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# Metabolomic Analysis of *Ranunculus* spp. as Potential Agents Involved in the Etiology of Equine Grass Sickness

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Supporting Information

**ABSTRACT:** Identification of toxic or harmful agents continues to be a key goal in agricultural chemistry. This paper reports a metabolomic analysis of *Ranunculus repens* and related species, which were recently postulated to be cocausative agents in the etiology of equine grass sickness (EGS). Specifically, samples collected at EGS sites were compared with those from non-EGS sites. Furthermore, interspecific and seasonal variations and the species' response to edaphic and climatic factors were investigated. $^1$H NMR spectroscopy in combination with multivariate data analysis was applied to the crude methanol extracts of the *Ranunculus* samples, as well as their chloroform fractions. Samples from EGS sites were significantly different from control samples. The metabolite composition varied greatly between different *Ranunculus* species. No significant changes could be observed between samples collected in different seasons. This work provides strong evidence that *Ranunculus* is involved in the etiology of EGS and has implications for agricultural management of pastures.

**KEYWORDS:** *Ranunculus* (buttercups), equine grass sickness, metabolomics, principal component analysis, partial least-squares, $^1$H NMR

## INTRODUCTION

Equine grass sickness (EGS) or equine dysautonomia is a degenerative polyneuropathy affecting horses mainly in Great Britain.[1] It is very likely that EGS has a multifactorial etiology, including intoxication with *Clostridium botulinum* type C.[2] As the name suggests, EGS is associated with grazing.[1] Recent research in our group has shown that a range of edaphic and botanical factors are strongly associated with EGS outbreaks including the regular occurrence of *Ranunculus* spp. on EGS sites.[3] Fresh *Ranunculus* contains ranunculin, a glycoside that is enzymatically hydrolyzed to produce protoanemonin,[4] a toxic metabolite that can cause blistering[5] and gastrointestinal irritation[6] (Supporting Information, Figure 1S).

The initial hypothesis was that ranunculin levels of *Ranunculus* samples collected from EGS sites are higher compared to those from control sites. HPLC quantification was therefore carried out and showed that ranunculin levels in samples from EGS sites were indeed significantly higher.[3]

However, confining the analysis to just a few metabolites to confirm or deny if *Ranunculus* species are involved in EGS would have been misleading, because the role of *Ranunculus* as a causal agent of this disease is likely to be more complex.

NMR metabolomics offers a more suitable approach, because it can be used to compare the whole plant metabolome of samples from EGS and non-EGS sites. It also allows taking into consideration interspecific variation and variation caused by environmental changes.

Metabolic profiling has been proposed as a method for the analysis of phytomedicines,[7−10] and food products [11,12] and it also has been used widely for understanding toxin- and stress-induced metabolomic changes[13−15] in mammals. A number of studies show how plants respond to their environment, for example, neighboring species,[16] temperature,[17] salt,[18] phosphorus,[19] and cadmium.[20] However, these studies are usually carried out under extreme conditions created artificially in the laboratory, whereas the influence of naturally occurring changes such as the changing of seasons or physiological ion levels in the soil has not been studied.

The aim of this work is to investigate (a) the differences between EGS and non-EGS samples using multivariate statistical methods such as principal component analysis (PCA) or partial least-squares−discriminant analysis (PLS-DA) and (b) the differences between *Ranunculus* species and seasonal changes in the metabolite composition by using PCA as well as (c) metabolic changes triggered by soil minerals (PLS).

## MATERIALS AND METHODS

**Chemicals.** All chemical reagents used were of analytical grade. Deuterated chloroform ($CDCl_3$, 99.8%) and deuterated methanol (MeOD, 99.8%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). 3-(Trimethylsilyl)propionic-2,2,3,3-$d_4$acid, sodium salt (TSP, 98%), and tetramethylsilane (TMS, 99.9%) were obtained from Sigma-Aldrich (St. Louis, MO).

