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LC-MS/MS Quantitation of Hop-Derived Bitter Compounds in Beer Using the ECHO Technique

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A new quantification method for hop-derived bitter compounds in beer was developed. By means of LC-MS/MS operating in the multiple reaction monitoring mode, a total of 26 hop-derived bitter compounds, namely, the post-, co-, n-, ad-, pre-, and adpre-congeners of iso-α-acids, α-acids, and β-acids, as well as the prenylflavonoid isoxanthohumol and the chalcone xanthohumol, could be simultaneously detected for the first time in a single HPLC run in authentic beer samples without any cleanup procedures. To compensate for the effect of coextracted matrix components in LC-MS/MS analysis, the so-called ECHO technique was applied for the first time as a suitable strategy for the quantitative analysis of the hop-derived bitter compounds in fresh and stored beer. On the basis of quantitative data, the remarkable instability of α-acids and *trans*-iso-α-acids was confirmed, and it was observed that the degradation of *trans*-iso-α-acids during the storage of beer is not dependent from the nature of the alkanoyl side chain of the congeners. In contrast, an increase of the concentrations of β-acids and of the prenylflavonoid isoxanthohumol as well as of the chalcone xanthohumol during the storage of beer was observed.

KEYWORDS: Beer; hop; iso-α-acids; α-acids; β-acids; LC-MS; quantification; ECHO technique

INTRODUCTION

Besides its sedative activity, beer has been attracting consumers over centuries due to its refreshing character, attractive aroma, and typical bitter taste. Aroma-active volatiles as well as nonvolatile bitter compounds of beers have been thoroughly investigated in recent decades, and it is agreed that the typical beer bitterness is caused by adding cones, pellets, or extracts of hop (*Humulus lupulus* L.) during wort boiling.

During the wort-boiling process, a number of isomerization processes have been identified to be of major importance for bitter taste development in the final beer product. The so-called isoxanthohumol (**1**, **Figure 1**), identified as a bitter compound in beer (*1*), was found to be generated from the hop-derived prenylated chalcon **2**, the so-called xanthohumol, during wort boiling (*2*). Moreover, the *trans*- and *cis*-iso-α-acids **3**−**14** (**Figure 1**) have been identified as the major bitter contributors in beer and were demonstrated to be generated upon a rearrangement reaction of their hop-derived precursors, the α-acids **15**−**20** (**Figure 1**) (*3*). Following the α-acids, the second major constituents of hop are the β-acids **21**−**26** (**Figure 1**), but there are almost no data available on the direct contribution of these compounds to beer bitterness or on their role in the generation of bitter-tasting conversion products during wort boiling.

The α-acids and the corresponding *iso*-α-acids, as well as the β-acids, each occur in six different congeners differing in the carbon skeleton of the alkanoyl side chain (**Figure 1**). Up to now, the chemical structures of only the quantitatively predominating derivatives bearing a 2-methylpropanoyl moiety such as cohumulone (**16**), a 2-methylbutanoyl moiety such as *n*-humulone (**17**), or a 3-methylbutanoyl moiety as present in adhumulone (**18**) were unequivocally determined by means of 1D/2D NMR spectrometric techniques (*4*−*6*). In contrast, the structures of the minor constituents bearing a propanoyl moiety such as the posthumulone (**15**), a 4-methylpentanoyl moiety as part of the prehumulone (**19**), or a hexanoyl residue as found in adprehumulone (**20**) have only been tentatively identified either by LC-MS analysis (*7*) or by GC-MS analysis of the isopropyl esters of the carboxylic acids corresponding to the side chains after oxidative cleavage (*8*), and only adprehumulone (**20**) was confirmed by ^{1}H NMR spectrometry (*9*).

Due to the importance of these compounds for beer bitterness, various approaches were taken in the past to quantitatively measure these bitter compounds in hops and beers. The most common procedure, mainly used by the brewing industry, is the determination of so-called "bitter units" according to a method of the European Brewing Convention (*10*). As this bitter unit is calculated as a converted absorption value (275 nm) from an isooctane extract prepared from acidified beer, it gives no detailed information on the exact composition of the hop-derived bitter compounds in beer samples. To overcome this limitation, multiple RP-HPLC methods with UV detection have been developed (*11*−*13*), but difficulties in the chromatographic

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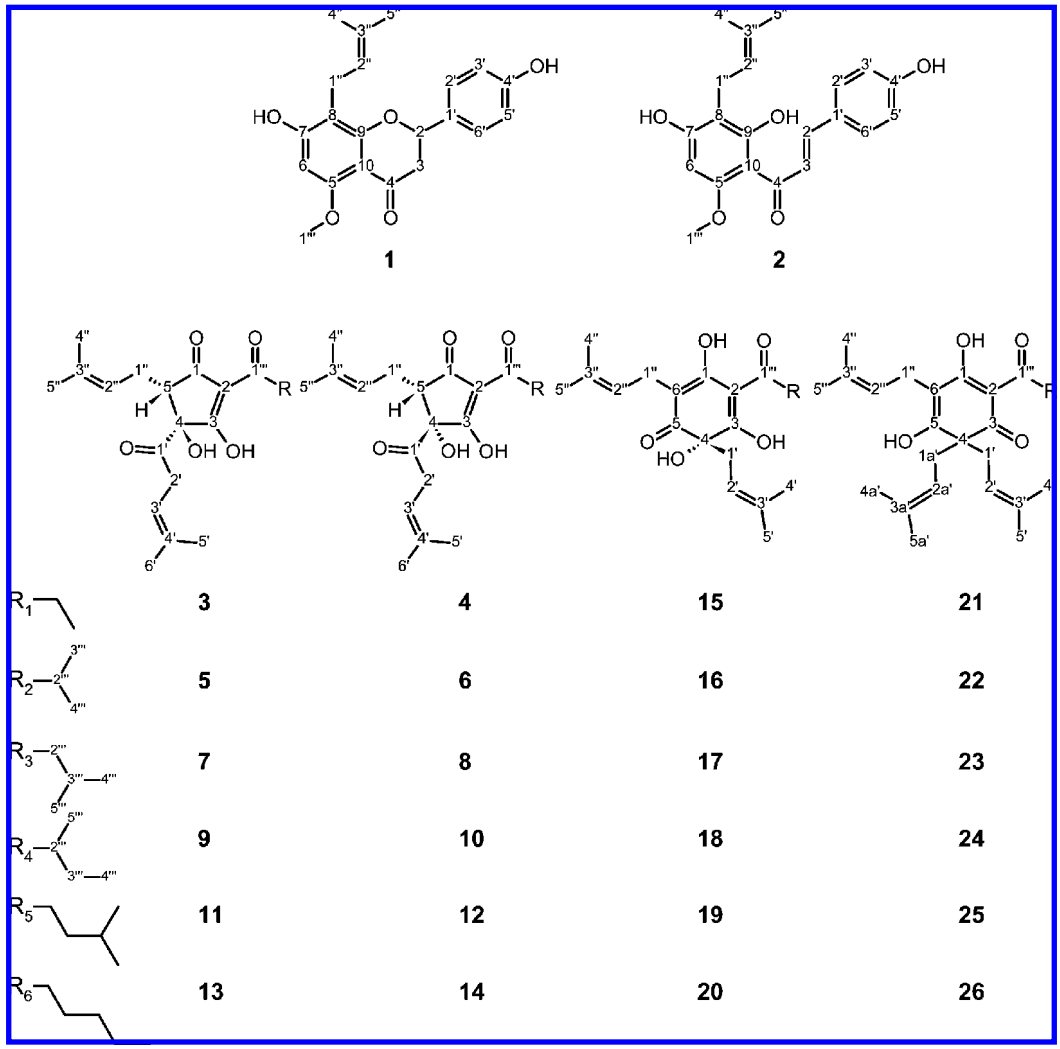

Figure 1. Structures of the prenylflavonoid isoxanthohumol (**1**), the chalcone xanthohumol (**2**), the iso-α-acids *trans*-isoposthumulone (**3**), *cis*-isoposthumulone (**4**), *trans*-isocohumulone (**5**), *cis*-isocohumulone (**6**), *trans*-isohumulone (**7**), *cis*-isohumulone (**8**), *trans*-isoadhumulone (**9**), *cis*-isoadhumulone (**10**), *trans*-isoprehumulone (**11**), *cis*-isoprehumulone (**12**), *trans*-isoadprehumulone (**13**), and *cis*-isoadprehumulone (**14**), the α-acids posthumulone (**15**), cohumulone (**16**), humulone (**17**), adhumulone (**18**), prehumulone (**19**), and adprehumulone (**20**), and the β-acids postlupulone (**21**), colupulone (**22**), lupulone (**23**), adlupulone (**24**), prelupulone (**25**), and adprelupulone (**26**).

