Extended abstract No. 291

HUMIC ACIDS FOR MEDICAL USE: 2. REPLACING HYDROCHLORIC ACID BY AN ORGANIC ACID IN THE PRECIPITATION OF HUMIC ACIDS

Guido Meyer[a,b], Renate Klöcking[a,c]
[a]Institut für Verfahrensentwicklung, Torf- und Naturstoff-Forschung, Friedrich-Schneider-Straße 26, D-02763 Zittau, Germany; Hochschule Zittau/Görlitz, Zittau, Germany.
[b]Phone: +493583-61-2315, email: gmeyer@hs-zigr.de
[c]Phone: +493583-61-2303, email: rkloecking@hs.zigr.de

SUMMARY

Two modifications of the standard procedure for the isolation of humic acids from peat samples have been explored. The modifications comprise a Soxhlet-extraction of the freeze-dried peat sample with an organic solvent and the use of organic acids for the precipitation of the humic acid fraction. The organic extraction effectively removes organo-soluble impurities (up to 12 %) without any obvious sign of alteration or loss of humic material (Meyer, 2011). The use of organic acids as precipitant aims at the halogen and mineral acid free preparation of humic acids. In this article, the quality of humic acids obtained using the modified procedure is compared to those obtained by traditional HCl precipitation.

KEYWORDS: Humic acid precipitation, organic acids, purification, organohalogen compounds**.**

INTRODUCTION

Motivation

During recent years, saxonian fish farms have been plagued by a highly contagious fish disease that affects the gills and leads to death in 80-100 % of cases. This epidemic causes great financial losses and affects both food-producing fish farms and producers of much beloved ornamental fish like Koi-carps (fig. 1).

Our interest was aroused when the pathogen was identified as a herpesvirus, the cyprinid herpesvirus 3 (Hedrick, 2000), and the disease was called Koi herpes virus infection (KHV-I), henceforth. From many years of experience in the field of humic substances, we knew that these are potent agents against human pathogenic herpesviruses. Therefore it is intriguing to try whether they are active against KHV, too.

