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# A new method for quantifying causative and diagnostic markers of methylenecyclopropylglycine poisoning

Johannes Sander[a,*], Michael Terhardt[a], Stefanie Sander[a], Sabine Aboling[b], Nils Janzen[a,c]

[a] Screening-Labor Hannover, Ronnenberg, Germany
[b] Institute for Animal Nutrition, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany
[c] Department of Clinical Chemistry, Hannover Medical School, Hannover, Germany

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ABSTRACT

*Background:* Up to now quantification of hypoglycin A in serum and urine in the range of nmols to µmols per liter plus the measurement of accumulated acyl conjugates have been used for the diagnosis of poisoning by fruits or seeds of*Sapindaceae* in humans and animals. A second poison, methylenecyclopropylglycine, however, is known to occur in this material. The objective of our study was to develop and evaluate a method for the quantification of this compound suitable for test materials obtained from animals and man.

*Method:* Methylenecyclopropylglycine was extracted from serum and urine of a volunteer by a methanolic solution containing labeled methylenecyclopropylglycine as internal standard. UPLC-MS/MS analysis was performed after butylation.

*Results:* Lower limits of detection and quantification were found at 0.5 and 2.5 nmol/L respectively in both urine and serum for each of two isomers, linearity of results ($r^2 > 0.998$) was demonstrated for the range of 0.5–500 nmol/L in urine and serum.

The method was applied to urine and serum of horses poisoned by *Acer* seeds, methylenecyclopropylglycine was found in addition to hypoglycin A. Methylenecyclopropylformyl glycine, a metabolite of methylenecyclopropylglycine, however, was present in much higher concentrations than methylenecyclopropylglycine in all but one samples.

*Conclusions:* Quantification of methylenecyclopropylglycine can be successfully integrated into our established analytical procedure used for clinical diagnosis of *Sapindaceae* poisoning. The extended method will improve disease evaluation in humans and animals.

## 1. Introduction

Fruits and seeds from plant species of the *Sapindaceae* family of plants, also called soapberry plants, were found to cause numerous cases of food-borne poisoning. For more than 100 years, it has been reported that ackee fruit triggers the so-called Jamaican vomiting disease [1] while a possible causation by certain trace elements could be largely excluded [2]. In recent years litchi fruit poisoning in children has received increasing medical and scientific attention [3–10]. In the veterinary literature numerous reports about the poisoning of horses by seeds or seedlings of *Acer negundo* or *Acer pseudoplatanus* were published since *Acer* seed intoxication was first reported [11–14].

*Sapindaceae* produce two toxic non-proteinogenic amino acids, methylenecyclopropylglycine (MCPG) and hypoglycin A (HGA) (Fig. 1) [15–19]. Both of them also occur as dipeptide conjugated with glutamic acid [20]. In the past, toxicological investigations have focused mainly on HGA, whereas MCPG received less scientific interest. However, Das et al. [6] 2015 postulated MCPG as a causative agent in seasonal outbreaks of hypoglycemic encephalopathy observed in children who stayed on Indian litchi farms during harvesting. The authors isolated MCPG from litchi pulp and identified it by LC–MS/MS analysis. MCPG as one of two specific poisons causing encephalopathy was confirmed by Isenberg et al. [21,22] who detected high levels of MCPG metabolites in the urine of children after excessive consumption of litchi. We showed that glycine and carnitine conjugates of methylenecyclopropylformate (MCPF), a metabolite of MCPG, were excreted following the ingestion of ackee fruit [23]. It therefore seems reasonable to include MCPG quantification in the investigation of cases of poisoning by fruit

*Abbreviations:* MCPG, methylenecyclopropylglycine; HGA, hypoglycin A; MCPF, methylenecyclopropylformate; AM, atypical myopathy; MCPF-G, methylenecyclopropylformyl glycine; MCPA-G, methylenecyclopropylacetyl glycine

* Corresponding author at: Screening-Labor Hannover, Postbox 91 10 09, 30430 Hannover, Germany.
*E-mail address:* j.sander@metabscreen.de (J. Sander).