**Plant Material.** Between April 2007 and March 2009 *Ranunculus* spp. samples were collected from 9 control sites and 12 EGA sites and identified by Prof. Michael Heinrich and Dr. Sarah Edwards. Furthermore, over the course of a year 10 samples were collected from one site

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**Table 1. Information on the Samples Collected on EGS (E) and Control Sites (C): Species, Number of EGS Cases Reported, Date of Collection, and County (see also Edwards et al.[3]).**

| | species | EGS cases | collection | county |
|---|---|---|---|---|
| E1 | *R. acris* | 1 | May 15, 2007 | Stirlingshire |
| E2 | *R. acris* | 1 | May 15, 2007 | Stirlingshire |
| E3 | *R. repens* | 2 | May 17, 2007 | Moray |
| E4 | *R. repens* | 5 | May 25, 2007 | Northamptonshire |
| E5 | *R. repens* | 5 | May 25, 2007 | Northamptonshire |
| E6 | *R. acris* | 4 | June 5, 2007 | Lincolnshire |
| E7 | *R. repens* | 1 | June 12, 2007 | Hampshire |
| E8 | *R. acris* | 4 | July 30, 2007 | Hertfordshire |
| E9 | *R. repens* | 4 | Aug 20, 2007 | Buckinghamshire |
| E10 | *R. bulbosus* | 4 | Aug 20, 2007 | Buckinghamshire |
| E11 | *R. repens* | 2 | Dec 13, 2007 | Lancashire |
| E12 | *R. repens* | 2 | Jan 29, 2008 | Powys |
| E13 | *R. bulbosus* | 2 | May 15, 2008 | Hertfordshire |
| E14 | *R. bulbosus* | 2 | May 15, 2008 | Hertfordshire |
| E15 | *R. repens* | 2 | May 15, 2008 | Hertfordshire |
| E16 | *R. repens* | 1 | May 19, 2008 | Berkshire |
| E17 | *R. repens* | 1 | May 19, 2008 | Berkshire |
| E18 | *R. repens* | 1 | July 28, 2008 | Suffolk |
| C1 | *R. acris* | 0 | Nov 28, 2007 | London |
| C2 | *R. repens* | 0 | June 11, 2007 | Oxfordshire |
| C3 | *R. acris* | 0 | June 11, 2007 | Oxfordshire |
| C4 | *R. repens* | 0 | July 8, 2007 | Northamptonshire |
| C5 | *R. repens* | 0 | July 15, 2007 | Angus |
| C6 | *R. repens* | 0 | July 23, 2008 | Berkshire |
| C7 | *R. acris* | 0 | July 23, 2008 | Berkshire |
| C8 | *R. acris* | 0 | July 23, 2008 | Oxfordshire |
| C9 | *R. repens* | 0 | July 28, 2008 | Buckinghamshire |
| C10 | *R. repens* | 0 | July 28, 2008 | Hertfordshire |
| C11 | *R. acris* | 0 | July 28, 2008 | Buckinghamshire |
| C12 | *R. repens* | 0 | Aug 29, 2008 | Buckinghamshire |

