JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Floral Markers of Cornflower (*Centaurea cyanus*) Honey and Its Peroxide Antibacterial Activity for an Alternative Treatment of Digital Dermatitis

Stefanie Oelschlaegel,*[,†] Laura Pieper,[§] Rudolf Staufenbiel,[#] Margit Gruner,[‡] Linda Zeippert,[†] Bernd Pieper,[⊥] Isabelle Koelling-Speer,[†] and Karl Speer*[,†]

[†]Food Chemistry, Technische Universität Dresden, Bergstrasse 66, 01069 Dresden, Germany

[§]Department of Population Medicine, Ontario Veterinary College, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1

[#]Ruminant and Swine Clinic, Free University of Berlin, Koenigsweg 65, 14163 Berlin, Germany

[‡]Organic Chemistry, Technische Universität Dresden, Bergstrasse 66, 01069 Dresden, Germany

[⊥]Technologie- und Produktentwicklung GmbH, Dorfstrasse 34, 16818 Wuthenow, Germany

ABSTRACT: Cornflower (*Centaurea cyanus*) honey can be characterized by a greenish yellow color and an intense flavor with a bitter aftertaste. Because cornflower honey contains only a limited amount of pollen for the verification of its floral origin, one objective was the characterization of its polyphenol and norisoprenoid contents to assign floral markers. Here, lumichrome (18.8−43.5 mg/kg), 7-carboxylumichrome, (*Z*/*E*)-3-oxo-retro-α-ionol, and 3-oxo-α-ionol appeared to be quite suitable for distinguishing cornflower honey from other unifloral honeys. Additionally, due to its comparably high hydrogen peroxide content (0.5−0.9 mM/h) and the associated antibacterial activity, cornflower honey was used as an alternative treatment of digital dermatitis on an organic dairy farm. Cows affected by this hoof disease often show severe lameness and a subsequent decline in milk yield and loss of body condition. The cows' hooves treated with cornflower honey showed significantly faster healing than the control group without any treatment.

KEYWORDS: *unifloral honey, norisoprenoids, peroxide antibacterial activity, digital dermatitis, dairy cow*

INTRODUCTION

Especially unifloral honeys, with their typical smell and taste, are increasingly favored by customers even though they are more expensive compared to polyfloral honeys. Furthermore, certain honeys such as manuka honey are used as wound-healing remedies.[1,2] Hence, in terms of quality assurance the botanical origin of honeys needs to be clearly recognizable. Because microscopic pollen analysis is applicable in a limited way, objective analytical methods that complement the pollen analysis in the determination of the botanical origin of honey were developed. Several compounds detected in honey such as volatiles,[3−5] norisoprenoids,[6,7] heterocyclics,[8,9] and phenolic substances[10,11] could be correlated to the floral origin. In this context, benzoic acid, phenylacetic acid, (±)-2-*cis*,4-*trans*-abscisic acid, and dehydrovomifoliol among others were assigned as markers for heather honey.[12,13] Nitrogen-containing compounds such as kynurenic acid, 2-aminoacetophenone, and 2-caboxy-4-quinolinone are proven to be characteristic for chestnut honey.[14,15] The flavonoids luteolin and tricetin are typical constituents of eucalyptus honey,[16] and citrus honey can be distinguished by its methyl anthranilate level of >2 mg/kg,[17] just to mention a few unifloral honeys and their chemical markers.

However, no literature data are available on the floral markers of cornflower honey. Because the greenish yellow honey reveals only a limited pollen amount for the verification of the floral origin,[18] one objective of this study was the characterization of its polyphenol and norisoprenoid contents to assign floral markers. In addition, with regard to its antibacterial activity, the hydrogen peroxide yield was determined in comparison to other kinds of honey.

The antimicrobial activity of honey has been attributed to osmolality, acidity, viscosity, phenolic compounds, and, likewise, peroxide produced by glucose oxidase.[19] The use of honey as a topical therapy seems to have beneficial effects on superficial wounds and burns.[20] Among animal diseases, hoof diseases are one of the main culling reasons in organic dairy cow herds in Germany.[21] Digital dermatitis is a localized circumscribed superficial epidermitis of the coronary margin or interdigital space, which can develop to an ulcerative granulomatous lesion. Affected cows often show severe lameness, subsequent decline of milk yield, and loss of body condition. The infectious, multifactorial disease is caused by spirochetes such as *Treponema* spp. and *Borrelia* spp., which colonize wet and fissured skin.[22] Gram-negative, anaerobe bacteria such as *Fusobacterium necrophorum* are also associated with digital dermatitis.[23] Antibiotics in local or systemic treatment and footbaths with nonantibiotic chemical additives are commonly used for therapy.[22] However, the use of

Received: August 27, 2012
Revised: November 6, 2012
Accepted: November 10, 2012
Published: November 10, 2012