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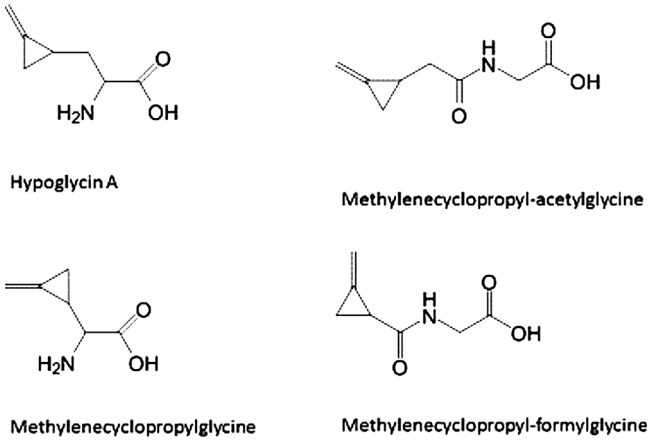


**Fig. 1.** Chemical structures of hypoglycin A (HGA), methylenecyclopropylglycine (MCPG), methylenecyclopropyl-acetylglycine (MCPA-G) and methylenecyclopropyl-formylglycine (MCPF-G).

and seeds of soapberry plants. The mechanism of action of soapberry toxins is based on the inhibition of ß-oxidation of fatty acids. If fatty acids are not available as source of energy, the energy supply is interrupted when the glycogen stocks are used up. In mammals ß-oxidation takes place in peroxisomes and mitochondria with different enzymes processing fatty acids of different chain length and structure [24]. Methylenecyclopropylacetyl-CoA (MCPA-CoA), derived from HGA, is known to inhibit acyl-CoA dehydrogenases, which catalyze the first step of the ß-oxidation in mitochondria. MCPF-CoA, derived from MCPG, mainly inhibits the second step which takes place in peroxisomes as well as in mitochondria [25–28]. In peroxisomes the isoenzyme responsible for the hydration of enoyl-CoA compounds is ECH 2, the equivalent enzyme in mitochondria is ECH 1. While the enzymatic reaction in the peroxisomes involves a 3R hydroxyacyl-CoA intermediate, the intermediate of enoyl hydratase 1 mediated catalysis is 3S hydroxyacyl-CoA. Stereoisomerism must therefore be taken into account. At the same time, the activity of enoyl-CoA hydratases (ECHs) toward substrates of different acyl chain lengths varies between species. Enzymes, however, may be equally susceptible to inactivation by both isomers of methylenecyclopropylformyl glycine (MCPF-G), as was shown for bovine liver ECH (crotonase) [28]. Thus, although in principle, similar toxicological effects are elicited by MCPG and HGA, the details of enzyme inhibition differ significantly.

The objective of our present study was to integrate the quantification of MCPG as a further analytical parameter into our established analytical procedure [23,29,30]. In analogy to the method established for the measurement of HGA and acyl conjugates, MCPG was quantified by UPLC-MS/MS after butylation together with a spectrum of acylcarnitines and acylglycines.

## 2. Material and methods

### 2.1. Reagents

MCPG (mixture of diastereomers) with chemical names (2S)-amino [(1S)-2-methylenecyclopropyl]acetic acid and 2-amino-3-(2-methylidenecyclopropyl)propanoic acid, unlabeled and [$^{13}C_3$]-labeled, were products of IsoScience, (King of Prussia, PA, USA). The purity of the unlabeled material was ≥97%, that of the isotopically labeled form was ≥99.3%. Both compounds contained almost exactly 50% of each isomer. All other reagents were used as described earlier [23] (Sander et al., 2016b).

### 2.2. Preparation of internal standards and calibrators

A stock solution of [$^{13}C_3$]-MCPG was prepared in methanol and stored at −20 °C. An equivalent was added to the internal standard solution. The final concentration of [$^{13}C_3$]-MCPG in the standard solution used for extraction was 52.2 nmol/L for each of the isomers. This solution also contained $d_3$-leucine as internal standard for HGA quantification as described earlier [23].

