

Development of an HPTLC method for determination of hypoglycin A in aqueous extracts of seedlings and samaras of *Acer* species.

J.A. Habyarimana¹, E. Baise², C. Douny³, M. Weber¹, F. Boemer⁴, P. De Tullio⁵, T. Franck⁶, C. Marcillaud-Pitel⁷, M. Frederich⁸, A. Mouithys-Mickalad⁶, E. Richard⁹, M.-L. Scippo³, D. Votion¹⁰ and P. Gustin¹

1. Department of functional Sciences, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of veterinary Medicine, University of Liege, 4000 Liege – Belgium
2. Department of animal Productions: Biostatistics, Economy and animal selection, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liege, 4000 Liege – Belgium
3. Department of Food Sciences, Laboratory of Food Analysis, FARAH - Veterinary Public Health, University of Liège, University of Liege, 4000 Liege – Belgium
4. Biochemical Genetics Laboratory, CHU, University of Liege, 4000 Liege – Belgium
5. Center for Interdisciplinary Research on Medicines (CIRM), Medicinal Chemistry, University of Liege, 4000 Liege – Belgium
6. Centre de l'Oxygène, Recherche et Développement (CORD) and Fundamental and Applied Research for Animals & Health (FARAH), University of Liege, 4000 Liege – Belgium
7. Réseau d'Epidémiologie et de Surveillance en Pathologie Equine (RESPE), 14000 Caen - France
8. Center for Interdisciplinary Research on Medicines (CIRM), Pharmacognosie, University of Liege, 4000 Liege – Belgium
9. Normandie Université, UNICAEN, SF 4206 ICORE / LABÉO Frank Duncombe, Caen Cedex 4, France
10. Equine Pole, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liege, 4000 Liege – Belgium

Hypoglycin A (HGA) is a toxin contained in seeds of the sycamore maple tree (*Acer pseudoplatanus*). Ingestion of this amino acid causes equine atypical myopathy (AM) in Europe. Another variety, *A. negundo*, is claimed to be present where AM cases were reported in the US. For unknown reasons, occurrence of this disease has increased. It is important to define environmental key factors that may influence toxicity of samaras from *Acer* species. In addition, the content of HGA in seedlings needs to be determined since AM outbreaks, during autumn period when the seeds fall but also during spring when seeds are germinating. The present study aims to validate a reliable method using high performance thin layer chromatography for determination and comparison of HGA in samaras and seedlings.

The working range of the method was between 20 µg HGA to 408 µg HGA per ml water, corresponding to 12 - 244 mg/kg fresh weight or 40 - 816 mg/kg dry weight, taking into account of an arbitrary average dry matter content of 30%. Instrumental limit of detection and limit of quantification were of 10 µg HGA/ml and 20 µg HGA/ml water, respectively. Instrumental precision was 4% (RSD on 20 repeated measurements) while instrumental accuracy ranged between 86% and 121% of expected value. The HGA recovery of the analytical method estimated from spiked samaras and seedlings samples ranged between 63 and 103%. The method was applied to 9 samples of samaras from *Acer pseudoplatanus*, *A. platanoides* and *A. campestre* and 5 seedlings samples from *A. pseudoplatanus*. The results confirm detection of HGA in samaras from *A. pseudoplatanus* and the absence of detection in samaras of other tested species. They also suggest that detected levels of HGA are highly variable. This confirmed the suitability of the method for HGA detection in samaras or seedling.

Keywords: Hypoglycin A, HPTLC, validation, equine atypical myopathy.

46 INTRODUCTION

47 Hypoglycin A (HGA) is assessed to be a potential inhibitor and a key ingredient involved in the metabolism
48 process of fatty acids. This nonproteinogenic amino acid is contained in ackee (*blighia sapida*), a fruit found
49 in Jamaica [1] and in seeds of some maple species [2], like the sycamore maple tree (*Acer pseudoplatanus*).
50 Ingestion of HGA causes equine atypical myopathy (AM) in Europe [3; 4]. These diseases are characterized
51 by the abrupt symptoms onset resulting from the acute necrosis of postural, respiratory muscles and the
52 myocardium [4]. Horses exhibit depression, weakness, stiffness, recumbency, trembling, sweating and
53 dyspnea [5; 6].

54 Data from epidemiological studies have shown the seasonality of AM with outbreaks in autumn that are often
55 followed by smaller outbreaks in the subsequent spring [6], resulting from HGA ingestion contained in seeds
56 and seedlings, respectively [7]. All the circumstances leading to AM are not yet fully established, with an
57 increasing number of cases in the recent years [8]. More than 1800 horses throughout Europe have suffered
58 from AM since 2006 (data from the Atypical Myopathy Alert Group website; [http://www.myopathie-](http://www.myopathie-atypique.be)
59 [atypique.be](http://www.myopathie-atypique.be)), with a mortality rate of 74% [6].

60 To our knowledge, no curative treatment has been so far documented. Therefore, preventive measures will be
61 of high importance and can only be managed by identifying the sources and the rate of exposure of grazing
62 horses to HGA as well as gaining a good understanding of the factors that influence the concentration of HGA
63 in the horse's environment. The seasonality of AM outbreak and occurrence of sporadic cases lead us to
64 question the importance of climatic conditions and pasture composition in the development of this disease.
65 These points deserve further investigations to improve preventive measures.