Table 1. Honey Samples, Floral and Geographical Origins, and Year of Production

honey sample	origin	year of production	honey sample	origin	year of production
acacia 1	Romania	2010	lavender 4	France	2009
acacia 2	Germany	2010	lavender 5	France	2010
acacia 3	unknown	2010	lime 1	Romania	2010
acacia 4	southeastern Europe	2010	lime 2	unknown	2010
acacia 5	Hungary	2010	lime 3	Romania	2010
buckwheat 1	Germany	2008	lime 4	Romania	2010
buckwheat 2	Germany	2008	lime 5	Romania	2010
buckwheat 3	Poland	2008	manuka 1	New Zealand	2010
buckwheat 4	China	2009	manuka 2	New Zealand	2010
buckwheat 5	Germany	2009	manuka 3	New Zealand	2010
chestnut 1	Italy	2008	manuka 4	New Zealand	2010
chestnut 2	Italy	2009	manuka 5	New Zealand	2010
chestnut 3	Italy	2009	orange 1	unknown	2008
chestnut 4	unknown	2009	orange 2	Mexico	2008
chestnut 5	Italy	2009	orange 3	Spain	2009
clover 1	Canada	2009	orange 4	unknown	2009
clover 2	unknown	2009	orange 5	Spain	2009
clover 3	unknown	2009	pine 1	unknown	2010
clover 4	unknown	2009	pine 2	Turkey	2010
clover 5	unknown	2009	pine 3	Mediterranean	2010
cornflower 1	Romania	2008	pine 4	unknown	2010
cornflower 2	Romania	2008	pine 5	Mediterranean	2010
cornflower 3	Romania	2008	rape 1	Romania	2009
cornflower 4	Germany	2008	rape 2	Germany	2009
cornflower 5	Germany	2008	rape 3	southeastern Europe	2009
eucalyptus 1	Uruguay	2009	rape 4	France	2009
eucalyptus 2	unknown	2009	rape 5	Croatia	2009
eucalyptus 3	unknown	2009	rosemary 1	Spain	2008
eucalyptus 4	Australia	2009	rosemary 2	Spain	2008
eucalyptus 5	unknown	2009	rosemary 3	Spain	2009
fir 1	Greece	2009	rosemary 4	Spain	2009
fir 2	Slovakia	2009	rosemary 5	Spain	2009
fir 3	Poland	2009	sage 1	Croatia	2008
fir 4	Germany	2010	sage 2	Croatia	2008
fir 5	unknown	2010	sage 3	Germany	2009
heather 1	Norway	2008	sage 4	Germany	2009
heather 2	France	2008	sage 5	unknown	2009
heather 3	Germany	2008	sunflower 1	unknown	2010
heather 4	Spain	2009	sunflower 2	Romania	2010
heather 5	France	2009	sunflower 3	Ukraine	2010
honeydew 1	Italy	2009	sunflower 4	eastern Europe	2010
honeydew 2	Italy	2009	sunflower 5	eastern Europe	2010
honeydew 3	unknown	2009	thyme 1	Greece	2008
honeydew 4	Germany	2009	thyme 2	Spain	2010
honeydew 5	Germany	2009	thyme 3	Spain	2010
lavender 1	France	2008	thyme 4	Spain	2010
lavender 2	France	2009	thyme 5	Greece	2010
lavender 3	France	2009			

antibiotics in organic farming is strictly regulated with long withdrawal times for milk and meat. Furthermore, the use of formalin and $CuSO_4$ for footbaths is controversial due to their potential environmental hazard and unclear regulations in Germany.

The use of honey for wound dressing is not yet a common practice in veterinary medicine;[24] however, licensed veterinary products are available. Even though cornflower honey is of less commercial relevance, it showed strong bacteriostatic effects, especially against *Staphylococcus aureus* and *Sacina lutea*, and is, thus, under discussion as a natural wound-healing remedy.[25,26] Hence, the aim of this study was to demonstrate the suitability of cornflower honey as an alternative treatment of digital dermatitis on an organic dairy farm.

MATERIALS AND METHODS

Honey Samples. For a comparative study 5 authentic samples of 19 kinds of honey (acacia, buckwheat, chestnut, clover, cornflower, eucalyptus, heather, lavender, lime, manuka, orange, pine, rape, rosemary, sage, sunflower, thyme, and honeydew) were analyzed. Most of the 95 honey samples (Table 1) were provided by Quality Services International GmbH (Bremen, Germany) and stored at 8 °C in darkness until analyzed. According to the provided data of the

sensory and microscopic pollen analyses, all samples were declared as unifloral honeys.

Chemicals. Methanol of HPLC grade, acetic acid 100% glacial, and sodium chloride were purchased from VWR (Darmstadt, Germany); acetonitrile and methanol of LC-MS grade were ordered from Fisher Scientific (Schwerte, Germany); and DMSO-d_6 was from Armar Chemicals (Doettingen, Switzerland). Kojic acid, methyl syringate, daidzein, 4-hydroxyquinoline, kynurenic acid, and (±)-2-*cis*,4-*trans*-abscisic acid were acquired from Alfa Aesar (Karlsruhe, Germany). Ethyl acetate anhydrous, formic acid of LC-MS grade, 1-hydroxy-4-keto-2-ionone (dehydrovomifoliol), 4-hydroxybenzoic acid, 4-methoxy-C13,d_3-benzoic acid-d_4, caffeic acid, 5-hydroxymethylfurfural, β-phenyllactic acid, caffeine, lumichrome, methyl anthranilate, *p*-coumaric acid, phenylalanine, pinobanksin, pinocembrin, protocatechuic acid, *trans*-ferulic acid, tyrosin, and tryptophan were purchased from Sigma-Aldrich (Steinheim, Germany); apigenin and chrysin were obtained from Roth (Karlsruhe, Germany). All purchased chemicals were of analytical grade. The compounds 3-oxo-α-ionone (MS purity = 94.3%) and desoxyabscisic acid (MS purity = 79.5%) were isolated from honey, and the structures were elucidated via NMR and MS. Bidistilled water was generated by the Bi-Distilling Apparatus Bi 18E from QCS GmbH (Maintal, Germany).

Solid Phase Extraction (SPE) and UPLC-PDA-MS/MS Detection. Two grams of honey was dissolved in 3 mL of 2% NaCl, and the pH was adjusted to 1. The final volume after SPE was adjusted to 5 mL. The SPE procedure on the Chromabond HR-X cartridge (Macherey-Nagel, Düren, Germany) and the UPLC separation of the methanolic extract on a Nucleodur C18 Pyramid column (100 × 2.1 mm, 1.8 μm; Macherey-Nagel, Düren, Germany), using 0.1% formic acid and methanol with 0.05% formic acid as eluents as well as the PDA-MS/MS-detection of the analytes were performed as reported by Oelschlaegel et al.[27]

Quantitation. The quantitative data of the polyphenols and norisoprenoids were obtained by MS/MS measurements. To quantitate the analytes, a calibration was set up in a model honey solution. Therefore, a model honey consisting of 40% glucose, 40% fructose, and 20% water was subjected to SPE, and the standard mixtures were subsequently spiked to the extract. All of the standards were dissolved in methanol (HPLC grade). The quantitation was accomplished via a time-scheduled selected reaction monitoring (SRM) method with at least two transitions per compound (Table 2). The quantitation limits of the analytes were determined on the basis of the signal-to-noise ratio of 10:1. The linearity range of the analytes and their recoveries for two spiking levels referring to the sample weight of the SPE method were determined as well (Table 3).