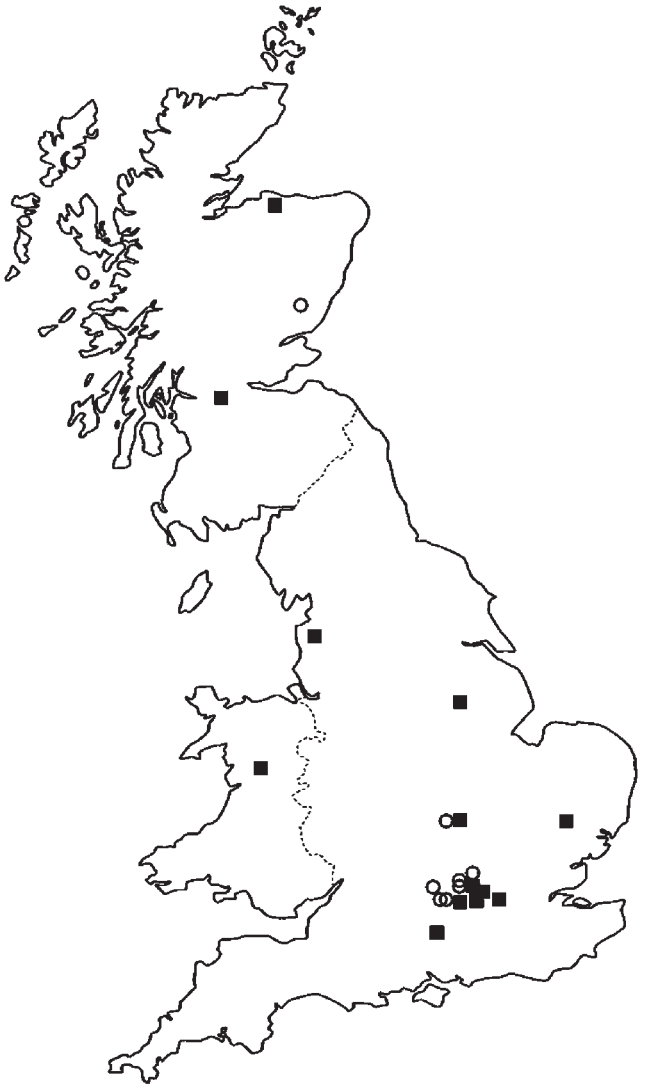

**Figure 1.** Locations of EGS (■) and control sites (○).

in Oxfordshire. In total, 30 samples were collected. Eighteen were identified as *Ranunculus repens* L., 9 as *Ranunculus acris* L., and 3 as *Ranunculus bulbosus* L. (Ranunculaceae; Table 1). All sites are located in Great Britain (Figure 1).

**Extraction.** Ground plant material was extracted in a glass column with methanol for 6 h. The extracts were evaporated to dryness below 40 °C. Ten percent of the crude methanol extract was dried under nitrogen and used for $^1$H NMR measurements.

Ninety percent of the crude methanol extract was partitioned between 50 mL of 90% methanol and 50 mL of hexane three times. The hexane fractions were discarded. Water was added to the methanol fraction to obtain 70% methanol. The 70% methanol fraction was partitioned with 50 mL of chloroform three times. The chloroform fractions were evaporated to dryness below 40 °C, dried under nitrogen, and used for $^1$H NMR measurements. The extraction was made in triplicates for each sample.

**NMR Experiments.** Samples for NMR analysis were prepared by dissolving the methanol extracts in MeOD containing 0.01% TSP and the chloroform extracts in $CDCl_3$ containing 0.01% TMS to a concentration of 10 mg/mL. The solutions were transferred into 5 mm NMR tubes. $^1$H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400.13 MHz. For each sample 128 transients were recorded as 65K data points with a spectral width of 8278 Hz using 3.96 s acquisition time, 1.0 s relaxation delay, and a 30° pulse angle. FIDs were Fourier transformed with LB = 0.3 Hz. The spectra were referenced to the internal TMS or TSP. $^1$H NMR spectra were manually corrected for phase and baseline distortions using Topspin (v 1.3., Bruker Biospin).

**Data Reduction and Statistical Analysis.** The spectra in the range of 0.2−10.5 ppm were divided into 258 regions ("buckets") of 0.04 ppm using AMIX software (v. 3.5.5., Bruker Biospin), and the signal intensity in each region was integrated. This bucket size was chosen to allow the small signal shift compensation that occurred. Water signals (4.6−5.0 ppm in MeOD spectra) and residual proton signals corresponding to MeOD (3.24−3.4 ppm) and $CDCl_3$ (7.2−7.4 ppm) were excluded. The data could then be imported into Microsoft Excel for the addition of labels and normalization. The spectral areas were normalized to the total sum of the spectra integral to avoid dilution effects. PCA, PLS-DA, and PLS were carried out on the normalized and centered NMR data set using the software SIMCA P+ (v. 12, Umetrics, Umea, Sweden). PCA is an unsupervised pattern recognition technique,[21] which can be used to reduce the complexity of a data set and visualize the data in two- or three-dimensional plots. PLS is a regression technique for relating the measured data ($X$) and the response variable ($Y$) by a linear multivariate model.[22] PLS with discriminant analysis (PLS-DA) is