For the preparation of MCPG calibrators, serum and urine (diluted to 7600 µmol/L creatinine) from a volunteer were spiked with MCPG stock solution to give concentrations of 0.5, 1.25, 2.5, 5, 50 and 500 nmol/L each of the isomers.

### 2.3. Preparation of samples

For the evaluation of MCPG measurement, spiked urine (diluted to 7600 µmol/L creatinine) and serum of a healthy volunteer were used. Linearity and intraday precision were determined in serial dilutions of MCPG prepared in urine and serum. For the determination of interday precision we analyzed serum of a poisoned horse containing a low concentration of MCPG.

To test the applicability for toxicological routine diagnosis in cases of atypical myopathy (AM) we analyzed seven urine samples and eleven serum samples of horses diseased with AM. Samples were collected for diagnostic analysis. Urine and serum samples 1–3 were from the same horses and were collected simultaneously. The owners had agreed to the use of residual material for scientific purpose. Control material was taken from horses examined for diseases not related to AM. In addition to MCPG, HGA and glycine conjugated metabolites of both poisons were analyzed. To estimate the degree of inhibition of fatty acid ß-oxidation we also included quantification of two glycine and two carnitine conjugates of medium chain fatty acids. Analytical details for these compounds have been described earlier [23]. In case of very high concentrations of these compounds a second analysis was performed using 1:100 prediluted samples.

### 2.4. Analysis

Serum (25 µL) and urine (25 µL) undiluted as well as diluted were extracted with 300 µL methanolic internal standard solution, vortexed and centrifuged for 10 min at 17,000 RCF. Of the clear supernatant 250 µL were transferred to a microtiter plate and dried at 65 °C under a gentle stream of nitrogen. The residue was treated with 50 µL 3 N butanolic HCl for 15 min at 65 °C and dried again at 65 °C. To resolve the dry material 70 µL methanol/water (80:20 vol/vol) were used, these were further diluted 1:2 with water in order to improve the chromatographic separation of early eluting compounds. Of this solution 90 µL were transferred to a 384-well microtiter plate, again centrifuged at 17,000 RCF in order to sediment any particles and the supernatant then used for ultrahigh performance chromatography-tandem mass spectrometry (UPLC-MS/MS). 5 µL of the solution were injected onto an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm, Waters, Eschborn, Germany) for gradient chromatography (gradient A: water plus 0.1% formic acid and 0.01% trifluoro acetic acid, gradient B: acetonitrile plus the same additives, starting with 80% A, reaching 1% A after 10 min). The analysis was done as described earlier (Sander et al., 2016a) on a Xevo UPLC-MS/MS system (Waters).

The analyzed transitions *[m/z]* were 184.0 > 110.7 for the butyl ester of MCPG and 187.0 > 113.7 for butylated [$^{13}C_3$]-MCPG. The butyl esters were detected in ESI positive mode by multiple reaction monitoring (MRM). A ratio was calculated from the signal height obtained for both MCPG isomers and the height of signals of the respective isomers of the internal standard [$^{13}C_3$]-MCPG.