Liquid Extraction and Semipreparative HPLC for the Fractionation of Unknown Compounds. To identify unknown polyphenols or norisoprenoids, about 800 g of cornflower honey was extracted with ethyl acetate, and the compounds of concern were isolated with a semipreparative HPLC-DAD system equipped with a fraction collector (VWR) as reported by Oelschlaegel et al.[27]

HPLC-MS Q-TOF Analysis. To determine the exact mass, the isolated compounds were analyzed on a Dionex Ultimate 3000 RS HPLC system using a Nucleodur C18 column (150 mm × 4.6 mm, 5 μm, Macherey Nagel, Düren, Germany). The HPLC conditions were as follows: flow rate, 0.3 mL/min; solvent A, H_2O (0.1% formic acid); solvent B, acetonitrile (0.1% formic acid); gradient elution program, 0−1 min, 10% B; 1−6 min, 10−90% B; 6−7 min, 90% B; 7−8 min, 90−10%; equilibration for 12 min. The samples (5 μL) were then analyzed by ESI in the positive and negative modes using a Bruker maxis 3 g time-of-flight (TOF) MS (Bruker Daltonic GmbH, Bremen, Germany). Mass spectral data were acquired in the range of m/z 75−600 with an acquisition rate of 1 spectra/s, averaging three transients. The source parameters were as follows: drying gas temperature, 200 °C; drying gas flow rate, 8 L/min; nebulizer pressure, 2 bar; end plate offset voltage, 500 V; capillary voltage, 4500 V; and transfer time, 30−50 μs.

NMR Spectroscopy. The isolated fractions were concentrated to dryness and subsequently resolubilized in DMSO-d_6. To elucidate the structure of the unknown compounds, a set of NMR experiments were performed on a Bruker Avance-600 spectrometer (Bruker Bio Spin GmbH, Rheinstetten, Germany). The ^{1}H and ^{13}C spectra were recorded as solutions in DMSO-d_6 at 25 °C using TMS as an internal standard for ^{1}H and ^{13}C with δ = 0 ppm, respectively. The ^{15}N

Table 2. Identified Compounds in Cornflower Honey, Retention Time (t_R), Molecular Ion, SRM Transitions, and UV Maxima (UV_{max})

compound[a]	t_R (min)	$[M + H]^+$	SRM transitions	UV_{max} (nm)
tyrosin	0.76	182.02	91.15, 136.09, 165.06	274
kojic acid	1.30	143.06	69.30, 97.21	266
phenylalanine	1.64	166.04	77.18, 103.14, 120.14	256
5-hydroxymethylfurfural	2.00	127.05	53.32, 81.17, 109.09	284
tryptophan	2.62	205.02	118.11, 146.05, 188.04	278
4-hydroxyquinoline	3.80	146.05	77.20, 91.18, 118.12	316/326
4-hydroxybenzoic acid	4.12	139.06	PDA detection	255
hydroxymonoterpenic acid[b]	5.21	183.03	93.16, 137.09	306
kynurenic acid	6.79	190.04	89.23, 116.19, 144.15	330
dehydrovomifoliol	7.53	223.07	93.16, 121.10, 205.07	241
vomifoliol[b]	7.53	225.14	95.23, 149.15, 207.15	242
dehydromonoterpenic acid[b]	8.73	165.06	91.16, 119.14	302
methyl syringate	10.38	213.03	121.09, 154.03, 181.02	274
3-oxo-α-ionone[b]	11.87	207.08	43.28, 91.15, 137.11	242
methyl anthranilate	11.91	152.14	92.08, 119.94, 134.93	242/330
lumichrome	12.60	243.07	172.15, 198.12	259/352
(±)-2-*cis*,4-*trans*-abscisic acid	13.73	265.05	201.08, 229.03, 247.07	264
(*Z*)-3-oxo-retro-α-ionol[b]	13.89	209.13	123.18, 149.13, 165.14	295
(*E*)-3-oxo-retro-α-ionol[b]	14.22	209.12	149.15, 151.14, 191.16	291
3-oxo-α-ionol[b]	14.22	209.13	133.15, 137.14, 191.15	242
desoxyabscisic acid[b]	15.48	249.04	119.12, 147.07, 231.11	266
apigenin	15.82	271.01	91.14, 119.07, 153.03	266/340
pinocembrin	16.41	257.02	77.17, 103.16, 131.08	288
chrysin	16.87	255.05	77.31, 103.24, 153.11	266/316
compound[a]	t_R (min)	$[M - H]^-$	SRM transitions	UV_{max} (nm)
protocatechuic acid	2.73	152.96	91.22, 108.90, 109.20	259/291
caffeic acid	5.58	179.06	107.16, 134.10, 135.12	323
β-phenyllactic acid	6.91	165.01	101.12, 103.15, 119.12	256
p-coumaric acid	7.42	163.04	93.18, 119.14, 162.26	309
7-carboxylumichrome[b]	8.12	271.05	184.13, 227.13	266/348
pinobanksin	14.70	271.01	161.05, 197.06, 253.02	288

[a]Compounds identified via reference substances if not stated otherwise. [b]Compounds identified via isolated substance, elucidated by NMR and MS.

Table 3. Linearity Range, Correlation Coefficient, Limit of Detection (LoD), Limit of Quantitation (LoQ), and Recovery of Analytes Used for Quantitation

	linearity range				recovery (R)			
compound[a]	mg/kg	R^2	LoD (mg/kg)	LoQ (mg/kg)	level 1 (mg/kg)	level 2 (mg/kg)	$R_{Level\ 1}$ (%)	$R_{Level\ 2}$ (%)
3-oxo-α-ionone[b]	0.30−100	0.999	0.14	0.47	0.5	2.5	99	99
4-hydroxybenzoic acid	0.60−20	0.993	0.18	0.60	3.5	20.0	103	91
4-hydroxyquinoline	0.10−31	0.999	0.03	0.10	0.6	6.0	105	97
apigenin	0.20−47	0.993	0.10	0.20	0.1	1.0	80	74
β-phenyllactic acid	0.25−1250	0.999	0.08	0.25	187.5	750.0	98	99
caffeic acid	0.05−25	0.999	0.03	0.10	5.0	20.0	96	99
chrysin	0.10−125	0.997	0.10	0.20	0.1	1.0	99	94
(±)-2-*cis*,4-*trans*-abscisic acid	0.03−125	0.994	0.01	0.03	5.0	50.0	103	104
dehydrovomifoliol	0.05−1560	0.996	0.02	0.05	62.5	250.0	102	101
desoxyabscisic acid[b]	0.30−60	0.995	0.10	0.35	0.5	30.0	102	101
5-hydroxymethylfurfural	0.03−125	0.996	0.01	0.03	2.3	45.0	98	99
kojic acid	0.13−125	0.993	0.08	0.25	0.5	20.0	93	95
kynurenic acid	0.03−500	0.993	0.02	0.05	nd	35.0	nd	95
lumichrome	0.03−190	0.996	0.01	0.03	7.5	30.0	105	108
methyl syringate	0.03−140	0.998	0.15	0.50	5.8	115.0	105	105
methyl anthranilate	0.05−125	0.999	0.05	0.25	0.1	1.5	101	94
p-coumaric acid	0.25−125	0.992	0.05	0.25	12.5	50.0	105	107
phenylalanine	0.10−125	0.998	0.04	0.20	0.1	75.0	95	96
protocatechuic acid	0.03−62	0.991	0.02	0.05	3.8	15.0	91	102
tryptophan	0.10−125	0.998	0.03	0.13	0.1	12.5	93	94
tyrosin	0.10−125	0.995	0.04	0.20	0.1	25.0	92	87

[a]Reference substances if not stated otherwise. [b]Isolated substance, elucidated by NMR and MS.