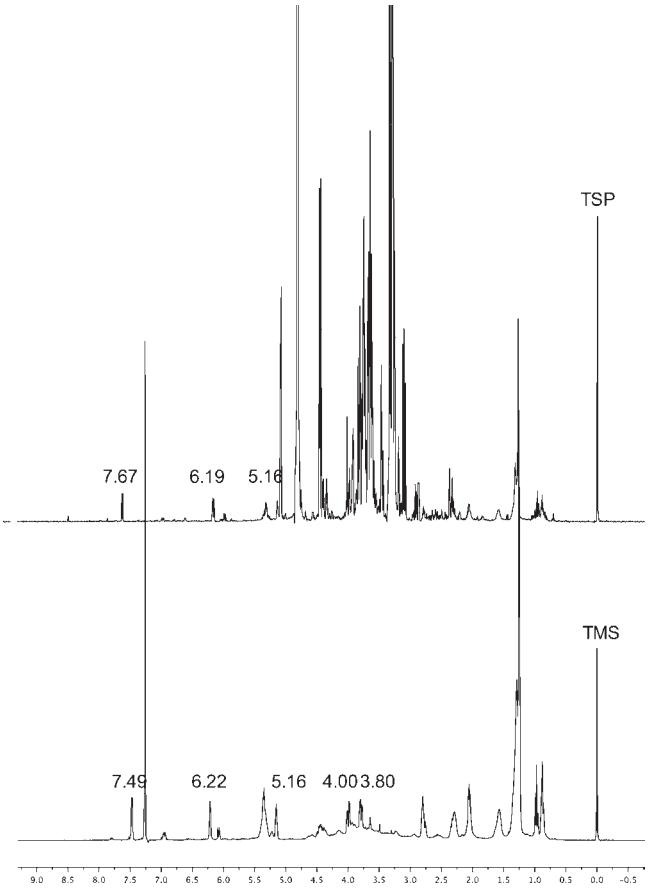


**Figure 2.** Typical $^1$H NMR spectra of methanol and chloroform extracts. Highlighted peaks correspond to resonance signals for ranunculin aglycon.[6]

an analysis useful in classification, where the *Y* variable is chosen to represent the class membership.[23] Validation of the models was performed using full cross-validation and permutation tests (200 repeats). The quality of all PCA, PLS-DA, and PLS models was measured using the correlation coefficient $R^2$ and a cross-validated correlation coefficient $Q^2$ (goodness of prediction).[22] Hotelling's T2 region is shown as an ellipse in the scores plots. It defines the 95% confidence interval of the modeled variation.[24]

## RESULTS AND DISCUSSION

**Identification of Metabolites in the Extracts.** $^1$H NMR spectra of methanol and chloroform extracts were inspected visually. Well-defined signals were present in the aromatic region of the spectra. The following peaks corresponding to ranunculin aglycon could be found in the $^1$H NMR spectra of methanol extracts: 7.67 (H-3, dd, $J$ = 1.51, 5.77 Hz), 6.19 (H-2, dd, $J$ = 2.01, 5.75 Hz), and 5.16 (H-4, m) ppm. With regard to the chloroform extracts, the signals shifted from 7.67 to 7.49 ppm (H-3, dd, $J$ = 1.51, 5.77 Hz) and from 6.19 to 6.22 ppm (H-2, dd, $J$ = 2.01, 5.75 Hz), respectively. Additional peaks could be identified at 4.00 (H-5A, dd) and 3.80 (H-5B, dd) ppm (Figure 2). The signals were assigned to the resonance peaks of ranunculin aglycon by comparison with data of the literature.[6] The ranunculin aglycon was found to be the major ranunculin metabolite in all extracts. Freeze-drying of the extracts led to inactivation of the enzymes responsible for the biotransformation of ranunculin into its toxic metabolite protoanemonin (Supporting Information, Figure 1S).

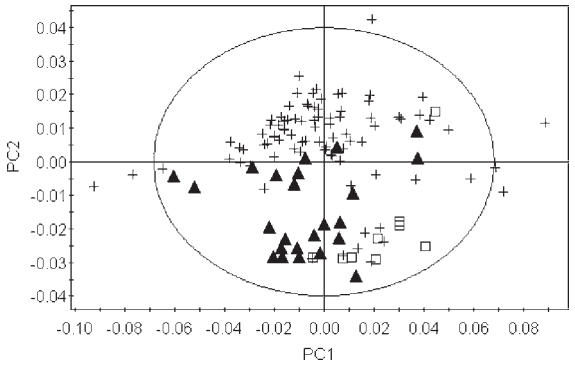


**Figure 3.** Scores plot of a two-component PCA model of the $^1$H NMR spectral data of all methanol extracts ($\delta$ 4.6−5.0 and 3.24−3.5 regions excluded, 0.04 ppm buckets, data mean-centered): □, *R. bulbosus*; ▲, *R. acris*; +, *R. repens*.