Table 1. Assignment of ^{1}H NMR Signals (400 MHz, CD_3OD) of Compounds **5**−**10**[a]

	compound											
	5		**6**		**7**		**8**		**9**		**10**	
proton at carbon	δ[b]	M[c]	δ	M	δ	M	δ	M	δ	M	δ	M
H−C(5)	2.96	[dd, 1H]	3.17	[dd, 1H]	2.95	[dd, 1H]	3.15	[dd, 1H]	2.88	[dd, 1H]	3.16	[dd, 1H]
Hα−C(2′)	3.43	[dd, 1H]	3.48	[d, 1H]	3.43	[dd, 1H]	3.48	[d, 2H]	3.43	[dd, 1H]	3.48	[d, 2H]
Hβ−C(2′)	3.45	[dd, 1H]			3.45	[dd, 1H]			3.45	[dd, 1H]		
H−C(3′)	5.24	[dd, 1H]	5.23	[dd, 1H]	5.23	[dd, 1H]	5.23	[dd, 1H]	5.24	[dd, 1H]	5.23	[dd, 1H]
H−C(5′)	1.59	[s, 3H]	1.64	[s, 3H]	1.59	[s, 3H]	1.60	[s, 3H]	1.58	[s, 3H]	1.59	[s, 3H]
H−C(6′)	1.73	[s, 3H]	1.73	[s, 3H]	1.73	[s, 3H]	1.72	[s, 3H]	1.72	[s, 3H]	1.72	[s, 3H]
Hα−C(1″)	2.26	[ddd, 1H]	2.37	[ddd, 1H]	2.26	[ddd, 1H]	2.36	[ddd, 1H]	2.23	[ddd, 1H]	2.36	[ddd, 1H]
Hβ−C(1″)	2.49	[m, 1H]	2.44	[ddd, 1H]	2.50	[m, 1H]	2.45	[ddd, 1H]	2.50	[m, 1H]	2.46	[ddd, 1H]
H−C(2″)	5.17	[m, 1H]	5.12	[dd, 1H]	5.18	[m, 1H]	5.11	[dd, 1H]	5.17	[m, 1H]	5.09	[dd, 1H]
H−C(4″)	1.51	[s, 3H]	1.60	[s, 3H]	1.51	[s, 3H]	1.60	[s, 3H]	1.51	[s, 3H]	1.60	[s, 3H]
H−C(5″)	1.67	[s, 3H]	1.64	[s, 3H]	1.67	[s, 3H]	1.64	[s, 3H]	1.66	[s, 3H]	1.64	[s, 3H]
Hα−C(2‴)	3.53	[m, 1H]	3.48	[m, 1H]	2.69	[dd, 1H]	2.72	[d, 2H]	3.48	[m, 1H]	3.40	[m, 1H]
Hβ−C(2‴)					2.77	[dd, 1H]						
Hα−C(3‴)	1.14	[d, 3H]	1.13	[d, 3H]	2.12	[m, 1H]	2.11	[m, 1H]	1.41	[m, 1H]	1.42	[m, 1H]
Hβ−C(3‴)									1.76	[m, 1H]	1.73	[m, 1H]
H−C(4‴)	1.09	[d, 3H]	1.09	[d, 3H]	0.95	[d, 3H]	0.95	[d, 3H]	0.91	[dd, 3H]	0.91	[dd, 3H]
H−C(5‴)					0.97	[d, 3H]	0.95	[d, 3H]	1.06	[d, 3H]	1.07	[d, 3H]

[a] Arbitrary numbering according to structures **5**−**10** in **Figure 1**. [b] Chemical shift [ppm] of proton in relation to CD_3OD. [c] M, multiplicity of signal.

separation of the individual congeners as well as the lack of commercially available reference materials consisting of single components allowed the determination of only the total amount of coeluting derivatives.

Table 2. Assignment of Homonuclear $^1H,^1H$ Connectivities (400 MHz, CD_3OD) of **5**−**10**[a]

proton at carbon	compound (connectivity[b] with proton)					
	5	**6**	**7**	**8**	**9**	**10**
H−C(5)	Hα−C(1″); Hβ−C(1″)	Hα−C(1″); Hβ−C(1″)	Hα−C(1″); Hβ−C(1″)	Hα−C(1″); Hβ−C(1″)	Hα−C(1″); Hβ−C(1″)	Hα−C(1″); Hβ−C(1″)
Hα−C(2′)	Hβ−C(2′); H−C(3′;5′;6′)	Hβ−C(2′); H−C(3′;5′;6′)	Hβ−C(2′); H−C(3′;5′;6′)	H−C(3′;5′;6′)	Hβ−C(2′); H−C(3′;5′;6′)	H−C(3′;5′;6′)
Hβ−C(2′)	Hα−C(2′); H−C(3′;5′;6′)	Hα−C(2′); H−C(3′;5′;6′)	Hα−C(2′); H−C(3′;5′;6′)		Hα−C(2′); H−C(3′;5′;6′)	
H−C(3′)	H−C(2′;5′;6′)	H−C(2′;5′;6′)	H−C(2′;5′;6′)	H−C(2′;5′;6′)	H−C(2′;5′;6′)	H−C(2′;5′;6″)
H−C(5′)	H−C(2′;3′;6′)	H−C(2′;3′;6′)	H−C(2′;3′;6′)	H−C(2′;3′)	H−C(2′;3′;6′)	H−C(2′;3′)
H−C(6′)	H−C(2′;3′;5′)	H−C(2′; 3′;5′)	H−C(2′;3′;5′)	H−C(2′;3′)	H−C(2′;3′;5′)	H−C(2′;3′)
Hα−C(1″)	Hβ−C(1″); H−C(5;2″;4″;5″)	Hβ−C(1″); H−C(5;2″;4″;5″)	Hβ−C(1″); H−C(5;2″;4″;5″)	Hβ−C(1″); H−C(5;2″;4″;5″)	Hβ-C(1″); H−C(5;2″;4″;5″)	Hβ−C(1″); H−C(5;2″;4″;5″)
Hβ−C(1″)	Hα−C(1″); H−C(5;2″;4″;5″)	Hα−C(1″); H−C(5;2″;4″; 5″)	Hα−C(1″); H−C(5;2″;4″;5″)	Hα−C(1″); H−C(5;2″;4″;5″)	Hα−C(1″); H−C(5;2″;4″;5″)	Hα−C(1″); H−C(5;2″;4″;5″)
H−C(2″)	Hα−C(1″); Hβ−C(1″); H−C(4″;5″)	Hα−C(1″); Hβ−C(1″); H−C(4″;5″)	Hα−C(1″); Hβ−C(1″); H−C(4″;5″)	Hα−C(1″); Hβ−C(1″); H−C(4″;5″)	Hα−C(1″); Hβ−C(1″); H−C(4″;5″)	Hα−C(1″); Hβ−C(1″); H−C(4″;5″)
H−C(4″)	Hα−C(1″); Hβ−C(1″); H−C(2″;5″)	Hα−C(1″); Hβ−C(1″); H−C(2″;5″)	Hα−C(1″); Hβ−C(1″); H−C(2″;5″)	Hα−C(1″); Hβ−C(1″); H−C(2′)	Hα−C(1″); Hβ−C(1″); H−C(2″;5″)	Hα−C(1″); Hβ−C(1″); H−C(2′)
H−C(5″)	Hα−C(1″); Hβ−C(1″); H−C(2″;4″)	Hα−C(1″); Hβ−C(1″); H−C(2″;4″)	Hα−C(1″); Hβ−C(1″); H−C(2″;4″)	Hα−C(1″); Hβ−C(1″); H−C(2″)	Hα−C(1″); Hβ−C(1″); H−C(2″;4″)	Hα−C(1″); Hβ−C(1″); H−C(2″)
Hα−C(2‴)	H−C(3‴;4‴)	H−C(3‴;4‴)	Hβ−C(2‴); H−C(3‴)	H−C(3‴)	Hα−C(3‴); Hβ−C(3‴); H−C(5‴)	Hα−C(3‴); Hβ−C(3‴); H−C(5‴)
Hβ−C(2‴)			Hα−C(2‴); H−C(3‴)			
Hα−C(3‴)	H−C(2‴)	H−C(2‴)	Hα−C(2‴); Hβ−C(2‴); H−C(4‴;5‴)	H−C(2‴;4‴;5‴)	Hβ−C(3‴); H−C(2‴;4‴)	Hβ−C(3‴); H−C(2‴;4‴)
Hβ−C(3‴)					Hα−C(3‴); H−C(2‴;4‴)	Hα−C(3‴); H−C(2‴;4‴)
H−C(4‴)	H−C(2‴)	H−C(2‴)	H−C(3‴)	H−C(3‴)	Hα−C(3‴); Hβ−C(3‴)	Hα−C(3‴); Hβ−C(3‴)
H−C(5‴)			H−C(3‴)	H−C(3‴)	H−C(2‴)	H−C(2‴)

[a] Arbitrary numbering according to structures **5**−**10** in **Figure 1**. [b] Homonuclear $^1H,^1H$ connectivities obtained by means of a DQF-COSY experiment.

Recently, the optimization of the chromatographic separation parameters as well as the advantage of mass spectrometric detection significantly improved selectivity and sensitivity of the analysis of α-/β-acids and *iso*-α-acids, but the limited linearity of the MS detection as well as the influence of matrix effects is well accepted to require the use of internal standards for accurate quantitative analysis of target compounds (*14*, *15*). As the synthesis of stable isotope labeled analogues of α-/β-acids and *iso*-α-acids to be used as suitable internal standards is extremely laborious and challenging, the so-called ECHO technique was identified as a promising strategy. This analytical technique was developed to compensate for the effect of coextracted matrix components in LC-MS/MS analysis of pesticide residues in plants by using the nonlabeled target compound as an internal standard, which was injected into the HPLC-MS system after a short time period as the "echo" of the analyte (*16*).