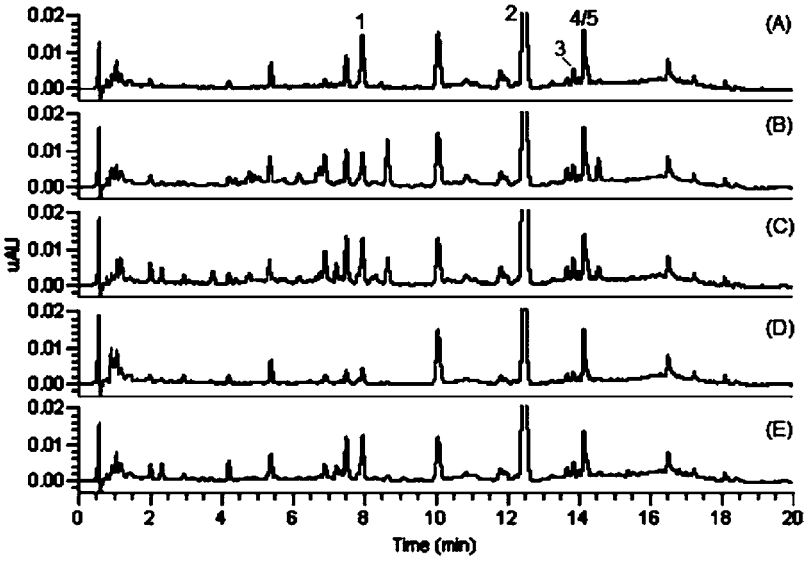


Figure 1. UPLC chromatograms at λ = 254 nm of cornflower honeys. Geographical origin: (A−C) Romania, (D, E) Germany. Peaks: (1) 7-carboxylumichrome; (2) lumichrome; (3/4) (*Z*/*E*)-3-oxo-retro-α-ionol; (5) 3-oxo-α-ionol.

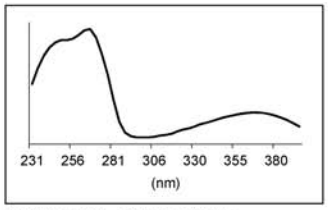
chemical shifts were related to CD_3-$^{15}NO_2$ with δ = 0 ppm as an internal standard. The following 2D NMR experiments were carried out: COSY, $^1H/^{13}C$ HSQC, $^1H/^{15}N$ HSQC, $^1H/^{13}C$ HMBC, NOESY, and ROESY, using a BBI probe with pulsed *z*-gradients and inverse technique.

Photometric Determination of Hydrogen Peroxide. A stock solution of peroxidase from horseradish (type I, Sigma-Aldrich, Germany) of 20 μg/mL and *o*-dianisidine (Sigma-Aldrich, Germany) 50 μg/mL was prepared in 10 mM sodium dihydrogen phosphate buffer, pH 6.5.[28] One gram of honey was dissolved in 5 mL of bidistilled water and incubated at 37 °C for 60 min to release the hydrogen peroxide. To degrade the hydrogen peroxide, 675 μL of the peroxidase/*o*-dianisidine solution was added to 200 μL of the incubated honey solution. The mixture was subsequently incubated at room temperature for 5 min. For this, *o*-dianisidine served as redox indicator, leading to a reddish color measured at 540 nm (HEλIOS photometer, Thermo Spectronic, Wuppertal, Germany). To stabilize the occurring color, 600 μL of 6 M sulfuric acid was added prior to the analysis. The hydrogen peroxide (30%, Prolabo VWR, Germany) calibration prepared in bidistilled water from 0.17 to 8.50 μg/mL revealed a coefficient of determination of 0.999. The reproducibility of the method was determined to CV 3.9% and the repeatability to CV 6.9%.

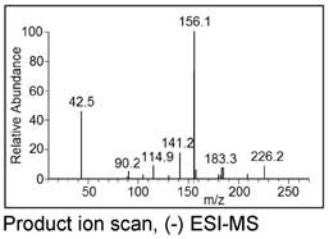
Animal Study. The clinical trial was conducted on an organic dairy farm in northeastern Germany. The herd size was approximately 300 Holstein dairy cows, which were kept in straw barns; the straw was replaced daily. In fall 2008, lameness and hoof disorders were increasingly observed.

Table 4. Chemical Characteristics of 7-Carboxylumichrome, (*Z*/*E*)-3-Oxo-retro-*α*-ionol, and 3-Oxo-*α*-ionol Detected in Cornflower Honey[a]

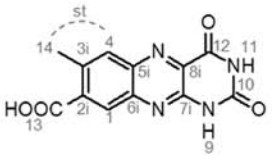
7-carboxylumichrome



UPLC-PDA, UV spectrum

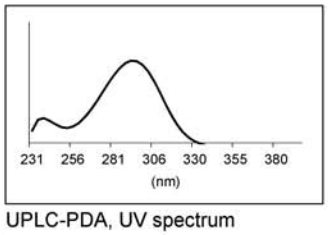


Product ion scan, (-) ESI-MS

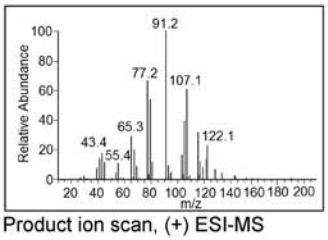


No.	δ_H	δ_C	$^{2,3}J_{CH}$-HMBC correlation
1	8.39	131.04	C-1/ H-14 w
2	-	137.11	C-2/ H-4 st, 14 m
3	-	143.27	C-3/ H-1 m, 14 st
4	7.73	127.77	C-4/ H-14 st
9	12.00	-	NH, br, no $\delta(^{15}N)$
11	11.76	-	NH, $\delta(^{15}N)$ = -220 ppm
13	12.30	168.20	C-13/ H-1 w
14	2.70	21.70	C-14/ H-4 st