Therefore, it is likely that the main metabolite to be found in the plant extracts is either ranunculin or its aglycon. Ranunculin was present in only trace amounts in some of the samples and had spontaneously degraded into its aglycon due to long storage times.[6]

Furthermore, the $^1$H NMR spectra of the methanol extracts contained a variety of primary metabolites such as $\alpha$-glucose (5.10 ppm, d, $J$ = 3.66 Hz), $\beta$-glucose (4.46 ppm d, $J$ = 9.82 Hz), and protons from fatty acids: 5.34 ppm (−C**H**═C**H**− groups), 2.31 ppm (═CHC**H**2-CH═), 2.07 ppm (−**H**2C−CH═CH−), 1.59 ppm (−C**H**2-$CH_2$−COOH), 1.31 ppm ($CH_2$ protons), and 0.9 ppm ($CH_3$ protons). The chloroform extracts, on the other hand, contained mainly ranunculin aglycon and fatty acids.

**Metabolomic Classification of *Ranunculus* Species Using PCA.** For the data set consisting of all samples (EGS, control, and 10 samples from the same Oxfordshire control site) a two-component model explained 67% of the variance. As observed in Figure 3, there is a discrimination possible between *R. repens* and *R. acris* and *R. bulbosus* by PC2. The extracts of *R. acris* and *R. bulbosus* form a cluster in the lower half of the scores plot, whereas most *R. repens* samples are located in the upper half of the scores plot. The loadings plot (data not shown) shows that the most influential chemical shifts are in the lipid and sugar regions. Differentiation between *R. repens* and the other two species was caused by higher levels of $\beta$-glucose, fatty acids, and an unknown aromatic compound (7.12 and 6.08 ppm in *R. repens* samples and higher amounts of $\alpha$-glucose (5.09 ppm) and other unidentified compounds in the sugar region (4.32, 3.64, and 2.52 ppm) for *R. acris* and *R. bulbosus* samples. However, ranunculin aglycon chemical shifts are not among the important influences that can be identified in the loadings plot. An ANOVA analysis confirmed that differences in the relative signal intensities for ranunculin aglycon between the three *Ranunculus* species were not statistically significant.

**Differentiation between EGS and Control Samples Using PCA and PLS-DA.** Due to the observed interspecies variation, only *R. repens* samples were used for determining if there is a significant difference between EGS and control site samples. Those collected from the same Oxfordshire control site over the year were also excluded to balance the number of EGS and control samples and to avoid bias caused by the fact that they are likely to have similar properties. A two-component PCA model based on all *R. repens* EGS and control samples explained 78% of the variance. As observed in Figure 4a, all control samples with

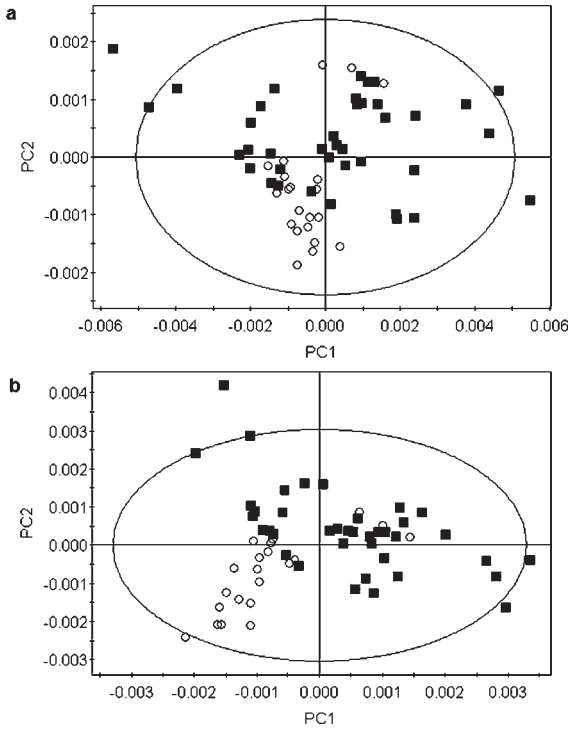


**Figure 4.** Scores plots of PCA (a) and PLS-DA (b) models of the $^1$H NMR spectral data of *R. repens* methanol extracts ($\delta$ 4.6−5.0 and 3.24−3.5 regions excluded, 0.04 ppm buckets, data mean-centered): ○, control sites; ■, EGS sites.