To meet the demand for a rapid, accurate, and sensitive method for the quantitation of hop-derived bitter compounds in beer, the objective of the present investigation was to develop a robust HPLC-MS/MS method based on the ECHO technique.

MATERIALS AND METHODS

Chemicals and Materials. The following chemicals were obtained commercially: formic acid, hydrochloric acid, and sodium hydroxide (Grüssing, Filsum, Germany); acetonitrile, ethyl acetate (Merck, Darmstadt, Germany); and dicyclohexylamine of puriss grade (Fluka, Neu-Ulm, Germany). Deuterated solvents were from Euriso-top (Saarbrücken, Germany). Deionized water used for chromatography was purified by means of a Milli-Q Gradient A10 system (Millipore, Billerica, MA). Commercial beer samples I−IX (Pilsner-type) and X (wheat beer) were obtained from the German brewing industry. The hop-free beer ("zero beer") was provided by the Bitburger brewery (Bitburg, Germany). To study the influence of aging on bitter compounds in beer, bottled beer samples I−III were stored under forced conditions for 8 months at 28 °C in the dark prior to opening. An iso-α-acid extract (30%) prepared by preisomerization of a hop extract, a crude xanthohumol extract, a carbon dioxide extract, and an ethanol extract of hop were provided by the Hallertauer Hopfenveredelungsgesellschaft mbH (Mainburg, Germany).

Isolation of α- and *β*-Acids. An aliquot (2.0 g) of the ethanolic hop extract was dissolved in methanol (5.0 mL) and, after filtration, separated by semipreparative RP-HPLC. The effluents of the six major peaks detected were collected, individually freed from solvent in vacuum, and freeze-dried twice. By means of UV−vis, LC-MS/MS, and 1D/2D NMR experiments, the structures of cohumulone (**16**), humulone (**17**), adhumulone (**18**), colupulone (**22**), lupulone (**23**), and adlupulone (**24**) were unequivocally confirmed in this elution order (RP-HPLC).

Cohumulone, **16**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 283 and 320 nm; LC-TOF-MS, found *m/z* 347.1855, calcd for $[C_{20}H_{28}O_5-H^+]^-$ 347.1864; LC-MS (ESI^-) *m/z* (%) 347 (100) $[M - H^+]^-$; MS/MS (−30 V) *m/z* (%) 347 (100), 278 (94), 234 (35), 207 (29); ^1H NMR (400 MHz CD_3OD, COSY) δ 1.08 [d, 3H, *J* = 6.8 Hz, H−C(4‴)], 1.15 [d, 3H, *J* = 6.8 Hz, H−C(3‴)], 1.50 [s, 3H, H−C(5′)], 1.61 [s, 3H, H−C(4′)], 1.66 [s, 3H, H−C(4″)], 1.73 [s, 3H, H−C(5″)], 2.51 [d, 2H, *J* = 7.9 Hz, H−C(1′)], 3.00 [dd, 1H, *J* = 7.0, 14.2 Hz, Hα−C(1″)], 3.09 [dd, 1H, *J* = 7.6, 14.2 Hz, Hβ−C(1″)] 3.80 [m, 1H, *J* = 6.8 Hz, H−C(2‴)], 4.95 [dd, 1H, *J* = 7.9, 7.9 Hz, H−C(2′)], 5.13 [dd, 1H, *J* = 7.5, 7.0 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.0 [C(5′,5″)], 17.3 [C(4‴)], 18.6 [C(3‴)], 20.5 [C(1″)], 24.3 [C(4′,4″)], 34.7 [C(2‴)], 41.0 [C(1′)], 78.4 [C(6)], 108.3 [C(2)], 116.4 [C(2′)], 121.7 [C(2″)], 135.8 [C(3″)], 136.0 [C(3′)], 172.4 [C(1)], 189.5 [C(3)], 196.0 [C(5)], 205.1 [C(1‴)].

Humulone, **17**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 283 and 320 nm; LC-TOF-MS, found *m/z* 361.1998, calcd for $[C_{21}H_{30}O_5-H^+]^-$ 361.2020; LC-MS (ESI^-)

Table 3. Assignment of ^{13}C NMR Signals (125 MHz, CD_3OD; DEPT-135) of **5**−**10**[a]

	compound											
	5		**6**		**7**		**8**		**9**		**10**	
carbon	δ[b]	M[c]	δ	M	δ	M	δ	M	δ	M	δ	M
C(1)	203.6	[C]	205.2	[C]	204.0	[C]	204.9	[C]	203.9	[C]	205.3	[C]
C(2)	111.4	[C]	110.6	[C]	112.4	[C]	111.4	[C]	112.0	[C]	111.3	[C]
C(3)	198.3	[C]	197.4	[C]	198.5	[C]	197.6	[C]	199.1	[C]	197.9	[C]
C(4)	90.4	[C]	88.3	[C]	90.8	[C]	88.2	[C]	90.5	[C]	88.1	[C]
C(5)	57.1	[CH]	52.2	[CH]	57.6	[CH]	52.3	[CH]	57.9	[CH]	52.1	[CH]
C(1′)	210.0	[C]	210.1	[C]	210.0	[C]	210.1	[C]	210.6	[C]	210.0	[C]
C(2′)	39.9	[CH_2]	38.1	[CH_2]	39.8	[CH_2]	37.8	[CH_2]	39.9	[CH_2]	38.1	[CH_2]
C(3′)	116.5	[CH]	116.7	[CH]	116.5	[CH]	116.3	[CH]	116.8	[CH]	116.5	[CH]
C(4′)	136.2	[C]	136.4	[C]	136.3	[C]	135.7	[C]	135.9	[C]	136.0	[C]
C(5′)	18.2	[CH_3]	17.8	[CH_3]	18.3	[CH_3]	17.5	[CH_3]	18.3	[CH_3]	17.7	[CH_3]
C(6′)	25.9	[CH_3]	25.6	[CH_3]	25.9	[CH_3]	25.3	[CH_3]	25.9	[CH_3]	25.6	[CH_3]
C(1″)	24.9	[CH_2]	26.0	[CH_2]	24.8	[CH_2]	25.6	[CH_2]	25.2	[CH_2]	26.1	[CH_2]
C(2″)	122.3	[CH]	121.6	[CH]	122.3	[CH]	121.4	[CH]	122.9	[CH]	121.5	[CH]
C(3″)	134.9	[C]	134.9	[C]	134.8	[C]	134.6	[C]	134.1	[C]	134.7	[C]
C(4″)	18.1	[CH_3]	17.8	[CH_3]	18.0	[CH_3]	17.5	[CH_3]	18.1	[CH_3]	17.7	[CH_3]
C(5″)	25.9	[CH_3]	25.8	[CH_3]	25.9	[CH_3]	25.6	[CH_3]	25.9	[CH_3]	25.8	[CH_3]
C(1‴)	204.3	[C]	205.6	[C]	198.8	[C]	200.0	[C]	204.0	[C]	204.8	[C]
C(2‴)	36.3	[CH]	36.7	[CH]	45.9	[CH_2]	46.5	[CH_2]	43.0	[CH]	42.9	[CH]
C(3‴)	18.3	[CH_3]	17.9	[CH_3]	27.6	[CH]	26.7	[CH]	27.5	[CH_2]	26.4	[CH_2]
C(4‴)	18.4	[CH_3]	18.1	[CH_3]	22.7	[CH_3]	22.3	[CH_3]	12.2	[CH_3]	11.7	[CH_3]
C(5‴)					22.9	[CH_3]	22.3	[CH_3]	16.3	[CH_3]	15.4	[CH_3]

[a] Arbitrary numbering according to structures **5**−**10** in **Figure 1**. [b] Chemical shift [ppm] of carbon atoms in relation to CD_3OD. [c] M, multiplicity of signal.

m/z (%) 361 (100) [M − H^+]$^-$; MS/MS (−30 V) *m/z* (%) 361 (100), 292 (98), 249 (31), 221 (31); ^1H NMR (400 MHz CD_3OD, COSY) δ 0.95 [d, 3H, *J* = 6.7 Hz, H−C(5‴)], 0.98 [d, 3H, *J* = 6.7 Hz, H−C(4‴)], 1.51 [s, 3H, H−C(5′)], 1.60 [s, 3H, H−C(4′)], 1.67 [s, 3H, H−C(4″)], 1.73 [s, 3H, H−C(5″)], 2.12 [m, 1H, H−C(3‴)], 2.51 [dd, 2H, *J* = 7.3, 7.3 Hz, H−C(1′)], 2.73 [dd, 1H, *J* = 6.6, 13.6 Hα-C(2‴)], 2.82 [dd, 1H, *J* = 7.4, 13.6 Hβ−C(2‴)], 3.01 [dd, 1H, *J* = 7.0, 14.1 Hz, Hα−C(1″)], 3.10 [dd, 1H, *J* = 7.5, 14.1 Hz, Hβ−C(1″)], 4.93 [dd, 1H, *J* = 7.3, 7.3 Hz, H−C(2′)], 5.13 [dd, 1H, *J* = 7.5, 7.0 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.5 [C(5′,5″)], 20.5 [C(1″)], 21.6 [C(4‴)], 21.6 [C(5‴)], 24.7 [C(4′)], 24.8 [C(4″)], 25.8 [C(3‴)], 40.9 [C(1′)], 48.9 [C(2‴)], 78.2 [C(6)], 108.5 [C(2)], 116.2 [C(2′)], 121.7 [C(2″)], 130.8 [C(3″)], 136.0 [C(3′)], 170.9 [C(1)], 190.2 [C(3)], 196.0 [C(5)], 199.7 [C(1‴)].