66 Clarifying the role of maples in AM [9] requires a validated methodology for quantifying HGA in plant
67 extracts. Thus, high performance thin layer chromatography (HPTLC) can be used as a cheap and quick
68 diagnostic tool to measure HGA toxin in a reliable way in maple extracts and useful for valid comparisons
69 between samples collected in a standardized way.

70 A literature survey concerning HGA problematic revealed that no readily available method, being both
71 practical and cheap, has been developed until now. Indeed, the literature reports conventional chemistry
72 techniques, such as high performance liquid chromatography (HPLC) [10; 11] for determination or

73 quantification of HGA in biological samples [12] in ackee fruit [13], but not in maple samaras, nor in other
74 plant extracts, such as seedlings or leaves. Determination and quantification of HGA in plant extracts using
75 HPTLC, under standardized conditions appeared to be suitable for quick screening. This quantification was
76 done without any special pre-treatment of the sample and using relatively cheap material.

77 **OBJECTIVES**

78 The present study aims to develop a high performance thin layer chromatography (HPTLC) technique as a
79 cheap and quick diagnostic tool to determine HGA toxin in a reliable way in maple plant material extracts.
80 The technique will be tested on seedlings of *A. pseudoplatanus* and various samaras samples collected on
81 maple trees (*A. pseudoplatanus*, *A. platanoides* and *A. campestre*).

82 **STUDY DESIGN**

83 Hypoglycin A was extracted from seedlings and samaras using methanol. After methanol evaporation, the
84 dry extracted was solubilized in water before HPTLC analysis. Different known concentrations of solutions
85 of a commercial HGA standard were used to establish a calibration curve, by plotting the signal intensity
86 against the concentration. Quantification of HGA in plant samples was performed by measuring the signal
87 intensity of the dot on the HPTLC plate corresponding to HGA. This analytical procedure was validated for
88 parameters such as linearity, limit of detection and quantification, precision and accuracy. The analysis of 14
89 plant samples (supposed to contain or not HGA) was also performed.

90 **METHODS**

91 **REAGENTS AND PREPARATION OF STANDARD SOLUTIONS**

92 Methanol of analytical grade was obtained from VWR International® (Leuven, Belgium). Water was purified
93 using a Millipore® filtering system (Merck Millipore). S-Hypoglycin A (HGA, 85%) was provided by Toronto
94 Research Chemicals Inc. (TRC®, Canada). Ninhydrin (powder, 99%) and acetonitrile of analytical grade
95 (98%) were provided by UCB, Leuven in Belgium.

96 **PLANT MATERIAL**

97 Samaras are considered as indehiscent dry fruits called akene. In *Acer*, samaras are associated by pair. Each
98 samara contains one seed. Samaras are especially produced with a pericarp protrusion shaped like a
99 membranous wing. This wing form has a seed spreading function. The shape of the pericarp varies from one
100 species to another. This set (seed and pericarp) is considered as a whole unit and is named “samara” in this
101 study (Fig 1).

102 Specific sites were selected in the areas of pastures where cases of AM were clinically confirmed. Samaras
103 were collected from June to October 2016 directly on the tree and analyzed within 2 days. Seedlings from *A.*
104 *pseudoplatanus* (2 leaves stage) (Fig 1) were collected from March to May 2016 and stored directly at -80°C
105 until analysis. They were collected around mature identifiable and recognizable trees of the same species.
106 The developed HPTLC method was applied to 9 samples of samaras (five from *A. pseudoplatanus* species,
107 four from *A. plantanoides* and one from *A. campestre*) and five samples of seedlings from *A. pseudoplatanus*
108 at early stage.

109 **HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY ANALYSIS**

110 Chromatographic separation was performed using silica plates (60Å F254, 20 × 10 cm or 10 × 10, Merck
111 Darmstadt, Germany). Three microliters of standards and samples were applied as bands of 4 mm wide, 0.3
112 mm high and 6 mm apart, using a CAMAG Linomat 5[®] sample applicator equipped with a 100 µl Hamilton[®]
113 syringe. Samples were applied at 15 mm from the bottom edge of the chromatographic plate. The plates were
114 allowed to dry for 15 min before elution using a solution of methanol/acetic acid/water (70:20:10, v/v/v). The
115 developing distance was 85 mm, measured from the lower edge of the plates. Post-chromatographic
116 derivatization was obtained by three thin sprays of ninhydrin solution (0.2% in methanol) in 3 different
117 directions [14]. The plate was incubated at 110 °C for 5 min for visualization of the spots. Migration distance
118 was measured and retention factor (RF) was calculated. Chromatograms were obtained by reading the plate at
119 490 nm using a CAMAG[®] TLC scanner 3 and the WinCATS[®] 4.3 software (CAMAG, Muttenz, Switzerland).
120 A negative control (procedure blank) and a positive control (samara sample containing 150 mg HGA/kg fresh
121 weight) are analyzed on each plate. Unknown samples extracts are spotted in triplicate (Fig 2).