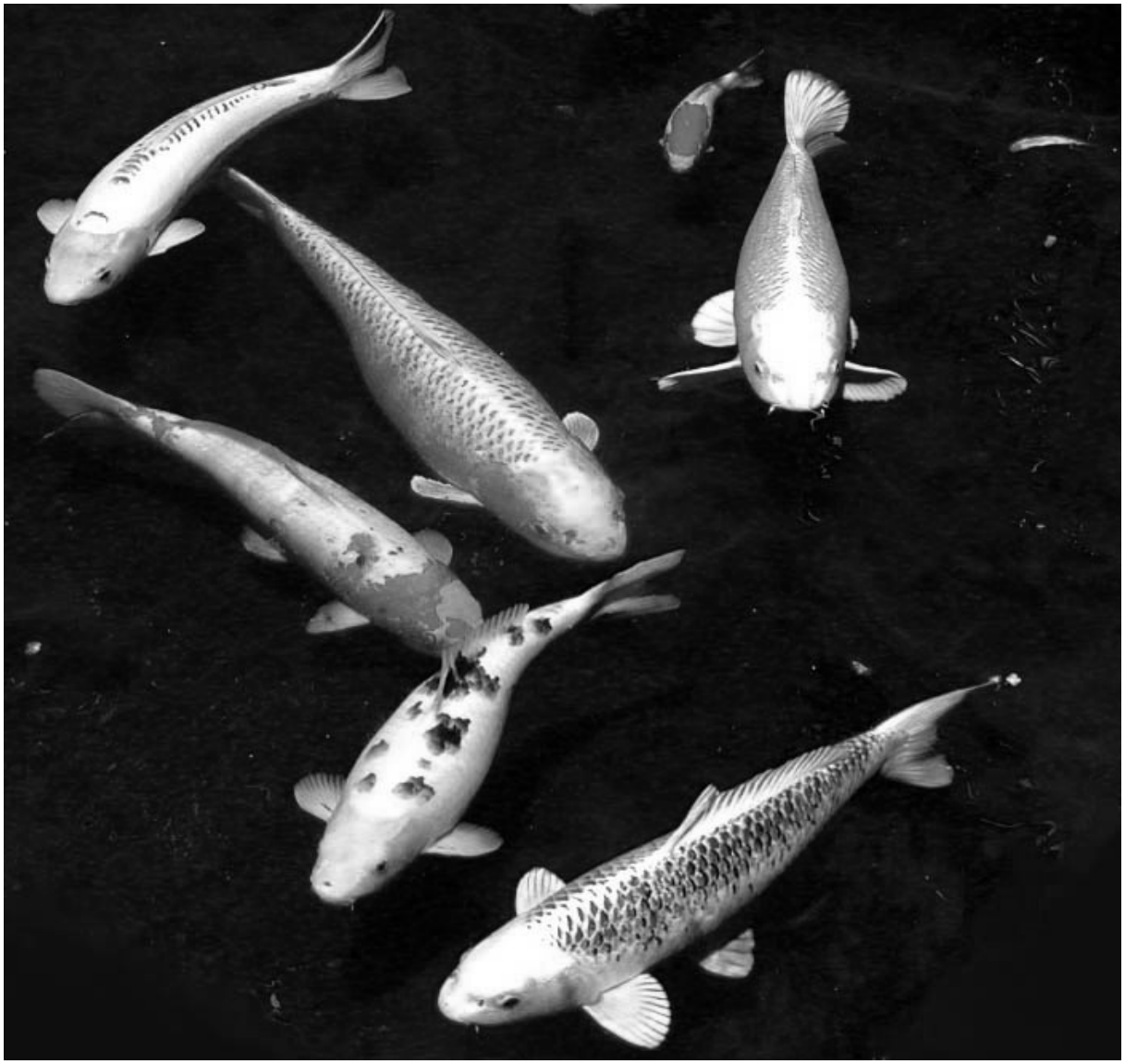

Fig. 1. Six types of Koi carps (Black and white version of Stan Shebs, http://en.wikipedia.org/wiki/File:Six_koi.jpg)

Biocompatibility of humic acids

During the last two decades more and more skeptical reports regarding the use of humic acids for medical purposes were published. It was recognised that humic substances have a high affinity towards many toxic heavy metals and aromatic organic compounds, and that they make an important contribution to storage and distribution of environmental contaminants (Murphy, 1995). Moreover, humic acids are highly active compounds that can catalyse many reactions. In the past, these properties have lead to unpleasant surprises, an example being the formation of toxic trichloromethane from humic acids upon treatment with chlorine during the preparation of drinking water (Adin, 1991). These problems prompted us to develop a protocol for the isolation of humic substances that focuses on minimizing the incorporation of toxic organic and halogenated compounds into the isolated humic substances. The general outline of this procedure is shown in figure 2 and will be explained in more detail later.

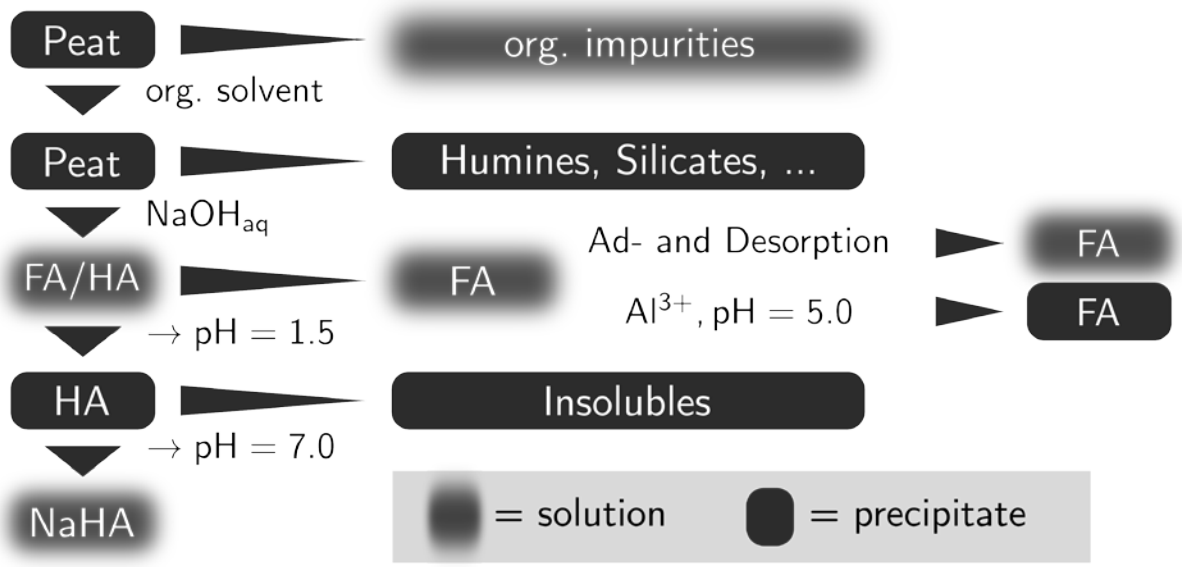

Fig. 2. Schematic drawing of the proposed isolation protocol for humic (HA) and fulvic acids (FA).

