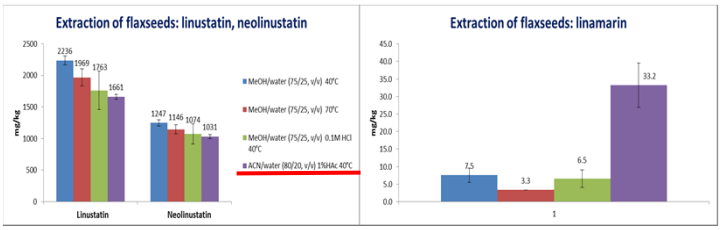

Figure 3. Optimization of the extraction of linustatin, neolinustatin and linamarin from flaxseed.

After the first extraction with 2 mL for 30 min, the extract is centrifuged, the supernatant collected and the residue extracted a second time. After centrifugation, the supernatants are merged and the volume adjusted to 5 mL. The extract is 10-fold diluted before LC-MS/MS analysis.

Results: analysis of samples: LC-MS/MS vs CEN 16160

Table 2. Occurrence of CNGs in almonds samples.

Sample	LC-MSMS mg amygdalin/kg	LC-MSMS mg HCN/kg	CEN 16160 mg HCN/kg
Almonds roasted	72	5.0	5.0
Almonds	894	53	66
Almonds flaked	19	1.1	2.2
Almond flour	118	6.9	15

Table 3. Occurrence of CNGs in flaxseed samples.

Sample	LC-MSMS Linustatin mg/kg	LC-MSMS Neolinustatin mg/kg	LC-MSMS Linamarin mg/kg	LC-MSMS Lotaustralin mg/kg	LC-MSMS mg HCN/kg	CEN 16160 mg HCN/kg
Flaxseed oil	-	-	-	-	0.0	0.0
[1]Flaxseed	1347	3253	112	484	341	410
[1]Flaxseed broken	1772	1069	44	39	194	241
[1]Flaxseed whole	1790	2447	147	290	319	386
Flaxseed golden	1170	1641	144	22	220	275

[1]Brown flaxseed

- LC-MS/MS gave slightly lower concentration of HCN than CEN. (Possible explanations: lotaustralin is semi-quantified, presence of other compounds that produce HCN, interference in the CEN method).

Conclusions

- Optimum extraction conditions may depend on compound and matrix.
- LC-MS/MS is a promising alternative for HCN determination.
- Further research is needed to explain differences between the LC-MS/MS and the CEN method.

Acknowledgment: This research was financially supported by the Dutch Ministry of Economic Affairs

RIKILT Wageningen UR
P.O. Box 123, 6700 AB Wageningen
Contact: patricia.lopezsanchez@wur.nl
T + 31 (0)317 48 02 81
www.wageningenUR.nl/en/Expertise-Services/Research-Institutes/rikilt.htm