122 CALIBRATION CURVE

123 Ten concentration points were used, fulfilling the criteria required by international conference on
124 harmonization (ICH) concerning the calibration curve [15]. The calibration curve was constructed using HGA
125 solutions in water at respective concentrations of 0, 10, 20, 40, 85, 97, 147, 199, 302, 408 and 510 $\mu\text{g/ml}$ from
126 which 3 μl were loaded on the plate.

127 VALIDATION PARAMETERS

128 The analytical procedure was validated according to the guidelines from the ICH of technical requirements for
129 registration of pharmaceuticals for human use [15]. According to these guidelines, the following parameters
130 are to be assessed: specificity, linearity, range, accuracy, precision (intra-day and inter-day precision),
131 detection limit and, quantification limit. In addition, recovery was assessed from HGA spiked samara and
132 seedlings samples.

133 Specificity was assessed by analyzing procedure blanks (the whole analytical procedure, but without sample).
134 Instrumental precision and accuracy was evaluated by the repeated ($n=5$) analysis of solutions of HGA in
135 water at 3 different concentrations (57; 177 and 283 $\mu\text{g/ml}$), on 4 different days. For each level of
136 concentration, precision was expressed as the coefficient of variation (CV) associated to the mean
137 concentration calculated from the repeated measurement, while for accuracy, the mean measured
138 concentration was expressed as a percentage of the theoretical concentration, considered as the “true” value.

139 According to ICH guidelines [16], limit of detection (LOD) was visually evaluated based on calibration curve
140 and selected value tested for reliability. Limit of quantification (LOQ) corresponded to the first point of the
141 calibration curve which was quantified with acceptable accuracy and precision.

142 Recovery of the method was determined using the standard addition method [17]. Samaras and seedlings (5
143 g fresh weight), spiked with two amounts of HGA (212 and 408 μg , respectively, corresponding to 127 and
144 245 mg/kg fresh weight, respectively) as well as unspiked, were extracted as described above (5 repeated
145 analysis). The amount of HGA measured from the unspiked samples was subtracted from the amount
146 measured in the spiked sample. The resulting amount was compared to the spiking level and the recovery
147 was expressed as a percentage of the spiking level.

148 *Stability of HGA during the chromatographic analysis*

149 The stability of HGA during the chromatographic analysis was investigated. This was assessed with standard
150 solution of HGA (200 µg/ml) in water and tested by a 2-dimensional HPTLC development. This was
151 performed by two successive HPTLC developments, in 2 perpendicular directions. At the end of these two
152 consecutive operations, components were detected and located in relation to the diagonal axis of the
153 chromatogram (Fig 3). No additional spot was observed.

154 **OPTIMIZATION OF EXTRACTION PROCEDURE**

155 *Determination of the adequate solvent*

156 Plant material (samara or seedlings) were precisely weighed and crushed. They were mixed with three
157 different solvent (methanol, water or acetonitrile) in three separate sealed vials. A permanent agitation was
158 ensured in order to promote a good contact with solvent. Three arbitrary set extraction periods were planned
159 for solvent collection before analysis of the samples by HPTLC. The concentration of HGA was then
160 calculated and necessary corrections were applied, as for the volume (Fig 4A).

161 *Determination of contact time with adequate solvent*

162 Time contact between plant materials was assessed by placing precise amount of samples in a sealed
163 container in presence of the selected solvent. After determined periods, some solvent was collected and
164 analyzed to determine HGA concentration. Eighteen measures were performed in a period of time spaced
165 from 15 min to 72 hours. Decision was taken to work at laboratory temperature to ensure comfortable and
166 repeatable work conditions and avoid any HGA heat-related alteration (Fig 4 B and C).

167 *Stability of HGA during extraction process*

168 A solution of HGA was kept in a sealed vial, in the same conditions than applied with plant material for
169 extraction (laboratory temperature and permanent shaking). Several samples were then collected and
170 analyzed in a period of time from 15 min to 72h (Fig 4 D).

171 **DETERMINATION OF THE RECOVERY RATE**

172 The determination of the HGA recovery rate was performed by using the standard addition method
173 technique [17]. Three increasing amounts of HGA were added to 3 containers, corresponding to

174 three different levels of HGA concentrations, compatible with the HGA concentrations reported in
175 the literature. The analysis was performed in triplicate. .

176 **EXTRACTION OF SAMARA AND SEEDLINGS EXTRACTS**

177 Five grams of fresh weight sample were mechanically grinded using a household mixer (Moulinex,
178 Moulinette[®]), were mixed with 25 ml of methanol and extracted during 24 hours at laboratory temperature
179 (about 20°C) under permanent mild shaking. After centrifugation (10000 g, 10 min), 12,5 ml of supernatant
180 was evaporated to dryness, and the dry residue was dissolved in 3 ml of water.

181 For each sample, dry matter was measured independently after 72 hours drying in an oven at 70 °C. The
182 amount of HGA in the sample was expressed in mg/Kg dry matter.

183 **RESULTS AND DISCUSSION**

184 **CHARACTERISTICS AND VALIDATION OF THE ANALYTICAL METHOD**

185 HGA showed a RF of 0.51 ± 0.01 , determined from the analysis of 10 measurements of HGA solutions (170
186 $\mu\text{g/ml}$). Appropriate separation is commonly accepted when $0.1 < R_f < 0.9$ [18]. Figure 2 shows a picture of
187 the plate after revelation loaded with a calibration curve (Table 1), a negative (procedure blank) and a positive
188 control and samples from *A. pseudoplatanus*. The procedure blank showed no spot at the RF of HGA, and
189 generated no peak after 490 nm reading, showing the good specificity of the method.