MATERIALS AND METHODS

Chemicals - Solvents used for the extractions were of synthesis grade. Water ($\sigma < 5.7$ µS/m), used for HPLC/SEC, or humic acid isolations, was purified using a *SG Reinstwassersystem Typ Clear UV*. Organic solvents used for HPLC/SEC were of HPLC grade or better. Other chemicals were of analysis grade. All chemicals were used as received. *Organic extraction* - The freeze-dried peat sample was extracted in a Soxhlet extractor with a mixture of methyl acetate and *c* hexane (3:2) until the extract was colourless. Residue and extract were dried in vacuum. *Aqueous extraction* - A suspension of the dried peat in water (10 ml per g wet peat) was warmed to 30 °C under stirring. pH was adjusted to 9.0 with NaOH and stirred under pH control for 2 h. The sludge was separated by centrifugation (2,800 x g, 10 min), and floating particles were removed by filtration. *Humic acid precipitations with oxalic acid* - 1.0 l of the aqueous extract was warmed to 30 °C, after which 0.33 l of an 0.50 M aqueous solution of oxalic acid was added. After stirring for 30 min, the precipitate was collected by centrifugation and washed with water until it was free of oxalic acid (typically 3-4x, controlled by HPLC). The residue was suspended in water and dissolved by adjusting to pH 7.0. The resulting solution was centrifuged (16,500 x g, 3 h) to remove undissolved material and then freeze-dried. *Humic acid precipitations with hydrochloric acid* - 1.0 l of the aqueous extract was warmed to 30 °C and the pH adjusted to 1.5-2.0 by stepwise addition of 10% hydrochloric acid. After stirring for 30 min, the precipitate was collected by centrifugation and washed with water once. The residue was suspended in water and dissolved by adjusting to pH 7.0. The resulting solution was centrifuged (16,500 x g, 3 h) to remove undissolved material and then freeze-dried. *HPSEC/HPLC* - A *Varian Prostar HPLC system* equipped with autosampler, gradient pump, column oven, and diode array detector was used. HPSEC was performed on a PSS BIO MCX column (1,000 Å, 5 µm) that was calibrated using polysulfonate standards (77 k, 32 k, 17 k, 8 k, 4 k, 2 k, 1 k) (PSS GmbH; Mainz; Germany). Additionally Alizarin S Red (320), *p*-amino-salicylic acid (153) and *p*-hydroxy-benzoic acid (138) were used as low molecular weight standards. A solution of 283.9 mg Na_2HPO_4, 87.7 mg ethylenediaminetetraacetic acid and 467.5 mg NaCl in 700 mL water was filtered, mixed with 300 mL of *n*-propanol, degassed, and used as eluent. $NaNO_3$ and

[Fe(EDTA)]Cl were used as flow markers, respectively. The detection was performed at 254 nm. HPLC was performed on a LiChroCART® 250–4; RP-18 (5 µm) column (Merck). A solution of 7.80 g NaH_2PO_4 in 1.00 l water was adjusted to pH 2.8, filtered, degassed and used as fluent. The detection was performed at 202 nm.

RESULTS

Since a pH below 2 is necessary to ensure a complete precipitation of humic acids, a strong acid has to be used. Possible candidates are listed in table 1. We did not want to use the very strong mineral acids as we plan to explore the possibility of a pH-dependent fractionation of humic acids in future. Trifluoroacetic, trichloroacetic and amidosulfonic acid would be very promising candidates but cannot be used due to the risk of halogen and nitrogen incorporation, respectively. Therefore, we tested the next two candidates, oxalic acid and citric acid in the precipitation of humic acids isolated from peat of the Altteicher Moor and found them both capable of precipitating humic acids quantitatively. Both acids have anions of low toxicity towards fish (e.g. sodium oxalate: LC_{50}(Danio rerio) = 630 mg/l, 96 h; citric acid: LC_{50}(Leuciscus idus melanotus) = 440 mg/l, 48h, (MSDS, 2012)) and water-soluble and hence can be removed by washing the precipitated humic acids with water. Since oxalic acid is quantified by HPLC more easily, it will be used in further experiments.

Table 1. Water soluble organic acids that are candidates for the precipitation of humic acids.