Adhumulone, **18**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 283 and 320 nm; LC-TOF-MS, found *m/z* 361.2023, calcd for [$C_{21}H_{30}O_5$−H^+]$^-$ 361.2020; LC-MS (ESI$^-$) *m/z* (%) 361 (100) [M − H^+]$^-$; MS/MS (−30 V) *m/z* (%) 361 (100), 292 (98), 249 (31), 221 (31); ^1H NMR (400 MHz CD_3OD, COSY) δ 0.95 [dd, 3H, *J* = 7.4; 7.4 Hz, H−C(5‴)], 1.01 [d, 3H, *J* = 6.8 Hz, H−C(4‴)], 1.40 [m, 1H, Hα−C(3‴)], 1.45 [s, 3H, H−C(4′)], 1.56 [s, 3H, H−C(5′)], 1.61 [s, 3H, H−C(5″)], 1.68 [s, 3H, H−C(4″)], 1.76 [m, 1H, Hβ−C(3‴)], 2.46 [d, 2H, *J* = 7.9 Hz, H−C(1′)], 2.99 [dd, 1H, *J* = 7.0, 14.2 Hz, Hα−C(1″)], 3.10 [dd, 1H, *J* = 7.5; 14.2 Hz, Hβ−C(1″)], 3.66 [m, 1H, H−C(2‴)], 4.91 [m, 1H, *J* = 7.9, 7.9 Hz, H−C(2′)], 5.08 [m, 1H, *J* = 7.5, 7.0 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 11.9 [C(5‴)], 16.6 [C(3‴)], 17.9 [C(4″,5′)], 21.8 [C(1″)], 26.0 [C(4′,5″)], 28.1 [C(4‴)], 42.0 [C(2‴)], 42.3 [C(1′)], 80.2 [C(6)], 110.6 [C(2)], 117.7 [C(2′)], 123.2 [C(2″)], 132.9 [C(3″)], 138.1 [C(3′)], 172.9 [C(1)], 192.4 [C(3)], 197.6 [C(5)], 206.1 [C(1‴)].

Colupulone, **22**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 275 and 332 nm; LC-TOF-MS, found *m/z* 399.2547, calcd for [$C_{25}H_{36}O_4$−H^+]$^-$ 399.2547; LC-MS (ESI$^-$) *m/z* (%) 399 (100) [M − H^+]$^-$; MS/MS (−30 V) *m/z* (%) 287 (100), 399 (75), 330 (30); ^1H NMR (400 MHz CD_3OD, COSY) δ 1.09 [d, 6H, *J* = 7.3 Hz, H−C(3‴,4‴)], 1.55 [s, 6H, H−C(5′,5a′)], 1.57 [s, 6H, H−C(4′,4a′)], 1.67 [s, 3H, H−C(4″)], 1.73 [s, 3H, H−C(5″)], 2.59 [m, 4H, H−C(1′,1a′)], 3.10 [d, 2H, *J* = 6.8 Hz, H−C(1″)], 3.98 [m, 1H, *J* = 7.3 Hz, H−C(2‴)], 4.77 [dd, 2H, *J* = 7.6, 7.6 Hz, H−C(2′,2a′)], 5.02 [dd, 1H, *J* = 6.7, 6.7 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.5 [C(4′,4a′,5″)], 17.8 [C(3‴,4‴)], 20.2 [C(1″)], 24.2 [C(4″,5′,5a′)], 36.0 [C(2‴)], 37.3 [C(1′,1a′)], 57.6 [C(6)], 110.9 [C(2)], 117.7 [C(2′,2a′)], 121.5 [C(2″)], 131.0 [C(3″)], 134.3 [C(3′,3a′)], 172.8 [C(1)], 189.3 [C(3)], 196.8 [C(5)], 207.0 [C(1‴)].

Lupulone, **23**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 274 and 331 nm; LC-TOF-MS, found *m/z* 413.2707, calcd for [$C_{26}H_{38}O_4$−H^+]$^-$ 413.2697; LC-MS (ESI$^-$) *m/z* (%) 413 (100) [M − H^+]$^-$; MS/MS (−30 V) *m/z* (%) 413 (100), 301 (44), 233 (11); ^1H NMR (400 MHz CD_3OD, COSY) δ 0.93 [d, 6H, *J* = 6.7 Hz, H−C(4‴,5‴)], 1.52 [s, 6H, H−C(5′,5a′)], 1.55 [s, 6H, H−C(4′,4a′)], 1.65 [s, 3H, H−C(4″)], 1.71 [s, 3H, H−C(5″)], 2.07 [m, 1H, H−C(3‴)], 2.34 [m, 4H, H−C(1′,1a′)], 2.82 [m, 2H, H−C(2‴)], 3.07 [d, 2H, *J* = 6.8, H−C(1″)], 4.74 [dd, 2H, *J* = 7.6, 7.6 Hz, H−C(2′,2a′)], 5.00 [dd, 1H, *J* = 6.8, 6.8 Hz, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 16.5 [C(4′,4a′,5″)], 20.2 [C(1″)], 21.6 [C(4‴,5‴)], 24.5 [C(4″,5′,5a′)], 32.6 [C(3‴)], 37.2 [C(1′,1a′)], 48.6 [C(2‴)], 57.2 [C(6)], 111.0 [C(2)], 117.5 [C(2′,2a′)], 121.4 [C(2″)], 131.3 [C(3″)], 134.4 [C(3′,3a′)], 172.8 [C(1)], 189.4 [C(3)], 197.6 [C(5)], 208.9 [C(1‴)].

Adlupulone, **24**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 280 and 332 nm; LC-TOF-MS, found *m/z* 413.2715, calcd for [$C_{26}H_{38}O_4$−H^+]$^-$ 413.2697; LC-MS (ESI$^-$) *m/z* (%) 413 (100) [M − H^+]$^-$; MS/MS (−30 V) *m/z* (%) 413 (100), 301 (44), 233 (11); ^1H NMR (400 MHz CD_3OD, COSY) δ 0.89 [dd, 3H, *J* = 7.4, 7.4 Hz, H−C(5‴)], 1.07 [d, 3H, *J* = 6.8 Hz, H−C(4‴)], 1.34 [m, 1H, Hα−C(3‴)], 1.54 [s, 6H, H−C(5′,5a′)], 1.57 [s, 6H, H−C(4′,4a′)], 1.67 [s, 3H, H−C(4″)], 1.71 [m, 1H, Hβ−C(3‴)], 1.73 [s, 3H, H−C(5″)], 2.59 [m, 4H, H−C(1′,1a′)], 3.09 [m, 2H, H−C(1″)], 3.89 [m, 1H, H−C(2‴)], 4.76 [m, 2H, H−C(2′,2a′)], 5.02 [m, 1H, H−C(2″)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 12.4 [C(5‴)], 16.9 [C(3‴)], 18.0 [C(4″,4′,4a′), 21.7 [C(1″)], 26.1 [C(5′,5a′,5″)], 30.8 [C(4‴)], 38.9 [C(1′,1a′)], 43.9 [C(2‴)], 81.7 [C(6)], 112.0 [C(2)], 119.4 [C(2′,2a′)], 123.3 [C(2″)], 132.6 [C(3″)], 136.0 [C(3″,3a′)], 174.8 [C(1)], 191.5 [C(3)], 198.4 [C(5)], 208.6 [C(1‴)].

Isolation of *cis*- and *trans*-Iso-α-acids. The iso-α-acid extract (35 g) was adjusted to pH 2.0 by the addition of aqueous hydrochloric acid (1 mol/L; 100 mL) and then extracted with ethyl acetate (3 × 200 mL). The organic extracts were pooled and dried over Na_2SO_4, and the solvent was removed in vacuum to obtain a mixture of the pure iso-α-acids (∼10 g). Following a literature protocol with some modifications (*17*), the mixture was dissolved in ethyl acetate (30 g) and dicyclohexylamine (5.1 g; 1.02 mol equiv) and maintained at room temperature. After 4 days, white crystals of the *trans*-iso-α-acid/dicyclohexylamine complex were filtered off, washed with ice-cold ethyl acetate (3 mL), and dried in vacuum. The crystals were dissolved in ethyl acetate (100 mL), and this solution was extracted three times