190 The validation parameters of the HPTLC analytical procedure are as follow: the dose response relationship
191 was linear in the working range of 20 to 408 $\mu\text{g/ml}$ HGA in water (corresponding 12-245 mg HGA/kg fresh
192 weight or 40-816 mg HGA/Kg dry matter of 30% content). Samples extracts displaying a response above the
193 upper limit of the working range are diluted and reanalyzed.

194 LOQ was set at 20 $\mu\text{g/ml}$, corresponding to the first point of the calibration curve determined with acceptable
195 accuracy and precision (7% and 19% respectively). Accuracy and precision were of 37% and 16%
196 respectively for the lowest concentration spotted on plate (10 $\mu\text{g/ml}$). This concentration was considered as
197 the LOD. Expressed in mg/kg of plant, the LOD was 6 mg/kg fresh weight and 20 mg/kg dry matter; taken
198 into account of a dry matter arbitrary set to 30% (dry matter content of samara and seedlings is highly

199 variable according to sampling conditions). The LOQ was 12 and 40 mg/kg fresh weight and kg dry matter,
200 respectively. These results are gathered in table 1.

201 The recovery of the method ranged from 78% to 103% for the levels of concentrations added to samara
202 samples, while 63% to 83% recovery was observed in seedlings. Recovery was tested with HGA
203 concentrations of 42 and 85 mg/kg fresh weight. These levels of recovery were considered as acceptable and
204 consistent with a diagnostic tool to determine the sources of HGA, as targeted in this study.

205 Intra-day and inter-day instrumental precision and accuracy of the method were calculated (Table 1). Intra-day
206 precision, expressed as a relative standard deviation (RSD), is ranging from 0 to 3%. As expected, inter-day
207 precision is a little bit higher (4%). For accuracy, inter-day accuracy appears to be better (89; 114% and 95%
208 of the introduced HGA concentration for HGA concentrations in water of 57; 177 and 283 µg/ml,
209 respectively) than intra-days accuracies, which ranged from 86 to 121%, depending on the target
210 concentration and the day of analysis. Generally, a range of 80 to 120% of the target value is considered as
211 acceptable for accuracy (Table 2).

212 During routine analysis of unknown samples, a positive control is analyzed on each plate, consisting of a
213 samara sample containing 150 mg HGA/kg fresh weight. The results of the plate are accepted only if the HGA
214 concentration measured in the positive control is in the range 120 - 180 mg/kg fresh weight.

215 ANALYSIS OF PLANT SAMPLES

216 Table 3 shows results of HGA concentration determined in samples of samara and seedlings as well as their
217 dry matter content. For Samaras from *A. platanooides* and *A. campestre*, no spot could be observed and no
218 peak could be detected in the area corresponding to the RF expected for HGA. This result is supported by
219 the literature [19]. In Samaras from *A. pseudoplatanus*, HGA concentrations ranged from 678.3 and 918
220 mg/kg fresh weight (or 2614 to 3104.6 mg/kg dry matter), while in seedlings of the same species, HGA
221 concentrations ranged from 729 and 3028.4 mg/kg fresh weight (or 2563.4 to 11397 mg/kg dry matter)-
222 These results show a high variability of HGA concentrations in *A. pseudoplatanus* samaras. Samples were
223 taken at different times through the season, regardless of factors like rain and morning dew. Effects such as
224 seeds leaching or sun drying cannot be seen in our results. The HGA concentrations found in samaras and

225 seedlings in this study are comparable to those found by Unger and co-workers [20], but are slightly lower
226 than those reported by Westermann and collaborators [19].

227 **MAIN LIMITATIONS**

228 No harmonized measure allows easy comparison of different studies, over a wide variety in results, whether
229 between different plant species or within the same group, or even because of collecting season. Laboratory
230 practice also matters: considering the whole samara or selected fragments allow to express results in so little
231 comparable way. The main limitation of the method lies in the fact that despite its precision it does not give
232 the amount of HGA ingested by the animal but provides an indicative value of its availability in the
233 environment of the horse.

234 **CONCLUSIONS**

235 A simple, cost effective, specific and accurate HPTLC method for detection, determination and
236 quantification of HGA in maple extracts from *A. species* has been developed and validated. This
237 standardized method was shown to be applicable as well on samaras as on seedlings.

238 It is also conceivable that with some proper adjustments and calibration, our method can quickly be adapted
239 for other vegetal samples as well as leaves or stems or adapted to other vegetal species as grass for example.
240 This precise and flexible method, easily adaptable, brings a valuable input and can become an interesting
241 tool in the research for a cheap, rapid test apparatus in the diagnosis of AM or seasonal pasture myopathy
242 resulting from HGA intoxication in horse.

243 **ACKNOWLEDGMENTS**

244 Dr Ursula Fogarty from the Irish Equine Center is gratefully acknowledged for advices regarding the
245 manuscript. The study was supported by the “Institut français du cheval et de l'équitation (*Ifce*)” of France
246 and by “Les Fonds Spéciaux pour la Recherche (FSR)” of Liege University (Belgium).