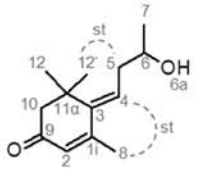
(Z)-3-oxo-retro-α-ionol



UPLC-PDA, UV spectrum

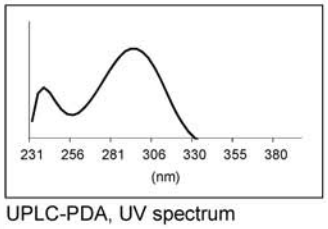


Product ion scan, (+) ESI-MS

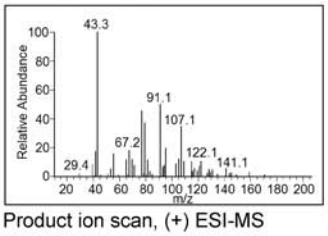


No.	δ_H	δ_C	$^{2,3}J_{CH}$-HMBC correlation
1	-	154.96	C-1/ H-4 st, 5 m, 8 st
2	5.84	124.61	C-2/ H-5 w, 8 st
3	7.68	141.61	C-3/ H-2 st, 4 m, 5 st, 8 st, 10 st, 12 st
4	7.95	133.24	C-4/ H-5 st, 6 w, 8 w
5	2.38	37.72	C-5/ H-6a-OH m, 7 st
6	3.72	66.10	C-6/ H-4 st, 5 st, 6a-OH m, 7 st
6a	4.65	66.10	-
7	1.22	23.44	C-7/ H-6a-OH st, 5 st
8	2.20	21.87	C-8/ H-2 st
9	-	197.92	C-9/ H-10 st, 12 w
10	2.36	53.41	C10/ H-2 m, 12 st
11α	-	39.14	C-11/ H-4 st, 5 w, 10 st, 12/12' st
12/12'	1.23	28.56	C-12,12'/ H-4 w, 5 w, 10 m, 12'/12 st

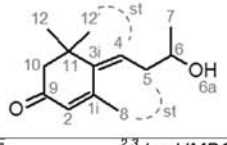
(E)-3-oxo-retro-α-ionol



UPLC-PDA, UV spectrum



Product ion scan, (+) ESI-MS



No.	δ_H	δ_C	$^{2,3}J_{CH}$-HMBC correlation
1	-	155.93	C-1/ H-4 st, 5 w, 8 st
2	5.83	127.91	C-2/ H-8 st
3	7.71	142.63	C-3/ H-2 m, 4 w, 5 st, 8 st, 10 st, 12/12' st
4	6.10	128.58	C-4/ H-5 st, 6 m, 8 w
5	2.48	36.01	C-5/ H-4 w, 6a-OH m, 7 st, 6 w
6	4.70	66.19	C-6/ H-4 st, 5 st, 6a-OH st, 7 st
6a	3.76	66.19	-
7	1.17	23.66	C-7/ H-5 st, 6a-OH st
8	2.03	24.29	C-8/ H-2 m
9	-	198.26	C-9/ H-10 st, 12 w
10	2.28	52.36	C-10/ H-2 w, 12 st
11	-	36.14	C-11/ H-4 st, 5 w, 10 m, 12/12' st
12/12'	1.23	27.72	C-12,12'/ H-10 st, 12'/12 st

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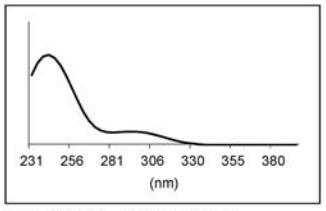
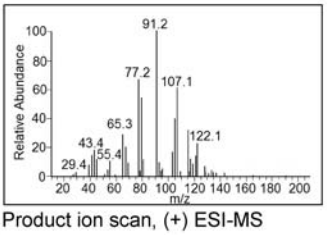
Table 4. continued

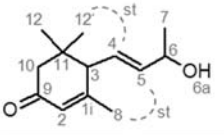
3-oxo-α-ionol



UPLC-PDA, UV spectrum



Product ion scan, (+) ESI-MS



No.	δ_H	δ_C	$^{2,3}J_{CH}$-HMBC correlation
1	-	166.99	C-1/ H-3 w, 4 w, 8 st
2	5.80	124.41	C-2/ H-8 st, 10 b w
3	2.57	54.53	C-3/ H-2 m, 4 m, 5 m, 8 st, 10 w, 12/12' st
4	5.49	126.57	C-4/ H-3 st, 5 m
5	5.61	139.15	C-5/ H-3 m, 4 m, 7 st
6	4.15	66.31	C-6/ H-4 m, 5 m, 7 st
7	1.12	23.03	C-7/ H-6 st
8	1.86	24.02	C-8/ H-2 m, 3 m
9	-	198.21	C-9/ H-10a,b m, 12 w
10a	2.38	47.19	C-10/ H-3 w, 12/12' st
10b	1.96	47.19	-
11	-	35.71	C-11/ H3 w, 10a,b m, 12/12' st
12/12'	0.88	27.44	C-12,12'/ H-3 w, 10a,b m, 12'/12 st

[a]w, weak; m, medium; st, strong; br, broad; numbering is independent of IUPAC numbering.

The affected hooves of the lame cows were inspected, trimmed, and cleaned weekly by professional hoof trimmers. On the first day of treatment, lame cows were randomly assigned to one of three treatment groups: group 1 (control; trimming and cleaning alone); group 2 (trimming and cleaning, bandage wrapped around hoof); group 3 (trimming, cleaning, about 10 g honey on lesion, and bandage around hoof). Each group had a minimum number of 20 animals initially enrolled. Once this number was reached in each group, the remaining animals were assigned to group 3. The treatment was repeated weekly. The hooves were considered to be cured once a complete epithelial layer was established on the lesion. All hooves with lesions other than digital dermatitis during the first examination were excluded from the analysis. The comparison of the treatment groups was terminated after the third week. Thereafter, the animals of all the groups as well as all newly infected hooves were treated with honey (like group 3). On the first day of treatment, the lesions were older and more severe than the lesions detected on later treatment days. Therefore, only hooves enrolled on the first day of treatment were considered for comparison of the treatment groups. The honey used for the treatment was a coldly spun cornflower honey obtained from a beekeeper located near the farm (Table 1, cornflower honey 5).