Table 4. Assignment of Heteronuclear ^{1}H,^{13}C Connectivities (400 MHz/100 MHz, CD_3OD) of **5**−**10**[a]

	compound (connectivity[b] with carbon)											
	5		**6**		**7**		**8**		**9**		**10**	
proton at	^1J	$^{2,3,4}J$	^1J	$^{2,3,4}J$	^1J	$^{2,3,4}J$	^1J	$^{2,3,4}J$	^1J	$^{2,3,4}J$	^1J	$^{2,3,4}J$
H−C(5)	C(5)	C(1;2;4;1′; 1″;2″)	C(5)	C(1;2;3;4; 1′; 1″;2″)	C(5)	C(1;2;4; 1′1″;2″)	C(5)	C(1;2;3;4;1′; 1″;2″)	C(5)	C(1;4;1′;1″;2″)	C(5)	C(1;2;3;4;1′; 1″;2″)
Hα−C(2′)	C(2′)	C(1′;3′;4′)	C(2′)	C(1′;3′;4′)	C(2′)	C(1′;3′;4′)	C(2′)	C(1′;3′;4′;5′;6′)	C(2′)	C(1′;3′;4′)	C(2′)	C(1′;3′;4′)
Hβ−C(2′)	C(2′)	C(1′;3′;4′)	C(2′)	C(1′;3′;4′)	C(2′)	C(1′;3′;4′)	C(2′)		C(2′)	C(1′;3′;4′)		
H−C(3′)	C(3′)	C(2′;5′;6′)	C(3′)	C(5′;6′)	C(3′)	C(5′;6′)	C(3′)	C(2′;5′;6′)	C(3′)	C(1′;2′;5′;6′)	C(3′)	C(5′;6′)
H−C(5′)	C(5′)	C(1′;3′;4′;6′)	C(5′)	C(1′;2′;3′;4′;6′)	C(5′)	C(1′;2′;3′;4′;6′)	C(5′)	C(1′;3′;4′;6′)	C(5′)	C(1′;2′;3′;4′;6′)	C(5′)	C(1′;3′;4′;6′)
H−C(6′)	C(6′)	C(1′; 3′; 4′; 5′)	C(6′)	C(1′; 2′; 3′; 4′; 5′)	C(6′)	C(1′; 3′; 4′; 5′)	C(6′)	C(1′; 3′; 4′; 5′)	C(6′)	C(1′; 3′; 4′; 5′)	C(6′)	C(1′; 3′; 4′; 5′)
Hα−C(1″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)
Hβ−C(1″)	C(1″)	C(4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)	C(1″)	C(4;5;2″;3″)	C(1″)	C(1;4;5;2″;3″)
H−C(2″)	C(2″)	C(4″; 5″)	C(2″)	C(4″; 5″)	C(2″)	C(4″; 5″)	C(2″)	C(5; 4″; 5″)	C(2″)	C(4″; 5″)	C(2″)	C(4″; 5″)
H−C(4″)	C(4″)	C(2″; 3″; 5″)	C(4″)	C(5; 2″; 3″; 5″)	C(4″)	C(5; 2″; 3″; 5″)	C(4″)	C(5; 2″; 3″; 5″)	C(4″)	C(5; 2″; 3″; 5″)	C(4″)	C(5; 2″; 3″; 5″)
H−C(5″)	C(5″)	C(2″;3″;4″)	C(5″)	C(5;2″;3″;4″)	C(5″)	C(5;2″;3″;4″)	C(5″)	C(5;2″;3″;4″)	C(5″)	C(5;2″;3″;4″)	C(5″)	C(5;2″;3″;4″)
Hα−C(2‴)	C(2‴)	C(1‴;3‴;4‴)	C(2‴)	C(1‴;3‴;4‴;5‴)	C(2‴)	C(2;1‴;3‴;4‴;5‴)	C(2‴)	C(2;1‴;3‴;4‴;5‴)	C(2‴)	C(1‴;3‴;4‴;5‴)	C(2‴)	C(1‴;3‴;4‴;5‴)
Hβ−C(2‴)					C(2‴)	C(2;1‴;3‴;4‴;5‴)						
Hα−C(3‴)	C(3‴)	C(1‴;2‴;4‴)	C(3‴)	C(1‴;2‴;4‴)	C(3‴)	C(1‴;2‴;4‴;5‴)	C(3‴)	C(1‴;2‴;4‴;5‴)	C(3‴)	C(1‴;2‴;4‴;5‴)	C(3‴)	C(1‴;2‴;4‴;5‴)
Hβ−C(3‴)									C(3‴)	C(1‴;2‴;4‴;5‴)	C(3‴)	C(1‴;2‴;4‴;5‴)
H−C(4‴)	C(4‴)	C(1‴;2‴;3‴)	C(4‴)	C(1‴;2‴;3‴)	C(4‴)	C(1‴;2‴;3‴;5‴)	C(4‴)	C(1‴;2‴;3‴;5‴)	C(4‴)	C(2‴;3‴)	C(4‴)	C(2‴;3‴)
H−C(5‴)					C(5‴)	C(1‴;2‴;3‴;4‴)	C(5‴)	C(1‴;2‴;3‴;4‴)		C(1‴;2‴;3‴)	C(5‴)	C(1‴;2‴;3‴)

[a] Arbitrary numbering according to structures **5**−**10** in **Figure 1**. [b] Homonuclear ^{1}H,^{13}C connectivities obtained by means of a HMQC (^1J) and a HMBC experiment ($^{2,3,4}J$), respectively.

Table 5. Optimized Mass Spectrometric Parameters for the Quantitative Analysis of Compounds **1**−**26**

compound[a]	Q1 mass [amu]	Q3 mass [amu]	DP[b] [V]	CE[c] [V]	CXP[d] [V]
1, **2**	352.9	118.8	−95	−42	−7
3, **4**	333.0	236.9	−90	−22	−11
5, **6**	347.0	251.0	−90	−22	−11
7−**10**	361.0	265.0	−90	−22	−11
11−**14**	375.1	279.0	−90	−22	−11
15	333.0	264.0	−65	−28	−17
16	347.1	277.9	−65	−28	−17
17, **18**	361.2	292.0	−65	−28	−17
19, **20**	375.0	306.0	−65	−28	−17
21	385.0	273.0	−80	−38	−15
22	399.2	286.9	−80	−38	−15
23, **24**	413.2	301.2	−80	−38	−15
25, **26**	427.0	315.0	−80	−38	−15

[a] Structures of compounds are given in **Figure 1**. [b] Declustering potential. [c] Collision energy. [d] Cell exit potential.

Figure 2. HPLC-MS/MS chromatogram of a beer sample. Signal intensity of each mass transition is normalized. Peak numbering refers to the chemical structures given in **Figure 1**.

Figure 3. HPLC-MS/MS chromatogram showing the quantitative analysis of selected bitter compounds in beer using the ECHO technique. The peak of the ECHO standard is labeled with an "e" prior to the substance number referring to the chemical structures given in **Figure 1**.

with aqueous hydrochloric acid (1 mol/L, 100 mL) to separate the dicyclohexylamine from the target compounds. After removal of the solvent in vaccum, the residue obtained was dissolved in a minimum amount of acetonitrile and the individual *trans*-iso-α-acids were isolated by means of RP18-HPLC. In addition, the supernatant of the *trans*-

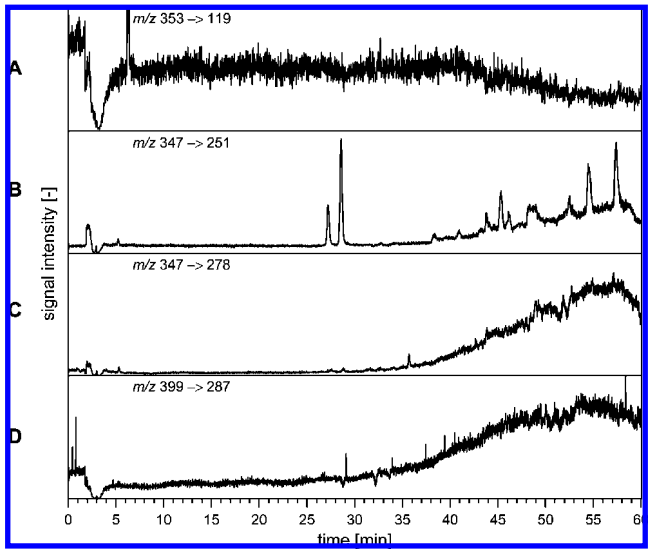

Figure 4. Influence of the matrix on the ionization of selected beer bitter compounds. HPLC-MS/MS (MRM) chromatograms were recorded for a beer sample while a continuous flow of isoxanthohumol (**A**), *trans*-isocohumolne (**B**), cohumulone (**C**), and colupulone (**D**), respectively, was introduced into the LC-MS/MS system by means of a syringe pump.