247 Conflict of Interest: authors disclose no conflict of interest.

248 REFERENCES

- 249 [1] Hassall, C.H. and Reyle, K. (1954) Hypoglycin A,B: biologically active polypeptides from *Blyghia*
250 *sapida*. *Nature* **173**, 356-357.
- 251
252 [2] Fowden, L. and Pratt, H.M. (1973) Cyclopropylamino acids of the genus *Acer*: Distribution and
253 biosynthesis. *Phytochemistry* **12**, 1677-1681.
- 254
255 [3] Votion, D.M., van Galen, G., Sweetman, L., Boemer, F., de Tullio, P., Dopagne, C., Lefere, L.,
256 Mouithys-Mickalad, A., Patarin, F., Rouxhet, S., van Loon, G., Serteyn, D., Sponseller, B.T. and
257 Valberg, S.J. (2014) Identification of methylenecyclopropyl acetic acid in serum of European horses
258 with atypical myopathy. *Equine veterinary journal* **46**, 146-149.
- 259
260 [4] Cassart, D., Baise, E., Cherel, Y., Delguste, C., Antoine, N., Votion, D., Amory, H., Rollin, F., Linden,
261 A., Coignoul, F. and Desmecht, D. (2007) Morphological alterations in oxidative muscles and
262 mitochondrial structure associated with equine atypical myopathy. *Equine veterinary journal* **39**,
263 26-32.
- 264
265 [5] Votion, D.M., Linden, A., Saegerman, C., Engels, P., Ercicum, M., Thiry, E., Delguste, C., Rouxhet, S.,
266 Demoulin, V., Navet, R., Sluse, F., Serteyn, D., van Galen, G. and Amory, H. (2007) History and
267 clinical features of atypical myopathy in horses in Belgium (2000-2005). *Journal of veterinary*
268 *internal medicine / American College of Veterinary Internal Medicine* **21**, 1380-1391.
- 269
270 [6] van Galen, G., Marcillaud Pitel, C., Saegerman, C., Patarin, F., Amory, H., Baily, J.D., Cassart, D.,
271 Gerber, V., Hahn, C., Harris, P., Keen, J.A., Kirschvink, N., Lefere, L., McGorum, B., Muller, J.M.,
272 Picavet, M.T., Piercy, R.J., Roscher, K., Serteyn, D., Unger, L., van der Kolk, J.H., van Loon, G.,
273 Verwilghen, D., Westermann, C.M. and Votion, D.M. (2012) European outbreaks of atypical
274 myopathy in grazing equids (2006-2009): spatiotemporal distribution, history and clinical features.
275 *Equine veterinary journal* **44**, 614-620.
- 276
277 [7] Baise, E., Habyarimana, J.A., Amory, H., Boemer, F., Douny, C., Gustin, P., Marcillaud-Pitel, C.,
278 Patarin, F., Weber, M. and Votion, D.M. (2015) Samaras and seedlings of *Acer pseudoplatanus* are
279 potential sources of hypoglycin A intoxication in atypical myopathy without necessarily inducing
280 clinical signs. *Equine veterinary journal*.
- 281
282 [8] van Galen, G., Saegerman, C., Marcillaud Pitel, C., Patarin, F., Amory, H., Baily, J.D., Cassart, D.,
283 Gerber, V., Hahn, C., Harris, P., Keen, J.A., Kirschvink, N., Lefere, L., McGorum, B., Muller, J.M.,
284 Picavet, M.T., Piercy, R.J., Roscher, K., Serteyn, D., Unger, L., van der Kolk, J.H., van Loon, G.,
285 Verwilghen, D., Westermann, C.M. and Votion, D.M. (2012) European outbreaks of atypical
286 myopathy in grazing horses (2006-2009): determination of indicators for risk and prognostic
287 factors. *Equine veterinary journal* **44**, 621-625.
- 288
289 [9] Gillman, J.H., Hegeman, A.D. and Sharp, R.G. (2014) Clarifying the role of maples in atypical
290 myopathy. *Equine veterinary journal* **46**, 135-136.