The data were analyzed with SPSS software (version 16.0, SPSS GmbH software, Munich, Germany). Individual hooves were treated as independent observations. A Fisher's exact test was used for the comparison of cured lesions after two treatments between treatment groups. For time to event (cure) assessment, Kaplan−Meier analyses were performed. Animals were censored if they were culled for any reason before they were diagnosed as cured. The log-rank test was used to determine differences in time to cure between treatment groups. Two-sided differences at $P < 0.05$ were considered to be significant for all of the analyses.

RESULTS AND DISCUSSION

Characterization of the UPLC-PDA Profile of Cornflower Honey. The investigated cornflower honeys originated from Romania and Germany and revealed great similarities among the UPLC chromatograms (Figure 1). On the basis of the recurring peak pattern and extraordinarily high intensities, five compounds, the fluorescing lumichrome and 7-carboxylumichrome and the norisoprenoids (*Z*)-3-oxo-retro-α-ionol, (*E*)-3-oxo-retro-α-ionol, and 3-oxo-α-ionol, appeared to be typical constituents of this kind of honey. Thereby, lumichrome was the most intense and characteristic peak in the PDA profile of cornflower honey, representing about 20% of the total peak area at 254 nm. For identification purposes of the other four profile-defining compounds, their peaks were isolated from cornflower honey by means of semipreparative HPLC and the structure was elucidated via UPLC-PDA-MS/MS and NMR spectroscopy (Table 4).

The degradation product of riboflavin 7-carboxylumichrome had previously been detected in milk and egg products.[29] To the best of our knowledge, this is the first identification of this substance in honey. Furthermore, the 3-oxo-retro-α-ionols had previously been detected in eucalyptus honeys[6] and in sulla (*Hedysarum coronarum* L.) honey.[30] 3-Oxo-α-ionol was likewise detected in eucalyptus honey[6,31,32] and, additionally, in New Zealand heather honey[33] and Asian longan honey.[34] These C_{13}-norisoprenoids are considered as precursors of numerous aroma compounds.[35,36] Additionally identified polyphenols and norisoprenoids in cornflower honey are given in Table 5.

Comparative Analysis of Useful Cornflower Honey Markers. The five potential markers for cornflower honey as well as other identified compounds were quantitated via UPLC-MS/MS (Tables 3 and 5). To estimate the feasibility of the assigned floral markers, a comparative quantitation study among 19 kinds of honey was executed. For comparison of the obtained yields in the different unifloral honeys, box plots were compiled (Figure 2).

Already after the comparison of the PDA profiles among the different unifloral honeys, the lumichrome intensities were conspicuous for cornflower honeys. The determined concentrations ranged from 19 to 44 mg/kg. Similar yields are reported only for thistle honey.[9] In regard to the analyzed kinds of honey, just the sage honeys contained considerable amounts of 12−18 mg/kg. The lumichrome yields detected in heather and manuka honeys did not exceed 7.0 mg/kg. Due to the structural differences and the associated great shift in retention time, the lumichrome derivate 7-carboxylumichrome was quantitated as lumichrome via UV detection at 254 nm. Aside from cornflower honeys, it was additionally detected in heather (<1.7 mg/kg) and manuka honeys (<0.7 mg/kg). The mean concentration in cornflower honey was determined to be 3.8 mg/kg, which is about one-tenth of the lumichrome yields

Table 5. Quantitated Compounds in Cornflower Honey, Minimum (min), Maximum (max), Mean, and Coefficient of Variation (CV)

compound	min (mg/kg)	max (mg/kg)	mean (mg/kg)	CV (%)
(*E*)-3-oxo-retro-α-ionol[a]	21.9	57.3	37.1	31.3
(*Z*)-3-oxo-retro-α-ionol[a]	30.5	95.9	58.7	36.0
3-oxo-α-ionol[a]	19.6	48.7	32.2	29.1
3-oxo-α-ionone	4.1	9.7	7.6	30.2
4-hydroxybenzoic acid	0.9	3.0	1.5	60.1
4-hydroxyquinoline	0.1	8.6	2.1	199.8
7-carboxylumichrome[b]	2.9	4.7	3.8	21.1
apigenin	<LoD	0.6	0.3	76.1
(±)-2-*cis*,4-*trans*-abscisic acid	1.1	2.4	1.9	27.2
caffeic acid	0.5	1.9	0.9	61.4
chrysin	0.4	2.0	1.1	62.2
dehydromonoterpenic acid[c]	<LoD	112.0	37.3	135.0
dehydrovomifoliol	3.4	6.6	5.4	24.2
desoxyabscisic acid	0.4	1.5	0.9	49.5
5-hydroxymethylfurfural	2.0	12.8	6.2	69.4
hydroxymonoterpenic acid[c]	1.7	5.3	3.0	49.4
kojic acid	4.3	12.5	6.8	48.6
kynurenic acid	0.1	0.5	0.3	42.4
lumichrome	18.8	43.5	31.6	29.2
methyl anthranilate	0.5	0.6	0.5	4.5
methyl syringate	3.1	6.1	5.0	24.2
p-coumaric acid	<LoD	11.8	3.8	120.0
phenylalanine	8.4	23.2	12.6	49.3
β-phenyllactic acid	107.1	422.7	255.9	44.4
pinobanksin[d]	0.5	1.9	1.1	56.5
pinocembrin[d]	0.3	2.2	1.0	76.4
protocatechuic acid	0.4	0.6	0.5	21.4
tryptophan	0.6	1.6	0.9	39.5
tyrosin	4.3	6.5	4.9	19.2
vomifoliol[e]	2.2	5.3	4.1	26.2

[a]Quantitated as equivalent of 3-oxo-α-ionone. [b]Quantitated as equivalent of lumichrome. [c]Quantitated as equivalent of caffeic acid. [d]Quantitated as equivalent of chrysin. [e]Quantitated as equivalent of dehydrovomifoliol.