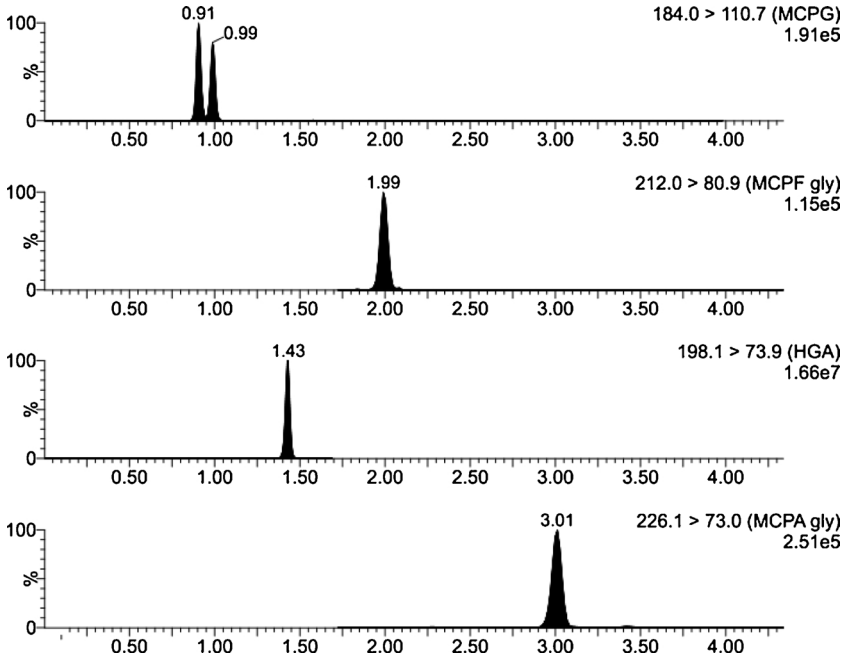


**Fig. 2.** Chromatographic separation of MCPG isomers, HGA and the metabolites MCPF-G and MCPA-G on an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm).

## 3. Results

### 3.1. Matrix effects

Using the method described by van Eeckhaut et al. [31] no significant matrix effect on quantitative results was observedduring the time window given by the chromatographic separation of MCPG and the other compounds included in the analysis. The background signal in the mass trace of MCPG reached a maximum height corresponding to 0.16 nmol at the time of elution of the MCPG isomers.

### 3.2. Method performance

#### 3.2.1. Chromatographic separation

As shown in Fig. 2, there was a clear separation of HGA and MCPG. MCPG was separated into two isomeric forms with slightly different retention times (0.93 and 1.01 min). Since no corresponding control material was available, we could not assign peaks A and B to the known 2S3R and 2S3S isomers. MCPF-G and MCPA-G were well separated.

#### 3.2.2. Linearity

Linearity of quantification was excellent in the range of concentrations tested (0.5–500 nmol/L) for the isomers of MCPG: the coefficient of determination $R^2$ was > 0.991for serum (Fig. 3) as well as for urine (Fig. 4) for both isomers. Due to the very low non-specific signal the lines run almost through the origin.

#### 3.2.3. Imprecision, limits of quantification and detection

Data for intraday imprecision of MCPG quantification in urine and serum are given in Table 1. Interday imprecision measured in a positive serum sample from a horse (22 measurements during a period of 252 days) resulted in a coefficient of variation (CV) of 17.9% for isomer A (concentration 4.45 ± 0.80 nmol/L) and CV 10.4% for isomer B (concentration 18.7 ± 1.94 nmol/L).

We set CV < 20% as standard to determine the lower limit of quantification (LOQ). This standard was met by our calibrator 2.5 nmol/L for both isomers. At this level the signal-to-noise ratio was > 10. The lower limit of detection (LOD) was defined as the concentration of the lowest calibrator showing a signal more than three times higher than the background. Since the lowest concentration which we used was 0.5 nmol/L we defined the LOD for each isomer as 0.5 nmol/L.