- 291
292 [10] Golden, K.D., Williams, O.J. and Bailey-Shaw, Y. (2002) High-performance liquid chromatographic
293 analysis of amino acids in ackee fruit with emphasis on the toxic amino acid hypoglycin A. *Journal*
294 *of chromatographic science* **40**, 441-446.
- 295
296 [11] Bowen-Forbes, C.S. and Minott, D.A. (2011) Tracking hypoglycins A and B over different maturity
297 stages: implications for detoxification of ackee (*Blighia sapida* K.D. Koenig) fruits. *Journal of*
298 *agricultural and food chemistry* **59**, 3869-3875.
- 299
300 [12] Kean, E.A. (1978) Assay of hypoglycin in biological samples. *Anal. Biochem.* **90**, 402-407.
- 301
302 [13] Gaillard, Y., Carlier, J., Berscht, M., Mazoyer, C., Bevalot, F., Guitton, J. and Fanton, L. (2011) Fatal
303 intoxication due to ackee (*Blighia sapida*) in Suriname and French Guyana. GC-MS detection and
304 quantification of hypoglycin-A. *Forensic Sci. Int.* **206**, e103-107.
- 305
306 [14] Dutt, M.C. and Poh, T.T. (1980) Use of ninhydrin as a spray reagent for the detection of some basic
307 drugs on thin-layer chromatograms. *Journal of chromatography* **195**, 133-138.
- 308
309 [15] ICH (2005) ICH Harmonised tripartite guidelines. International Conference on Harmonisation of
310 technical requirements for registration of pharmaceuticals for human use. *guideline Text and*
311 **methodology Q2 (R1)**.
- 312
313 [16] Olesen, H., Cowan, D., Bruunshuus, I., Klempel, K. and Hill, G. (1997) International Federation of
314 Clinical Chemistry and International Union of Pure and Applied Chemistry 2. Properties and Units in
315 the Clinical Laboratory Sciences. VI. Properties and Units in IOC Prohibited Drugs (IFCC-IUPAC
316 Recommendations 1997). *Clinica chimica acta; international journal of clinical chemistry* **268**, S5-
317 73.
- 318
319 [17] Cimetiere, N., Soutrel, I., Lemasle, M., Laplanche, A. and Crocq, A. (2013) Standard addition
320 method for the determination of pharmaceutical residues in drinking water by SPE-LC-MS/MS.
321 *Environmental technology* **34**, 3031-3041.
- 322
323 [18] Rashmin, P.M., Patel; Nitin, Dubey; Nidhi, Dubey; Bharat, Patel (2012) HPTLC Method Development
324 and Validation: Strategy to Minimize Methodological Failures. *Journal of Food & Drug Analysis* **20**.
- 325
326 [19] Westermann, C.M., van Leeuwen, R., van Raamsdonk, L.W. and Mol, H.G. (2016) Hypoglycin A
327 Concentrations in Maple Tree Species in the Netherlands and the Occurrence of Atypical Myopathy
328 in Horses. *Journal of veterinary internal medicine / American College of Veterinary Internal*
329 *Medicine* **30**, 880-884.
- 330
331 [20] Unger, L., Nicholson, A., Jewitt, E.M., Gerber, V., Hegeman, A., Sweetman, L. and Valberg, S. (2014)
332 Hypoglycin a concentrations in seeds of acer pseudoplatanus trees growing on atypical myopathy-
333 affected and control pastures. *J. Vet. Intern. Med.* **28**, 1289-1293.

334 **TABLES**

Characteristics	
DRY MATTER IN SAMARAS	66.3 % ± 0.1 (n = 15)
DRY MATTER IN SEEDLINGS	32.9 % ± 0.9 (n = 15)
RF OF HGA	0.51 ± 0.01 (n=15)
WORKING RANGE	20.2 - 425 µg/ml HGA in water, corresponding to In samara: 12.1 – 255 mg HGA/ Kg fresh weight In seedling: 30.3 – 637.5 mg HGA/ Kg fresh weight
LINEARITY	y = 47.11x + 2693.8 (R ² = 0.93)
LOD	6.0±0.2 µg/ml and, corresponding to In samara: 3.6 mg HGA/ Kg fresh weight In seedling: 9 mg HGA/ Kg fresh weight
LOQ	20.2±0.7 µg/ml corresponding to In samara: 12.1 mg HGA/ Kg fresh weight In seedling: 30.3 mg HGA/ Kg fresh weight
RECOVERY (%)	78 - 103 (samaras) 63 -83 (seedlings)

335 Table 1: The validation parameters of the HPTLC method for the quantification of HGA in plant extracts. The
336 two values for repeatability and recovery are respectively related to the two mean introduced concentration
337 values.

338

	HGA Introduced	HGA Measured		Precision (RSD)	Accuracy (CV)
	concentration (µg/ml)	Mean value	SD		
DAY 1 (N=5)	57	49	0.2	0%	86%
	177	191	3.4	2%	108%
	283	246	7.4	3%	87%
DAY 2 (N=5)	57	59	1.1	2%	104%
	177	206	1.5	1%	116%
	283	265	1.1	0%	94%
DAY 3 (N=5)	57	53	0.3	1%	93%
	177	193	2.5	1%	109%
	283	278	3.8	1%	98%
DAY 4 (N=5)	57	53	1.1	2%	93%
	177	215	4.5	2%	121%
	283	282	5.0	2%	100%
ALL RESULTS (N=20)	57	51	1.8	4%	89%
	177	201	7.2	4%	114%
	283	268	10.8	4%	95%

339 Table 2: Instrumental precision and accuracy of the HPTLC method to determine hypoglycin-A, calculated
340 from repeated analysis (n=5) of water solution of HGA at 3 concentrations, on 4 days. Precision is expressed

341 as the relative standard deviation (RSD) and accuracy as the ratio between introduced and measured
 342 concentration, expressed in percentage (CV).
 343