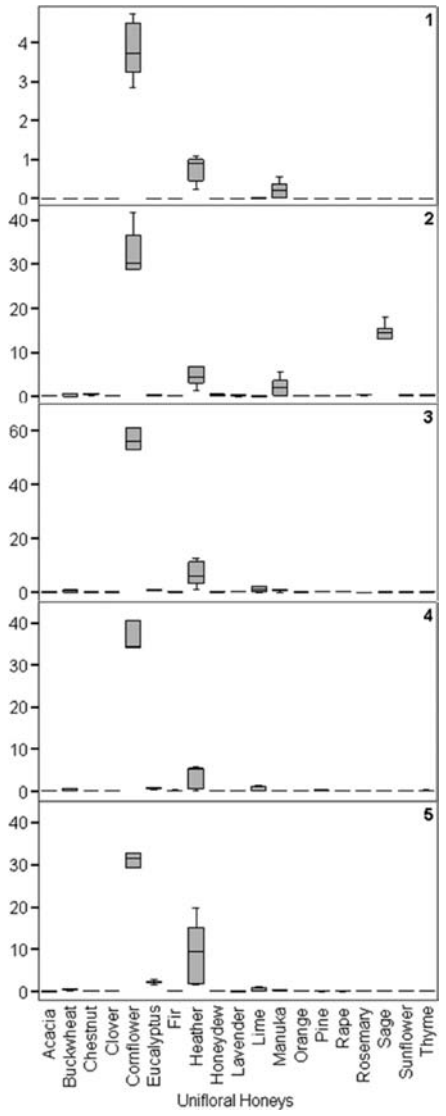


Figure 2. Box plots of useful markers of cornflower honey in comparison to 18 unifloral honeys (n = 5), yields in milligrams per kilogram; extreme values and outliers not shown; (1) 7-carboxylumichrome; (2) lumichrome; (3/4) (*Z*/*E*)-3-oxo-retro-α-ionol; (5) 3-oxo-α-ionol (line in box = median, box = 25 and 75% percentiles, whiskers = 95% percentiles).

determined. The ionol isomers were quantitated as 3-oxo-α-ionone equivalents. The highest yields were obtained for the cornflower honeys as well: (*Z*)-3-oxo-retro-α-ionol with 59 mg/kg, (*E*)-3-oxo-retro-α-ionol with 37 mg/kg, and 3-oxo-α-ionol with 32 mg/kg, on average. The investigated heather honey samples revealed far lower amounts of 1.0−12.6 mg/kg for (*Z*)-3-oxo-retro-α-ionol, 0.2−5.9 mg/kg (*E*)-3-oxo-retro-α-ionol, and 1.6−19.7 mg/kg for 3-oxo-α-ionol. The yields of these ionols in eucalyptus honey, reported[6,31,32] as well as detected, were <3.0 mg/kg. Lime honeys contained even less: 0.1−2.3 mg/kg (*Z*)-3-oxo-retro-α-ionol, 0.2−1.5 mg/kg (*E*)-3-oxo-retro-α-ionol, and 0.2−1.1 mg/kg 3-oxo-α-ionol. The ionol yields in the remaining unifloral honeys were generally <0.2 mg/kg. According to these quantitative data and the significantly (SPSS, ANOVA, Post-Hoc Tukey-HSD, α = 0.001) higher yields of lumichrome, 7-carboxylumichrome, (*Z*/*E*)-3-oxo-retro-α-ionol, and 3-oxo-α-ionol in cornflower honeys, they are suitable floral markers for this kind of honey.

Hydrogen Peroxide Contents of Unifloral Honeys. Glucose oxidase, which is added to the nectar by the bee's pharyngeal gland, catalyzes the conversion of glucose into gluconic acid and hydrogen peroxide.[37] The released peroxide prevents the spoilage of the unripe honey, where conditions such as a high water content would otherwise encourage microbial growth. During the ripening of the honey, the enzyme glucose oxidase is inactivated but regains its activity in diluted honey. Honey used as wound dressing is diluted by wound exudate or the osmotic withdrawal of wound fluid.[38] Thereby, the hydrogen peroxide levels present in honey promote an increased blood flow, and this stimulates wound healing.[39] Hence, besides the high sugar content and the associated osmotic effect, the low pH value, methylglyoxal, polyphenols, and bee defensin-1, hydrogen peroxide is well-known to contribute to the antibacterial activity of honey.[2,37,40] Honey has therefore been used for treating infected wounds since ancient times, and especially its potent activity against antibiotic-resistant bacteria leads to increased interest.

In 1979, cornflower honey was reported to exhibit considerably high peroxide yields, and that already low honey concentrations led to bacteriostatic or even antibacterial effects compared to rape or heather honey.[25] With regard to the hydrogen peroxide contents determined in this study, comparatively high peroxide levels for cornflower honeys could be confirmed (Figure 3). Among the investigated honeys,

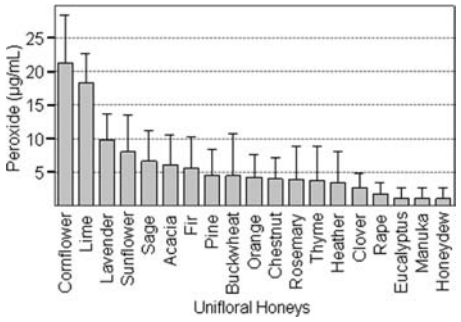


Figure 3. Hydrogen peroxide yields in 19 kinds of honey (n = 5) after 1 h, (bar = mean, whisker = upper 95% confidence limit).

cornflower honey showed the highest average yields with 21.31 μg/mL (0.63 mM) peroxide after 1 h. The mean peroxide contents among the remaining 18 kinds of honey ranged from 18.34 μg/mL (0.54 mM) for lime honeys to 1.07 μg/mL (0.03 mM) for the honeydew honeys. In general, the measured concentration range is consistent with other studies as reported by Bang et al.[38] Variations in the peroxide content among honeys of the same kind are most likely related to the presence of catalase and the decrease of peroxide by the inactivation of glucose oxidase.[41,42]

Because hydrogen peroxide is known to contribute to the antibacterial activity of honey and due to the fact that higher peroxide yields in honey are correlated with a stronger antibacterial property,[25,38] a regional cornflower honey was chosen as an alternative to antibiotics for the treatment of digital dermatitis on an organic dairy farm. The cornflower honeys investigated in this study showed great compositional similarities (Figures 1 and 3). Honey 5 was chosen due to the sufficient quantities for treating the animals.