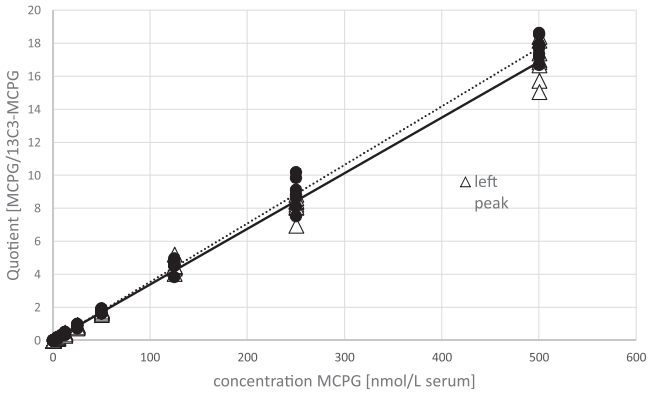


**Fig. 3.** Linearity of MCPG quantification in spiked serum from a volunteer, shown for the range 0 to 50 nmol/L.

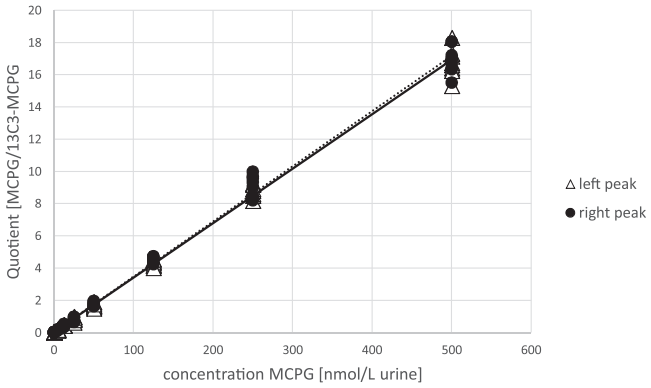


**Fig. 4.** Linearity of MCPG quantification in spiked urine from a volunteer, shown for the range 0 to 50 nmol/L.

At [*m/z*] 184.0 > 110.7, the mass transition of MCPG, we observed a non-specific signal of < 10% of the LOD and < 0.6% of the LOQ. Analyzing urine and serum samples of five horses not affected by acer

**Table 1**
Evaluation of intraday imprecision of MCPG quantification in urine and serum from a volunteer spiked with MCPG.

| MCPG per isomer nmol/L | Urine CV% Isomer A | Isomer B | Serum CV% Isomer A | Isomer B |
|---|---|---|---|---|
| 5 | 13.6 | 11.8 | 19.7 | 15.1 |
| 12.5 | 3.8 | 13.5 | 14.8 | 14.9 |
| 25 | 13.0 | 15.8 | 7.5 | 9.9 |
| 50 | 7.6 | 6.7 | 5.2 | 6.8 |
| 125 | 4.0 | 3.8 | 6.8 | 6.8 |
| 250 | 4.1 | 6.8 | 6.4 | 9.1 |
| 500 | 4.9 | 4.8 | 6.3 | 3.5 |

CV%: coefficient of variation in percent (8 measurements each).

seed poisoning and of two healthy human volunteers we observed no interfering signals exceeding the peak height mentioned before.

#### 3.2.4. Accuracy

As there are neither certified reference material nor results of a reference method available for measuring MCPG in urine and serum it was not possible to determine the accuracy of our new method. As a second piece of evidence supporting the ability of our method to quantify the analyte we measured the recovery of MCPG after known amounts had been added to urine and serum of a healthy individual not having ingested any soapberry fruit. MCPG was added to final concentrations of 10 and 100 nmol/L, resulting in measurements of 8.27 and 87.40 in the serum sample and 10.79 and 106.30 nmol/L in the urine specimen respectively (mean of 6 measurements).

#### 3.2.5. Stability

Stability of the processed samples was near 100% during the time interval of 12 h tested, exceeding the time required for the complete analysis including MS/MS measurement. The serum used for the measurement of interday precision was kept at −18 °C over a period of 252 days. Even though it was thawed and frozen 22 times, there was no detectable reduction in the concentrations of the two MCPG isomers.

## 4. Application for routine analysis

### 4.1. Serum

Both MCPG isomers were detected in 6 out of 11 serum specimens from diseased horses, however, some samples contained only traces of the analyte (Table 2). The isomers of MCPG were found in variable relative concentrations. In four cases only one isomer was present in a detectable or measurable concentration, in one sample the compound was not detected at all. In all but one of the cases the concentrations of MCPG were significantly less than those of its metabolite, the glycine ester of MCPF.

The concentrations of non-metabolized MCPG were much lower than those of HGA, differing from the latter by a factor of more than 100 in some of the samples. Inhibition of the ß-oxidation of fatty acids was demonstrated by elevated levels of medium chain acyl compounds but there was no direct correlation between the concentration of MCPG or HGA and the values obtained for acyl carnitines and glycines.

### 4.2. Urine

Unmodified MCPG was excreted with the urine in small quantities (Table 3), in one sample it was not detected. As in serum, concentrations of MCPG were lower than those of the higher homologue. The levels of the glycine esters of MCPF, however, exceeded those of the mother substance MCPG by orders of magnitude.