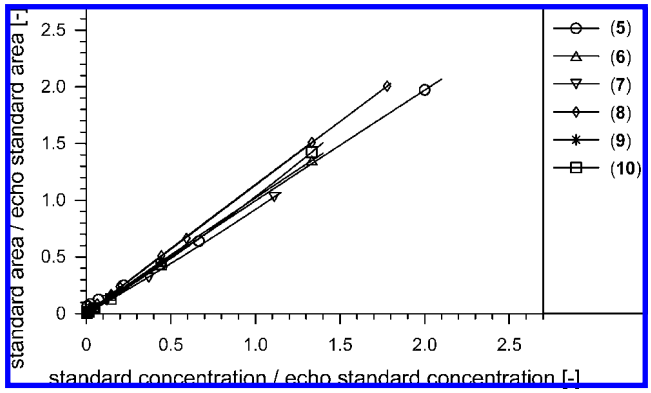

Figure 5. Calibration functions determined for *trans*-isocohumulone (**5**), *cis*-isocohumulone (**6**), *trans*-isohumulone (**7**), *cis*-isohumulone (**8**), *trans*-isoadhumulone (**9**), and *cis*-isoadhumulone (**10**), respectively.

iso-α-acid/dicyclohexylamine complex precipitation was separated from dicyclohexylamine by extraction with hydrochloric acid (1 mol/L, 100 mL), the solvent was removed under vacuum to yield a mixture consisting mainly of *cis*-iso-α-acids, which were individually isolated and purified by means of RP18-HPLC. The effluents of the individual peaks were collected, freed from solvent in vacuum, and freeze-dried to obtain the *cis*- and *trans*-iso-α-acids **5**−**10** in a purity of >97% (HPLC, ^{1}H NMR).

trans-Isocohumulone, **5**, **Figure 1**: UV−vis (1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 257 and 271 nm; LC-TOF-MS, found *m*/*z* 347.1871, calcd for $[C_{20}H_{28}O_5-H^+]^-$ 347.1864; LC-MS (ESI$^-$) *m*/*z* (%) 347 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 347 (10), 329 (10), 278 (15), 251 (80), 235 (25), 233 (35), 209 (30), 207 (25), 182 (100), 181 (10). 1D/2D NMR data are given in **Tables 1**−**4**.

cis-Isocohumulone, **6**, **Figure 1**: UV−vis (1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 257 and 265 nm; LC-TOF-MS, found *m*/*z* 347.1877, calcd for $[C_{20}H_{28}O_5-H^+]^-$ 347.1864; LC-MS (ESI$^-$) *m*/*z* (%) 347 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 347 (15), 329 (25), 278 (10), 251 (70), 235 (10), 233 (50), 209 (35), 207 (30), 182 (100), 181 (30). 1D/2D NMR data are given in **Tables 1**−**4**.

trans-Isohumulone, **7**, **Figure 1**: UV−vis (1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 257 and 277 nm; LC-TOF-MS, found *m*/*z* 361.2008, calcd for $[C_{21}H_{30}O_5-H^+]^-$ 361.2020; LC-MS (ESI$^-$) *m*/*z* (%) 361 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 361 (15), 343 (10), 292 (10), 265 (100), 247 (25), 235 (25), 223 (25), 221 (20), 196 (80), 195 (20). 1D/2D NMR data are given in **Tables 1**−**4**.

cis-Isohumulone, **8**, **Figure 1**: UV−vis (1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 257 and 272 nm; LC-TOF-MS, found *m*/*z* 361.2037, calcd for $[C_{21}H_{30}O_5-H^+]^-$ 361.2020; LC-MS (ESI$^-$) *m*/*z* (%) 361 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 361 (25), 343 (25), 292 (15), 265 (90), 247 (35), 235 (15), 223 (30), 221 (30), 196 (100), 195 (20). 1D/2D NMR data are given in **Tables 1**−**4**.

trans-Isoadhumulone, **9**, **Figure 1**: UV−vis (1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 257 and 274 nm; LC-TOF-MS, found *m*/*z* 361.2019, calcd for $[C_{21}H_{30}O_5-H^+]^-$ 361.2020; LC-MS (ESI$^-$) *m*/*z* (%) 361 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 361 (15), 343 (15), 292 (10), 265 (75), 247 (20), 235 (15), 223 (25), 221 (25), 196 (100), 195 (15). 1D/2D NMR data are given in **Tables 1**−**4**.

cis-Isoadhumulone, **10**, **Figure 1**: UV−vis (1% aqueous formic acid/acetonitrile; 20:80, v/v) λ_{max} = 257 and 268 nm; LC-TOF-MS, found *m*/*z* 361.2007, calcd for $[C_{21}H_{30}O_5-H^+]^-$ 361.2020; LC-MS (ESI$^-$) *m*/*z* (%) 361 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 361 (25), 343 (25), 292 (15), 265 (95), 247 (45), 235 (10), 223 (25), 221 (25), 196 (100), 195 (20). 1D/2D NMR data are given in **Tables 1**−**4**.

Isolation of Xanthohumol and Isomerization to Isoxanthohumol. For the isolation of xanthohumol, the commercial xanthohumol extract was separated by means of RP-HPLC to afford the target compound as a yellow powder with a purity of >98% (HPLC, ^{1}H NMR). For the preparation of isoxanthohumol, an aliquot (0.5 g) of the xanthohumol extract was dissolved in aqueous NaOH solution (80 mL, 0.1 mol/L) and was heated for 1 h at 100 °C in a closed vial. After cooling, the reaction was stopped by adjusting the pH value to 7.0 with aqueous hydrochloric acid (1 mol/L), the aqueous mixture was then extracted with ethyl acetate (3 × 100 mL), and the pooled organic extracts were dried over Na_2SO_4. After removal of the solvent in vacuum, pure isoxanthohumol was isolated from the residue by means of RP-HPLC.

Xanthohumol, **2**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 235 and 364 nm; LC-TOF-MS, found *m*/*z* 353.1394, calcd for $[C_{21}H_{22}O_5-H^+]^-$ 353.1394; LC-MS (ESI$^-$) *m*/*z* (%) 353 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 353 (80), 233 (100), 119 (50); ^{1}H NMR (400 MHz CD_3OD, COSY) δ 1.61 [s, 3H, H−C(4″)], 1.70 [s, 3H, H−C(5″)], 3.14 [d, 2H, *J* = 7.1 Hz, H−C(1″)], 3.87 [s, 3H, H−C(1‴)], 5.14 [dd, 1H, *J* = 7.1, 7.1 Hz, H−C(2″)], 6.09 [s, 1H, H−C(6)], 6.84 [d, 2H, *J* = 8.6 Hz, H−C(3′;5′)], 7.57 [d, 2H, *J* = 8.6 Hz, H−C(2′;6′)], 7.67 [d, 1H, *J* = 15.5 Hz, H−C(2)], 7.77 [d, 1H, *J* = 15.5 Hz, H−C(3)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 17.6 [C(4″)], 21.0 [C(1″)], 25.4 [C(5″)], 55.7 [C(1‴)], 90.9 [C(6)], 104.5 [C(10)], 107.3 [C(8)], 115.9 [C(3′;5′)], 123.0 [C(2″)], 123.7 [C(3)], 126.0 [C(1′)], 129.8 [C(3″)], 130.4 [C(2′;6′)], 142.4 [C(2)], 159.8 [C(4′)], 160.4 [C(5)], 162.3 [C(7)], 164.6 [C(9)], 191.6 [C(4)].

Isoxanthohumol, **1**, **Figure 1**: UV−vis (1% formic acid in water/acetonitrile; 20:80, v/v) λ_{max} = 238 and 287 nm; LC-TOF-MS, found *m*/*z* 353.1404, calcd for $[C_{21}H_{22}O_5-H^+]^-$ 353.1394; LC-MS (ESI$^-$) *m*/*z* (%) 353 (100) $[M - H^+]^-$; MS/MS (−30 V) *m*/*z* (%) 353 (80), 233 (100), 119 (50); ^{1}H NMR (400 MHz CD_3OD, COSY) δ 1.60 [s, 6H, H−C(4″;5″)], 2.62 [dd, 1H, *J* = 3.1, 16.5 Hz, Hα-C(3)], 2.80 [dd, 1H, *J* = 12.7, 16.5 Hz, H*β*-C(3)], 3.18 [d, 2H, *J* = 7.2 Hz H−C(1″)], 3.68 [s, 3H, H−C(1‴)], 5.10 [dd, 1H, *J* = 7.2, 7.2 Hz H−C(2″)], 5.15 [dd, 1H, *J* = 3.1, 12.7 Hz, H−C(2)], 5.89 [s, 1H, H−C(6)], 6.68 [d, 2H, *J* = 8.6 Hz, H−C(2′;6′)], 7.13 [d, 2H, *J* = 8.6 Hz, H−C(3′;5′)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC) δ 17.5 [C(4″)], 21.5 [C(1″)], 25.5 [C(5″)], 45.0 [C(3)], 55.8 [C(1‴)], 78.0 [C(2)], 93.0 [C(6)], 105.9 [C(10)], 106.5 [C(8)], 115.2 [C(3′;5′)], 121.0 [C(2″)], 127.4 [C(2′;6′], 131.1 [C(1′)], 135.0 [C(3″)], 155.2 [C(4′)], 160.4 [C(5)], 161.3 [C(9)], 162.1 [C(7)], 189.5 [C(4)].

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of two ProStar 210 type pumps (Varian, Middelburg, The Netherlands), a diode array detector ProStar 330, and an Rh 7725i injection valve with a 500 μL loop (Rheodyne, Bensheim, Germany). For chromatography, a semipreparative 250 × 10 mm, 5 μm, ODS Hypersil column (ThermoHypersil, Kleinostheim, Germany) equipped with a guard column of the same type was used as the stationary phase and aqueous formic acid (1% in water) as solvent A and acetonitrile containing 1% formic acid as solvent B. Monitoring the effluent flow

Table 6. Recovery Rates Determined for Selected Bitter Compounds in Beer

	added amount (μmol/L) and recovery rates (%) determined for compound						
expt[a]	**1**	**2**	**5**	**7**	**9**	**16**	**22**
1	0.37 μmol/L 98%	0.04 μmol/L 98%	0.62 μmol/L 111%	0.62 μmol/L 111%	0.62 μmol/L 98%	0.37 μmol/L 97%	0.02 μmol/L 85%
2	1.11 μmol/L 90%	0.11 μmol/L 97%	1.85 μmol/L 93%	1.85 μmol/L 102%	1.85 μmol/L 96%	1.11 μmol/L 99%	0.06 μmol/L 105%
3	3.33 μmol/L 99%	0.33 μmol/L 88%	5.56 μmol/L 110%	5.56 μmol/L 108%	5.56 μmol/L 100%	3.33 μmol/L 85%	0.17 μmol/L 110%
mean value	95%	94%	105%	107%	98%	94%	100%

[a] Hop-free "zero beer" was spiked with defined amounts of isoxanthohumol (**1**), xanthohumol (**2**), *trans*-isocohumulone (**5**), *trans*-isohumulone (**7**), *trans*-isoadhumulone (**9**), cohumulone (**16**), and colupulone (**22**) at three different concentration levels (expt 1−3) prior to quantitative analysis, and the amounts determined after spiking were compared with those found in the zero beer (control).