the exception of the three replicates of sample C2 form one group, whereas there appears to be much larger variation within the EGS samples. With regard to the loadings plot, the differentiation between the two groups is caused by fatty acids, glucose, and other carbohydrates, whereas the ranunculin aglycon does not contribute to the variability. PCA of the chloroform extracts showed a similar result (data not shown). However, ranunculin aglycon could be identified as one of the important influences in the PC1 loadings plot of the chloroform extracts. Positive loadings for ranunculin aglycon corresponded to EGS samples, indicating that the ranunculin aglycon content in EGS samples is higher. The negative loadings corresponding to control samples could be assigned as protons from fatty acids.

In addition to PCA, PLS-DA was carried out. The *Y* variable was defined as 1 for EGS samples and 0 for control samples. The PLS-DA scores plot for the methanol extracts show a clear discrimination between EGS and control samples (with the exception of the three replicates of sample C2) (Figure 4b). The correlation coefficient ($R^2Y$) for the three-component PLS-DA model was 0.65, with a $Q^2$ of 0.52. Furthermore, permutation tests were performed to validate the PLS-DA model. All $R^2$ and $Q^2$ values were lower than for the real model, indicating good predictability (Supporting Information, Figure 2S). The PLS-DA coefficients plot showed positive coefficients for ranunculin aglycon, as well as $\alpha$-glucose and protons corresponding to $CH_2$ groups of fatty acids (Figure 5).

To confirm whether EGS samples contained higher contents of ranunculin aglycon or not, we carried out a relative quantification by comparing the signal intensities for spectral region of the H-3 proton of ranunculin aglycon in the methanol extracts of all EGS and control samples. Ranunculin aglycon levels were

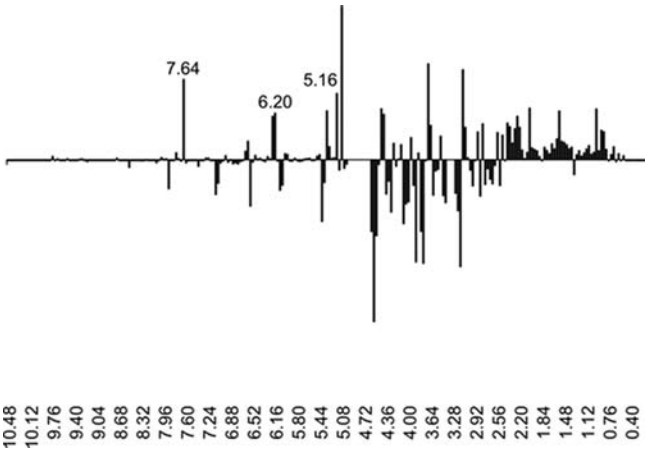


**Figure 5.** Coefficients plot of a three-component PLS-DA model of the $^1$H NMR spectral data of *R. repens* methanol extracts ($\delta$ 4.6−5.0 and 3.24−3.5 regions excluded, 0.04 ppm buckets, data mean-centered). Highlighted peaks correspond to resonance signals for ranunculin aglycon.[6]

0.33 ± 0.19 for EGS samples, which is significantly higher ($p$ < 0.01) than 0.18 ± 0.11% of the total integral for the control samples (Supporting Information, Figure 3S).

**Impact of Environmental Factors on the Metabolite Profile.** The chemical composition of plants can be influenced by many environmental factors, such as climatic conditions and the properties of the soil they grow on.

To investigate if seasonal changes have an impact on the chemical composition of the plant material, 10 samples were collected on one site at different times of the year. PCA was applied to their spectral data to see if groupings of samples collected in the same season could be observed in the scores plot. The results showed that there were no significant differences between samples collected in different seasons. The seasonal changes in the plants' metabolome were much lower than expected.