		HGA (mg/kg FW)	DRY MATTER (%)	HGA (mg/kg DM)	COLLECTED ON
SAMARAS <i>A. pseudoplatanus</i>	<i>Pseudoplatanus 1</i>	678.3	23%	2950.6	July 2016
	<i>Pseudoplatanus 2</i>	785.5	25%	3104.6	July 2016
	<i>Pseudoplatanus 3</i>	918.0	30%	3095.0	August 2016
	<i>Pseudoplatanus 4</i>	797.0	30%	2614.0	August 2016
SEEDLINGS <i>A. pseudoplatanus</i>	Seedlings 1	2162.3	25%	8798.2	March 2016
	Seedlings 2	3028.4	27%	11397.0	March 2016
	Seedlings 3	911.8	13%	6876.1	April 2016
	Seedlings 4	729.0	28%	2563.4	May 2016
	Seedlings 5	767.6	21%	3664.1	May 2016
OTHER SAMARAS	<i>Platanoides 1</i>	< LOD	49%	< LOD	Oct. 2016
	<i>Platanoides 2</i>	< LOD	46%	< LOD	Oct. 2016
	<i>Platanoides 3</i>	< LOD	54%	< LOD	Oct. 2016
	<i>Platanoides 4</i>	< LOD	53%	< LOD	Oct. 2016
	<i>Campestre</i>	< LOD	44%	< LOD	Oct. 2016

344 Table 3: hypoglycin A (HGA) concentrations measured in samara and seedling samples (LOD: limit of
 345 detection; FW: fresh weight; DM: dry matter).
 346

347

348

349 **FIGURES**

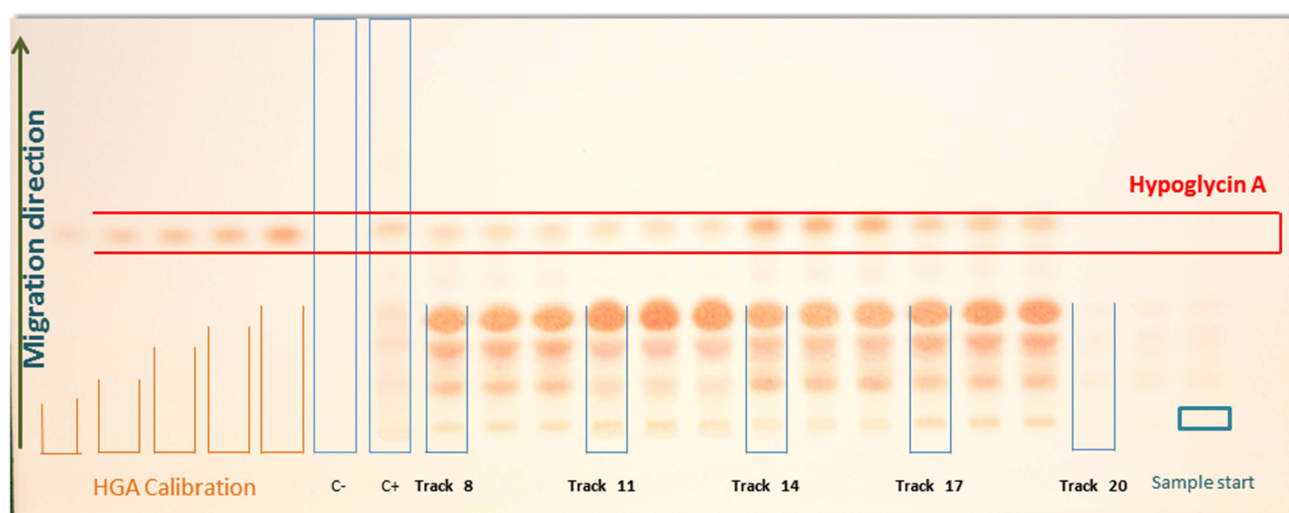
350 Figure 1: *Acer pseudoplatanus* samara and seedling. Samaras used in this study are complete (third position
351 from left to right) and seedlings are on early two-leaves stage of development (right side of picture).



352

353

354 Figure 2: Picture of an HPTLC plate after ninhydrin derivatization and revelation. Tracks 1 to 5: increasing
355 concentrations of hypoglycin A (HGA) in water (20; 40; 85; 199 and 408 $\mu\text{g}/\text{ml}$). Track 6: negative control
356 (C-). Track 7: positive control (samara sample containing 150 mg HGA/kg fresh weight). Track 8 to 22: five
357 samples from samaras of *Acer pseudoplatanus* spotted in triplicate.