Table 7. Concentrations of Hop-Derived Bitter Compounds in Fresh Beer Samples

	concn [μmol/L] in beer sample									
compound	I	II	III	IV	V	VI	VII	VIII	IX	X
					Prenylflavonoids					
isoxanthohumol (**1**)	2.90	0.17	2.48	3.90	5.73	6.14	2.85	1.18	2.25	4.03
xanthohumol (**2**)	0.051	0.008	0.043	0.043	0.129	0.097	0.030	0.013	0.025	0.387
ratio **2**/**1**	0.018	0.047	0.039	0.011	0.022	0.016	0.011	0.011	0.011	0.096
					Iso-α-Acids					
trans-isoposthumulone (**3**)	0.21[a]	0.13[a]	0.13[a]	0.13[a]	0.18[a]	0.22[a]	0.08[a]	0.13[a]	0.12[a]	0.03[a]
cis-isoposthumulone (**4**)	0.50[a]	0.32[a]	0.28[a]	0.29[a]	0.41[a]	0.58[a]	0.24[a]	0.28[a]	0.31[a]	0.11[a]
trans-isocohumulone (**5**)	13.16	15.53	9.20	13.33	12.11	12.10	7.82	13.53	11.09	3.50
cis-isocohumulone (**6**)	31.75	37.45	21.27	31.78	31.21	29.40	22.22	30.27	24.75	9.29
trans-isohumulone (**7**)	12.61	15.06	9.84	11.99	12.96	13.22	8.49	13.31	10.30	2.71
cis-isohumulone (**8**)	32.87	37.13	25.13	33.87	36.35	40.62	28.99	35.85	28.53	10.24
trans-isoadhumulone (**9**)	3.73	6.36	4.14	5.46	4.92	5.06	3.62	5.74	4.93	2.03
cis-isoadhumulone (**10**)	11.08	15.68	9.32	13.65	13.99	13.66	9.54	12.92	10.90	4.10
trans-isoprehumulone (**11**)	0.02[a]	0.04[a]	0.04[a]	0.05[a]	0.05[a]	0.04[a]	0.03[a]	0.05[a]	0.05[a]	0.01[a]
cis-isoprehumulone (**12**) + *trans*-isoadprehumulone (**13**)	0.21[a]	0.35[a]	0.30[a]	0.38[a]	0.40[a]	0.33[a]	0.24[a]	0.41[a]	0.38[a]	0.11[a]
cis-isoadprehumulone (**14**)	0.46[a]	0.63[a]	0.58[a]	0.67[a]	0.74[a]	0.69[a]	0.49[a]	0.76[a]	0.66[a]	0.24[a]
total *trans*/*cis*-iso-α-acids	106.60	128.70	80.20	111.60	113.30	115.90	81.80	113.30	92.00	32.40
trans/*cis* ratio	0.39	0.41	0.44	0.40	0.38	0.37	0.34	0.43	0.42	0.36
					α-Acids					
posthumulone (**15**)	0.024[a]	0.096[a]	0.082[a]	0.094[a]	0.071[a]	0.132[a]	0.027[a]	0.058[a]	0.039[a]	0.018[a]
cohumulone (**16**)	0.393	4.176	1.917	3.639	1.410	2.374	0.796	2.627	1.038	0.766
humulone (**17**)	0.648	4.926	2.207	3.225	1.354	3.527	1.145	3.527	1.248	1.120
adhumulone (**18**)	0.068	0.804	0.401	0.574	0.206	0.553	0.162	0.737	0.189	0.243
prehumulone (**19**)	0.0005[a]	0.0026[a]	0.0016[a]	0.0016[a]	0.0008[a]	0.0017[a]	0.0007[a]	0.0023[a]	0.0012[a]	0.0020[a]
adprehumulone (**20**)	0.0018[a]	0.0090[a]	0.0058[a]	0.0045[a]	0.0021[a]	0.0066[a]	0.0018[a]	0.0072[a]	0.0033[a]	0.0056[a]
iso-α-acid/α-acid ratio	94.0	12.8	17.4	14.8	37.2	17.6	38.4	16.3	36.5	15.0
					β-Acids					
postlupulone (**21**)	0.0020[a]	0.0014[a]	0.0023[a]	0.0029[a]	0.0015[a]	0.0013[a]	0.0013[a]	0.0025[a]	0.0020[a]	0.0024[a]
colupulone (**22**)	0.0689	0.0310	0.0536	0.0602	0.0290	0.0253	0.0320	0.0609	0.0523	0.0642
lupulone (**23**)	0.0546	0.0187	0.0360	0.0332	0.0181	0.0186	0.0209	0.0363	0.0339	0.0351
adlupulone (**24**)	0.0406	0.0047	0.0103	0.0088	0.0040	0.0054	0.0049	0.0079	0.0112	0.0119
prelupulone (**25**)	0.00002[a]	0.00001[a]	0.00004[a]	0.00002[a]	0.00001[a]	0.00006[a]	0.00002[a]	0.00003[a]	0.00003[a]	0.00002[a]
adprelupulone (**26**)	0.00004[a]	0.00006[a]	0.00019[a]	0.00010[a]	0.00004[a]	0.00022[a]	0.00006[a]	0.00009[a]	0.00014[a]	0.00010[a]

[a] Determined on the basis of the calibration curve of the corresponding co-derivative.

(4.5 mL/min) at 272 nm and, additionally at 360 nm for the detection of xanthohumol, chromatography was performed by increasing the amount of solvent B from 50 to 80% within 20 min and, then, to 100% within 5 min, thereafter, maintaining at 100% for additional 5 min.

High-Performance Liquid Chromatography−Mass spectrometry (HPLC-MS/MS). The Agilent 1100 series HPLC system consisted of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) and was connected to an API 4000 Q-TRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany) equipped with the electrospray ionization (ESI) source running in the negative ion mode. Samples were introduced by RP-HPLC with a solvent flow of 250 μL/min requiring the use of the turbo gas at a temperature of 400 °C. The ion spray voltage was set to −4500 V, and the declustering potential and the MS/MS parameters were optimized for each substance to induce fragmentation of the pseudomolecular ion $[M - H]^-$ to the corresponding target product ions after collision-induced dissociation. The dwell time for each mass transition was 44 ms. The declustering potential (DP), the cell exit potential (CXP), and the collision energy (CE) were set as given in **Table 5**. Nitrogen was used as the collision gas (4×10^{-5} Torr). The quantification was done using the MRM mode of the instrument with the fragmentation parameters optimized prior to analysis. Data processing and integration were performed by using Analyst software version 1.4.2 (AB Sciex Instruments). Chromatography was performed using a 250 × 4.6 mm, 5 μm, Pursuit C18 column (Varian) and aqueous formic acid (0.5% in water) as solvent A and acetonitrile with 0.5% formic acid as solvent B. Using a flow rate of 1.0 mL/min and a 1:4 split prior to MS detection, chromatography was performed by increasing solvent A from 55 to 60% within 20 min, then to 67% within 5 min, to 90% within 15 min and, finally, to 100% within 13 min.

Table 8. Influence of Storage on the Concentrations of Selected Hop-Derived Bitter Compounds in Beer

	sample I			sample II			sample III		
	concn[a] [μmol/L] in			concn[a] [μmol/L] in			concn[a] [μmol/L] in		
compound	fresh	stored[b]	change[c] [%]	fresh	stored	change [%]	fresh	stored	change [%]
				Prenylflavonoids					
isoxanthohumol (**1**)	2.90	4.44	53	0.17	0.22	29	2.48	4.36	76
xanthohumol (**2**)	0.051	0.123	141	0.008	0.010	25	0.043	0.117	21
				Iso-α-Acids					
trans-isocohumulone (**5**)	13.16	5.06	−62	15.53	6.87	−73	9.20	2.51	−56
cis-isocohumulone (**6**)	31.75	27.39	−14	37.45	33.20	−2	21.27	20.87	−11
trans-isohumulone (**7**)	12.61	3.96	−69	15.06	6.50	−68	9.84	3.15	−57
cis-isohumulone (**8**)	32.87	27.57	−16	37.13	32.88	−13	25.13	21.77	−11
trans-isoadhumulone (**9**)	3.73	1.05	−72	6.36	2.21	−72	4.14	1.14	−65
cis-isoadhumulone (**10**)	11.08	8.80	−21	15.68	14.75	−11	9.32	8.27	−6
total *trans*/*cis*-iso-α-acids	106.60	74.90	−30	128.70	97.50	−24	80.20	58.50	−27
trans/*cis* ratio	0.39	0.16		0.41	0.19		0.44	0.15	
				α-Acids					
cohumulone (**16**)	0.39	0.07	−81	4.18	0.20	−95	1.92	0.09	−96
humulone (**17**)	0.65	0.27	−59	4.93	0.45	−91	2.21	0.24	−89
adhumulone (**18**)	0.07	0.02	−69	0.80	0.09	−89	0.40	0.04	−89
				β-Acids					
colupulone (**22**)	0.069	0.086	25	0.031	0.126	307	0.054	0.134	151
lupulone (**23**)	0.055	0.076	38	0.019	0.108	478	0.036	0.129	260
adlupulone (**24**)	0.041	0.064	57	0.005	0.028	501	0.010	0.039	280

[a] Concentrations were determined by LC-MS/MS using the ECHO technique and are given as the mean of triplicates. [b] Prior to opening, the beer bottles were maintained in the dark for 8 months at 28 °C. [c] Change of the concentration of a bitter compound during storage.