Previous work in our group[3] suggests that soil nitrate levels at EGS sites are significantly higher than at control sites. Therefore, we wanted to investigate whether the levels of nitrate and other minerals present in the soil might have an impact on the metabolite composition of *Ranunculus*.

PLS was used to determine if there is a correlation between the content of soil minerals and the metabolite composition of the *Ranunculus* samples. The *Y* variable was defined as the level of minerals (nitrate nitrogen, ammonium nitrogen, phosphorus, potassium, magnesium, copper, zinc, total iron, available iron, lead, arsenic, cadmium, mercury, nickel, selenium, and fluoride) in soil samples collected at the same sites.[3] Low $R^2$ and $Q^2$ values of the PLS models indicate that the soil mineral content does not influence the metabolite composition of the *Ranunculus* samples. Whereas the correlation with nitrate levels in the soil is low, higher percentages of the variation in the methanol extracts can be explained by the variation in levels of available iron, arsenic, selenium, fluoride, and molybdenum (Figure 6). Interestingly, a positive correlation between ranunculin aglycon signals and available iron levels could be found, indicating that high available iron levels in the soil might trigger the biosynthesis of ranunculin (Supporting Information, Figure 4S).

We had previously postulated that the well-known irritant effect from eating *Ranunculus* combined with the consumption of

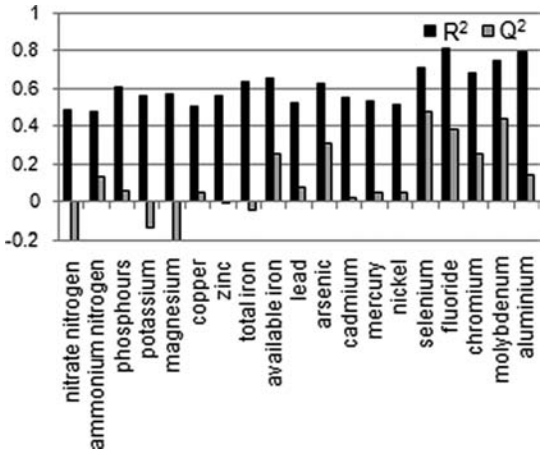


**Figure 6.** $R^2$ and $Q^2$ values of five-component PLS models showing the correlation between $^1$H NMR spectral data of *R. repens* methanol extracts ($\delta$ 4.6−5.0 and 3.24−3.5 regions excluded, 0.04 ppm buckets, data mean-centered) and levels of minerals in soil samples collected at the same sites.

herbage containing high amounts of heavy metals such as iron[3] or herbage with reduced antioxidant activity[1] might cause severe imbalances in a horse's gastrointestinal tract and therefore are likely to be responsible for the occurrence of EGS.

The results of this study support the above hypothesis that *Ranunculus* is involved in the etiology of EGS, but they also indicate that the content of toxic lactones is not the only difference between EGS and non-EGS samples. $^1$H NMR spectroscopy in combination with multivariate data analysis was able to show a clear discrimination between EGS and non-EGS samples. This work also highlights the importance of PCA and PLS for discriminating between different plant species and for understanding the implication of environmental factors on the plants' metabolome. It is evident that EGS has a very complex and multifactorial etiology. Therefore, it is unlikely that ingestion of *Ranunculus* alone can directly cause EGS. However, our data suggest that *Ranunculus* is an important factor in EGS development and is likely to be a cocausative environmental agent of this poorly understood disease.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Biotransformation of ranunculin, PLS-DA permutation tests, ranunculin aglycon levels, and PLS coefficients plot for available iron. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS USED

C, control samples; $CDCl_3$, deuterated chloroform; E, equine grass sickness samples; EGS, equine grass sickness; FID, free induction decay; LB, line broadening; MeOD, deuterated methanol; PCA, principal component analysis; PC, principal component; PLS, partial least-squares; PLS-DA, partial least-squares−discriminant analysis; TMS, tetramethylsilane; TSP, 3-(trimethylsilyl)-propionic-2,2,3,3-$d_4$ acid, sodium salt.

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