## 5. Discussion

The first to describe MCPG were Gray and Fowden, who in 1962 isolated it from seeds of *Litchi sinensis* by extraction with ethanol [15]. These authors detected the two stereo-isomeric forms of the molecule. Their approach, however, did not reveal any MCPG in the fleshy arils of ripe litchi fruit. In a 1985 study on rare amino acids from plants Minakata et al. [18] also isolated MCPG. Neither of the two groups attempted to establish a method of MCPG quantification.

Das et al. [6] measured MCPG by LC–MS/MS without derivatization. The compound was extracted from seeds and fruit pulp using ethanol. Quantitative results are published in the range of 0.18 µg/g dry weight of ripe fruit pulp to 1.80 µg/g of the litschi seeds. Recently a highly sensitive quantitative measurement was developed by Isenberg et al. [22] who extracted MCPG together with HGA from dehydrated soapberry arils in ethanol/water 80/20. Derivatization with dansyl chloride was followed by solid phase extraction (SPE) prior to analysis by HPLC-MS/MS. In contrast, our method, developed for diagnostic usage in clinical cases, uses derivatization with butanol HCl and does not apply a preanalytic SPE.

When comparing quality parameters of our method to those of Isenberg et al. [22] one has to keep in mind that this group used plant (rambutan) extract spiked with MCPG in variable concentrations for their analysis and quality assessment while we determined quality parameters in spiked urine and serum samples.

The lowest reportable result, the concentration of the lowest calibrator, in Isenberg's method was 1 ng/mL (8.26 nmol/L) [22,32]. These authors did not differentiate isomers. The LOD in our method for urine and serum samples was 0.5 nmol/L for each of the isomers, the LOQ,

**Table 2**
Analysis of serum:Concentrations of MCPG and HGA and degradation products of these toxins (nmol/L) plus concentrations of medium chain acyl conjugates (µmol/L) in serum of horses suffering from atypical myopathy. References are means found in samples of 5 unaffected horses.

| Sample | MCPG nmol/L isomers A | B | MCPF- glycine nmol/L | HGA nmol/L | MCPA- glycine nmol/L | Butyryl-carnitine µmol/L | Valeryl-glycine µmol/L | Hexanoyl-glycine µmol/L | Hexanoyl-carnitine µmol/L |
|---|---|---|---|---|---|---|---|---|---|
| Ref.s | < LOD | < LOD | < LOD | < LOD | < LOD | < 0.1 | < 0.5 | < 0.5 | < 0.1 |
| 1 | 361.8 | 32.7 | 166 | 12,175 | 634 | 248 | 3.9 | 7.1 | 8.0 |
| 2 | < LOD | 2.6 | 574 | 1,653 | 874 | 248 | 5.2 | 8.2 | 15.5 |
| 3 | trace | trace | 64.5 | 620 | 53.7 | 6.5 | 0.46 | 0.52 | 0.86 |
| 4 | 54.2 | 25.8 | 186 | 7,546 | 775 | 121 | 4.3 | 7.9 | 10.3 |
| 5 | 24.8 | 1.2 | 83,9 | 3,563 | 305 | 229 | 2.6 | 7.0 | 10.1 |
| 6 | 12.7 | 10.6 | 267 | 2,039 | 345 | 319 | 3.0 | 5.0 | 7.49 |
| 7 | 1.0 | 4.8 | 46,5 | 1,629 | 140 | 81,8 | 2.2 | 2.3 | 7.1 |
| 8 | 2.7 | < LOD | 129 | 1,101 | 209 | 108 | 1.7 | 3.2 | 7.7 |
| 9 | < LOD | trace | 118 | 2,805 | 419 | 121 | 3.5 | 13.6 | 9.5 |
| 10 | < LOD | < LOD | 173 | 1,151 | 237 | 127 | 1.4 | 3.5 | 10.0 |
| 11 | < LOD | 2.2 | 67 | 1,019 | 169 | 205 | 2.2 | 3.8 | 11.0 |

**Table 3**
Urine analysis Concentrations of MCPG isomers, hypoglycin A, glycine conjugates of MCPF and MCPA plus butyrylcarnitine, valerylglycine, hexanoylglycine and hexanoylcarnitine. References are means found in samples of 5 unaffected horses.