Animal Study Results. A total of 264 hooves were treated, 130 of them on the first day of treatment. Hind legs were affected in 83.0% of the cases. Digital dermatitis was the main cause for lameness in 75.0% of all cases, followed by sole ulcers (15.2%) and *Panaritium subcutaneum* (6.4%). A total of 70 hooves from the first day of treatment remained in the study after the exclusion of hooves with lesions other than digital dermatitis (group 1, n = 13; group 2, n = 14; group 3, n = 43), and 63 newly infected hooves were treated similarly to group 3 after the first day of treatment. After two treatments, group 3 showed more cured lesions compared to group 1 (40 vs 8%, p = 0.042). Numerically, group 2 also had more cured lesions compared to group 1 (29 vs 8%) but was statistically not significantly different from group 1 (p = 0.326) or group 3 (p = 0.538). The mean estimated times to cure were 2.9, 2.5, and 2.4 treatments in groups 1, 2, and 3, respectively (Figure 4; p = 0.035). Group 3 showed a significantly reduced time to cure relative to group 1 (p = 0.035); however, time to cure did not differ between groups 1 and 2 (p = 0.157) and groups 2 and 3 (p = 0.501). The sample size might have been too small and the follow-up time too short to establish significant differences

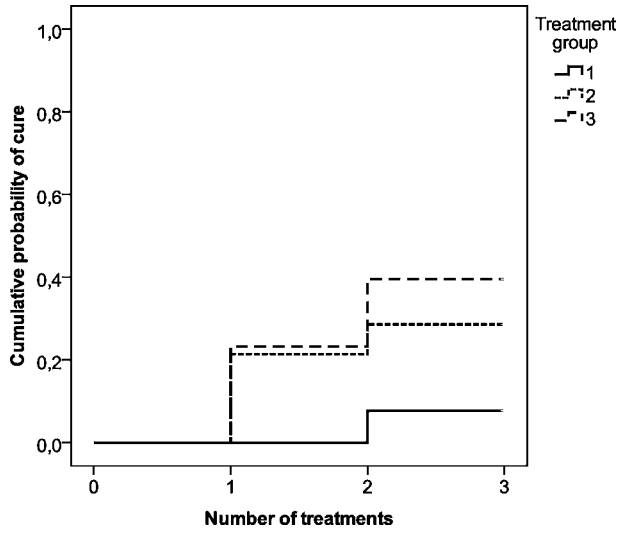


Figure 4. Time to event analysis of time to cure for digital dermatitis in organic dairy cows. Group 1, trimming and cleaning alone, n = 13; group 2, trimming, cleaning, and bandage around the hoof, n = 14; group 3,= trimming, cleaning, cornflower honey on lesion, and bandage around the hoof, n = 43. Treatments were repeated weekly; at 0 treatments, the probability of cure is 0% (100% probability of being diseased) in all groups, after two treatments the probability of cure increased to 40% in group 3 compared to 8% in group 1.

among all groups. A larger study with similar treatment groups is necessary to support our results. In group 3, fresh lesions showed a numerically decreased median time to cure when compared to old lesions (two vs three treatments; p = 0.193; Figure 5), indicating that it might be beneficial to immediately treat lesions to have fewer treatments. The absence of conventional treatment resulted in the manifestation of the clinical symptoms of digital dermatitis in >40% of the lactating dairy herd in the fall of 2008. In this study, the honey-treated group (group 3) showed significantly faster healing than the

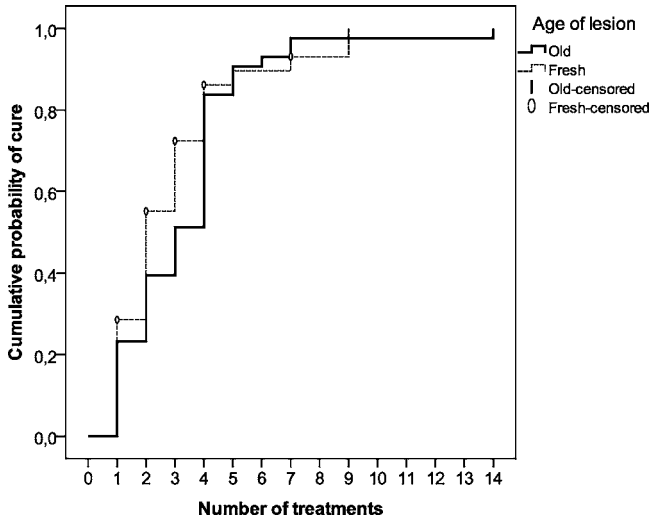


Figure 5. Time to event analysis of time to cure for digital dermatitis in organic dairy cows. Old lesions, treated at first day of treatment, n = 43; fresh lesions, newly infected hooves after first day of treatment, n = 63. Weekly treatment included trimming, cleaning, application of cornflower honey on lesion, and bandage around hoof; 50% of lesions are cured after two treatments for fresh lesions and after three treatments for old lesions.

control group, whereas Manske et al. found that glutaraldehyde under a bandage over a period of 1 week was no more effective than hoof trimming alone.[43] This indicates that cornflower honey may be an effective alternative treatment for digital dermatitis. One reason for the treatment effect might be the high peroxide activity of the cornflower honey (Figure 3).

Bandages were changed every 7 days. With dry flooring conditions, the hoof horn was still dry after 7 days under the bandage. However, the healing process might have been faster if the bandages had been changed and honey reapplied more frequently. Although hoof trimming and changing the bandage can be time-consuming, the costs for honey and dressing materials are relatively low compared to traditional therapies such as antibiotic administration and the costs for milk withdrawal. Therefore, the application of cornflower honey may be an economically viable alternative for the treatment of digital dermatitis on certified organic farms.

AUTHOR INFORMATION

Corresponding Author

*(S.O.) E-mail: Stefanie.Oelschlaegel@chemie.tu-dresden.de. (K.S.) Phone: +49 351 463-33132. Fax: +49 351 464-33132. E-mail: Karl.Speer@chemie.tu-dresden.de.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We sincerely thank Quality Services International GmbH, Dr. C. Luellmann (QSI Bremen, Germany), who provided most of the honey samples, and to the Max Rubner-Institut, Margarete Poehlmann and Céline Borchers (Kulmbach, Germany), for the TOF measurements. We greatly appreciate the effort of the farm staff from Dabergotzer Agrar GmbH and the professional hoof trimmers from Findeisen Klauenpflege GmbH & Co. KG that identified affected cows and helped to collect the data for the animal study.

ABBREVIATIONS USED

HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; COSY, correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating-fame Overhauser enhancement spectroscopy

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