Candidate acids	pK_a	Candidate acids	pK_a
Mineral acids	<0	Citric acid	3.1
Trifluoroacetic acid	0.2	Formic acid	3.8
Trichloroacetic acid	0.7	Lactic acid	3.9
Amidosulfonic acid	~1	Benzoic acid	4.2
Oxalic acid	1.3	Acetic acid	4.8

Peat from the Altteicher Moor shows a high degree of decomposition. In order to explore how oxalic acid performs in the isolation of humic acids from less decomposed peats, we used peat from a second sampling site, the Dierhäger Moor (DM) (see abstract no. 201/287).

The peat samples were freeze-dried and treated with methyl acetate/*c*-hexane to remove organo-soluble impurities. Then the humic acids were extracted with aqueous sodium hydroxide solution at pH 9, precipitated with hydrochloric and oxalic acid, respectively, thoroughly washed and converted to the corresponding sodium salts. The analytical data obtained so far are summarized in table 2. In all four cases 71-91 % of the material isolable via the HCl-procedure could be also isolated by our newly developed procedure. The chemical composition is within the limits of humic substances reported in the literature (Klavins, 2010), however, the C/N-ratio indicates a rather low N-content. The molecular mass

Table 2. Analytical data of sodium humates isolated from peat samples from the Dierhäger Moor (DM)

	M_w/kDa	M_n	PDI	C-Yield	C/N	E_4/E_6	E_2/E_2	A_{270}/µg C
DM-30	3.1	771	4.0	1.8 %	23	8.3	2.7	29
DM-30-HCl	2.6	705	3.7	2.4 %	22	8.3	2.8	30
DM-48	6.6	851	7.8	6.2 %	52	6.8	2.5	54
DM-48-HCl	4.5	789	5.6	7.4 %	57	7.7	2.5	37
DM-68	6.6	920	5.8	7.9 %	58	6.3	2.5	46
DM-68-HCl	6.7	846	7.9	8.7 %	55	6.0	2.5	40
DM-86	9.2	888	10.4	5.5 %	37	6.6	2.6	48
DM-86-HCl	5.1	803	6.4	7.7 %	44	6.3	2.6	31

distribution obtained by GPC indicate, that the developed method is biased against low molecular weight compounds. This is in agreement with the higher absorption at 254 nm obtained for the humic acids isolated using oxalic acid. The loss of material most likely occurs during the removal of the oxalic acid by washing as the later washings yield brown solutions. Further experiments to reduce this loss of material are under way.

CONCLUSIONS/DISCUSSION

A new method for the precipitation of humic acids from aqueous solution has been tested using humic acids isolated from the *Dierhäger Moor*. Compared to hydrochloric acid, oxalic acid is capable of precipitating 71-91 % of the humic acids. The analytical data of the obtained humic acids show a bias against low molecular weight humic acids.

ACKNOWLEDGEMENT

We thank the *Bundesministerium für Bildung und Forschung* for financial support.

REFERENCES

Adin, A., Katzhedler, J., Alkaslassy, D., Rav Acha, Ch., (1991), Trihalomethane Formation in Chlorinated Drinking Water: A Kinetic Model, *Water Research* **25**, 797-805.

Hedrick, R. P., et al., (2000), A Herpesvirus Associated with Mass Mortality of Juvenile and Adult Koi, a Strain of Common Carp., *Journal of Aquatic Animal Health* **12**, 44-57.

Klavins, M., (2010), *Variations of Humic Acid Properties Within Peat Profiles* in *Mires and peat*, Riga (Latvia), University of Latvia Press, p. 175-197.

Meyer, G., Klöcking, R., (2011), Optimising the Protocol for the Isolation of Humic Substances With Regards to Their Use in Biological Systems , *Proceedings of the Arbeitstagung Torf und Huminstoffe, Zittau, Germany, 28-30 September, 2011.*

MSDS, (2012), Material Safety Data Sheets, downloaded from *www.sigma-aldrich.com* on February 29, 2012.

Murphy, E. M., Zachara J. M., (1995), The Role of Sorbed Humic Substances on the Distribution of Organic and Inorganic Contaminants in Groundwater, *Geoderma* **67**, 103-124.

Shirshova, Ludmila T., Ghabbour, Elham A., Davies G., (2006) Spectroscopic Characterization of Humic Acid Fractions Isolated From Soil Using Different Extraction Procedures, *Geoderma* **133**, 214-216.

Stevenson, F. J., (1994), *Humus Chemistry: Genesis, Composition, Reactions*, John Wiley & Sons Ltd., New York.