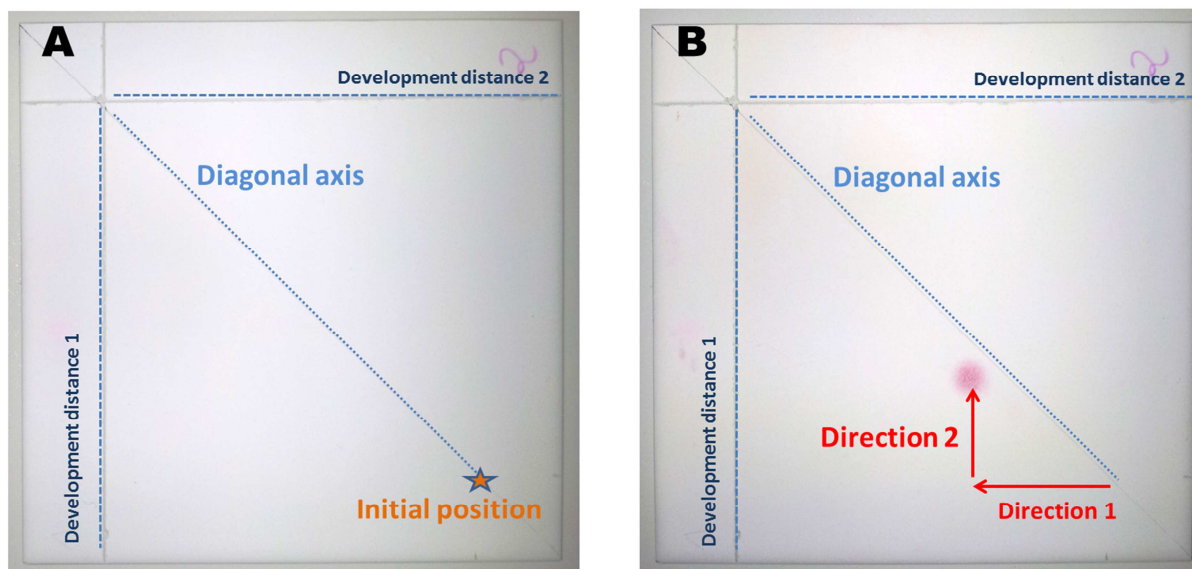


358

359

360

361 Fig 3: Picture of chromatogram. HP silica plate is used twice successively in two perpendicular directions.
362 The plate is dried before performing the second migration in a perpendicular direction (direction 1 and then 2).
363 Pictures A and B describe the situation before the first development and after the second development,
364 respectively.



365

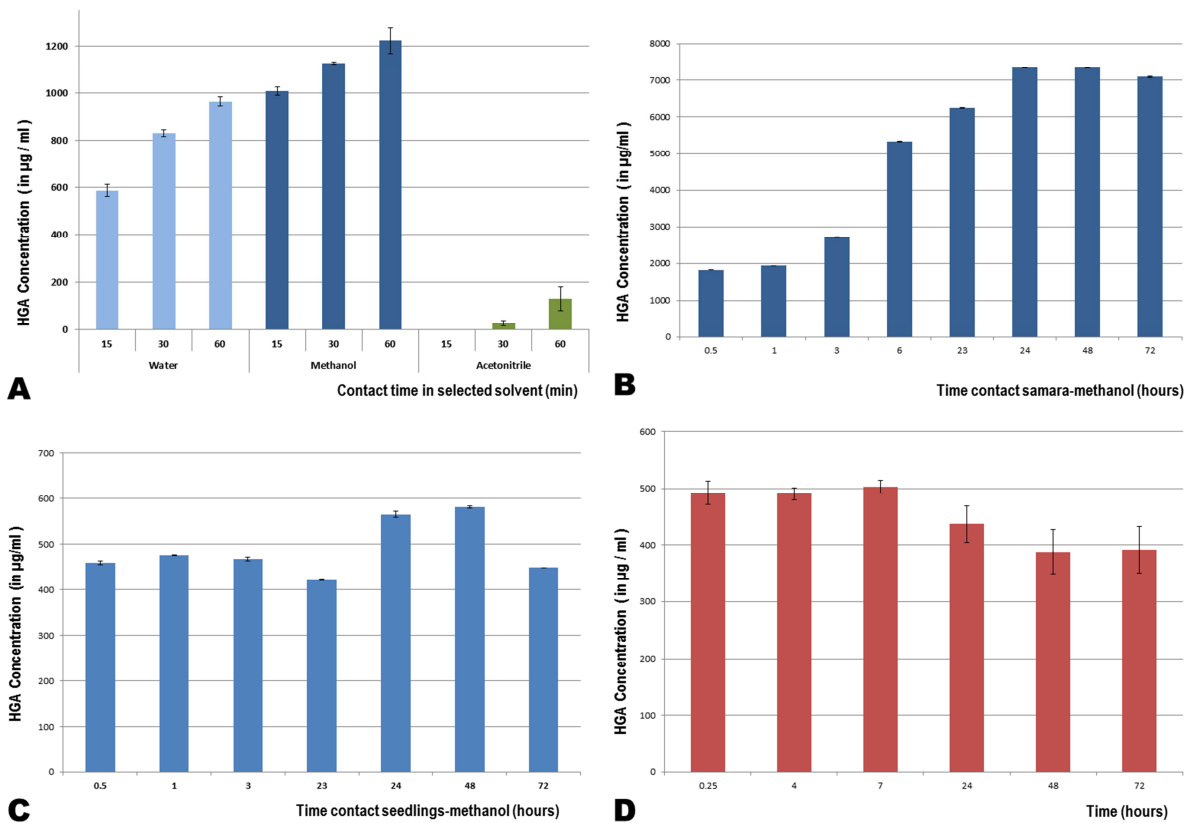
366

367 Fig 4: A. Figure summarizing the HGA extraction results on samara, performed on three different solvents
368 (filtered water, methanol and acetonitrile).

369 B. HGA diffusion from samaras. They're crushed and immersed in methanol. The whole mixture is placed in
370 a tube with constant and moderate agitation. 18 measures of HGA rates are carried over 72 hours.

371 C. HGA diffusion from seedlings. They're crushed then immersed in methanol. Measures of HGA rates are
372 carried over 72 hours.

373 D. HGA Stability. Standard solution of HGA is placed in a closed tube in laboratory room and under
374 permanent and moderate agitation (same condition as for plant extracts). HGA rate measures are performed
375 during 72 hours.



376

377