| | MCPG isomers[a] A | B | MCPF- glycine[a] | HGA[a] | MCPA- glycine[a] | Butyryl- carnitine[b] | Valeryl- glycine[b] | Hexanoyl- glycine[b] | Hexanoyl-carnitine[b] |
|---|---|---|---|---|---|---|---|---|---|
| Ref.s | < LOD | < LOD | < LOD | < LOD | < LOD | 0.5 | 1.0 | 0.5 | < 0.1 |
| Urine1 | 13.2 | 25.0 | 2,741 | 120 | 29,443 | 282 | 73.5 | 457 | 90 |
| Urine 2 | 2.0 | 3.8 | 8,574 | 96.8 | 34,219 | 274 | 303 | 497 | 80.8 |
| Urine 3 | 3.5 | < LOD | 4,187 | 0.60 | 3,276 | 6.5 | 16.6 | 14.7 | 0.26 |
| Urine 4 | 2.3 | 2.3 | 3,876 | 31.3 | 22,888 | 225 | 133 | 207 | 53.0 |
| Urine 5 | 1.4 | 0.83 | 4,321 | 45.8 | 31,840 | 233 | 341 | 525 | 80.5 |
| Urine 6 | < LOD | 0.3 | 3,875 | 130 | 21,614 | 404 | 131 | 444 | 90.0 |
| Urine 7 | < LOD | < LOD | 2,308 | 60.0 | 12,479 | 190 | 33.5 | 67.0 | 8.3 |

[a]nmol/mmol creatinine.
[b]µmol/mmol creatinine.

defined as a value measurable with a coefficient of variation below 20%, was 2.5 nmol/L in serum and urine. Thus, our method, primarily designed to be used on material for human medical or veterinary diagnostic purpose, is more sensitive than the one previously published.

High sensitivity for MCPG is necessary in diagnostic analyses since the concentrations to be expected in clinical material are very low. For clinical examination an important advantage of our method is that the two poisons and their derivatives are quantified together with the measurement of acyl conjugates. This allows not only the identification of the causative toxins, but also to estimate the degree of induced metabolic abnormalities. In the present study we limited the number of reported short and medium chain acyl conjugates to four compounds which we consider sufficient for this purpose. However, the range of compounds can easily be extended.

Analysis of serum and urine appear equally useful for the diagnosis of poisoning by seeds or fruits of *Sapindaceae*. The concentrations of metabolites, however, may differ considerably in serum versus urine. This is especially true for the glycine conjugates of MCPF and MCPA. In three horses (# 1–3 in Tables 2 and 3), for example, from which both, serum and urine were obtained at the same time, the absolute concentrations of these compounds measured in urine exceeded those found in serum by orders of magnitude.

The clinical relevance of a combined attack of the two different toxins responsible for soapberry poisoning is not yet clear. It has to be considered that the two isomers of MCPG show different specificity for ECH 1 and 2. These enzymes are either found in peroxisomes or in mitochondria, cell organellae responsible for different steps in the ß-oxidation of acyl compounds. In addition susceptibility of ECHs to inhibition by either one or both isomers of MCPG may vary among different species [27,33]. More investigations will be necessary to obtain a complete picture of the toxicological processes associated with the consumption of fruits, seeds or seedlings of *Sapindaceae*. The method described here can improve the monitoring of the clinical course of such toxicity in humans and animals. The simultaneous measurement of MCPG, HGA and their degradation products will allow an evaluation of the actual toxic effect caused by the natural plant toxins. For example, as MCPG seems to be metabolized rapidly, measuring concentrations of non-metabolized MCPG alone could lead to an underestimation of the clinical significance of that toxin. Measurement of its metabolites, which is part of our method, is therefore particularly important and recommended. In summary an analytical procedure is presented allowing to measure causative and metabolic markers of Soapberry poisoning in humans or animals. Sample acquisition and preparation are straightforward and all steps can easily be implemented in routine diagnostic analytics.

## Transparency document

The Transparency document associated with this article can be found in the online version.

## Declaration of Competing Interest

None.

## Acknowledgments

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