Quantitative Analysis by HPLC-MS/MS Using the ECHO Technique. Prior to HPLC-MS/MS analysis of the beer, the samples were degassed by ultrasonification for 2 min in a glass beaker. For quantitative analysis of the target compounds using the ECHO technique, the experiment was started by the injection of an aliquot (5 μL) of a mixture of xanthohumol (1 μmol/L; **e2**) and *trans*-isocohumulone (10 μmol/L; **e5**) as the first ECHO standards. After 1.5 min, an aliquot (5 μL) of the beer sample was injected, followed by the injection the ECHO standard of isoxanthohumol (1 μmol/L; **e1**) after 3 min, the ECHO standard of cohumulone (1 μmol/L; **e16**) after 17 min, and the ECHO standard of colupulone (0.1 μmol/L; **e22**) after a total of 31 min. When necessary, higher dilutions of the ECHO standards were used for the analysis of the analytes present in beer in low concentrations. For the screening experiments identical conditions were used with a single injection of an aliquot (5 μL) of the sample solution.

Investigation of Matrix Effects during HPLC-MS/MS. For the investigation of the matrix effects, the same HPLC-MS/MS parameters were used as given above but, in addition, a constant flow of 10 μL/min of solutions of either isoxanthohumol (**1**), 1 μmol/L; *trans*-isocohumulone (**5**), 1 μmol/L; cohumulone (**16**), 0.1 μmol/L; or colupulone (**22**), 0.1 μmol/L, was introduced by means of a PHD 4400 Hpsi type syringe pump (Harvard Apparatus) connected to the solvent flow via a three-way valve.

NMR. ^{1}H, ^{13}C, and 2D NMR data were acquired on a Bruker DMX-400 (Bruker BioSpin, Rheinstetten, Germany). CD_3OD was used as solvent, and chemical shifts were referenced to the solvent signal. For structural elucidation and NMR signal assignment, COSY, HMQC, and HMBC experiments were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany) as well as Mestre-C (Mestrelab Research, La Coruña, Spain).

RESULTS AND DISCUSSION

To accurately determine the amounts of the hop-derived bitter compounds in beer, an HPLC-MS/MS analysis using the ECHO technique should be developed. Because no pure standard substances were commercially available, first, the identity of the bitter substances reported in the literature needed to be verified and reference materials needed to be isolated and purified to be used as reliable ECHO standards.

Isolation of Reference Compounds. The α-acids **15**−**20** and the β-acids **21**−**26** were isolated and purified from a commercially available carbon dioxide extract of hop by means of HPLC; xanthohumol (**2**) was isolated from a commercially available crude xanthohumol extract; isoxanthohumol (**1**) was prepared by alkaline isomerization from **2** and purified by RP-HPLC; and the individual *cis*- and *trans*-iso-α-acids (**3**−**14**) were isolated from a commercially available iso-α-acid extract by dicyclohexylamine precipitation, followed by HPLC purification. By means of UV−vis, LC-MS/MS, and 1D/2D NMR experiments, the structures of compounds **1**, **2**, **5**−**10**, **16**−**18**, and **22**−**24** (**Figure 1**) were unequivocally determined and confirm data reported earlier (*4*−*6*, *18*, *19*). As there are no reports presenting the comprehensive and correct assignment of all protons and carbon atoms of the six major iso-α-acids **5**−**10**, these data are summarized in **Tables 1**−**4**. The post- (**3**, **4**, **15**, **21**), pre- (**11**, **12**, **19**, **25**), and adpre-congeners (**13**, **14**, **20**, **26**) are present in hops only in trace amounts and, in consequence, did not allow an unequivocal structure determination based on NMR spectroscopy. These compounds were therefore tentatively identified by means of their UV adsorption spectra and the characteristic RP-18 elution pattern, as well as their typical mass spectrometric fragmentation.

LC-MS/MS Detection of Bitter Compounds 1−**26 in Beer.** To analyze the target bitter compounds with high selectivity by using the triple-quadrupole mass spectrometer operating in the MRM mode, repeated manual sample injections were done to determine the pseudomolecular ion and daughter ions in full -scan mode in the range of 100−500 amu. Using flow injection and the automatic tuning option of the software, the settings were optimized between −95 and −65 for the declustering potential, between −42 and −22 for the collision energy, and between −17 and −7 for the cell exit potential, thus enabling the maximization of the product ion intensity (**Table 5**).

To screen for the hop-derived bitter compounds in beer, a degassed beer sample was analyzed by means of HPLC-MS/MS operating in the negative electrospray ionization and the MRM mode. As shown in the mass chromatograms in **Figure**

2, isoxanthohumol (**1**), xanthohumol (**2**), the α-acids (**15**−**20**), and the β-acids (**21**−**26**), as well as the *cis*- and *trans*-iso-α-acids (**3**−**14**) were identified in the beer sample without the need of any cleanup procedures. This is the first time that even the minor post-, pre-, and adpre-derivatives could be detected in authentic beer samples and, with the exception of *cis*-isoprehumulone (**12**) and *trans*-isoadprehumulone (**13**), all of these bitter terpenoids were chromatographically well separated.

LC-MS/MS Quantitation of the Bitter Compounds 1−**26 in Beer Using the ECHO Technique.** As the synthesis of stable isotope-labeled internal standards of the hop-derived bitter compounds is extremely challenging, the so-called ECHO technique should be applied as an alternative quantification strategy. To perform this technique and to compensate for the effect of coextracted matrix components in LC-MS/MS analysis, the isolated nonlabeled reference compounds of the analytes are used as quasi-internal standards and are injected into the HPLC-MS system before and/or after the target sample to target the elution time of this ECHO standard close to the retention time of the target analytes. To achieve this, one ECHO standard was used for each class of bitter compounds, namely, isoxanthohumol (**e1**) for the flavonone **1**, xanthohumol (**e2**) for the chalkone **2**, *trans*-isocohumulone (**e5**) for the iso-α-acids **3**−**14**, cohumulone (**e16**) for the α-acids **15**−**20**, and colupulone (**e22**) for the β-acids **21**−**26**, respectively. To compensate for the effect of coextracted matrix components in LC-MS/MS analysis, the time shift between analyte and corresponding ECHO standard was chosen to be as small as possible. As exemplified in **Figure 3**, the ECHO standard **e1** elutes shortly after the analyte **1**, whereas the ECHO standard **e5** was targeted to elute closely before the analyte **5** to prevent coelution with compound **6**.

Before quantitative analysis could be performed, it was necessary to confirm that coeluting matrix components do not affect differently the ionization of the analytes and the corresponding ECHO standards. To visualize such matrix effects, a constant flow of a solution of either isoxanthohumol, *trans*-isocohumulone, cohumulone, or colupulone was introduced into the LC-MS/MS system via a syringe pump during the analysis of a beer sample. As given in **Figure 4A**, a strong matrix-dependent suppression (>90%) of the ionization of isoxanthohumol (**1**) was observed between the retention times of 2 and 4 min. Such effects are known to be due to the huge amount of highly polar constituents eluting within the dead volume of the column and can be observed in many RP18 applications (*15*). Except for a short increase in the ionization yield at about 6 min, no further matrix effects could be observed throughout the mass chromatogram. In contrast, the ionization rate of the iso-α-acid *trans*-isocohumulone (**B**, **Figure 4**) and the α-acid cohumulone (**C**, **Figure 4**), as well as the β-acid colupulone (**D**, **Figure 4**) rose with increasing retention times above 35 min. For example, at a retention time of 55 min, the ionization yield of cohumulone (**16**) is increased by a factor of about 50. The ionization of iso-α-acid *trans*-isocohumulone (**B**, **Figure 4**) is strongly enhanced by some distinct less polar beer constituents. With this observation taken into account, the ECHO standard of *trans*-isocohumulone (**5**) was targeted to elute shortly before the iso-α-acids (**Figure 3**). In the case of the α- and β-acids (**C**,**D**), some minor matrix suppression effects (up to 20%) can be observed, but the analytes are not affected. As these matrix effects were found to be identical for multiple runs (data not shown) and the ECHO standards were targeted close to the retention times of the analytes, the ECHO standard and corresponding analyte should be affected by the matrix effects to a similar extent, thus making their quantitative analysis possible.

To enable the quantitation of the bitter compounds, calibration curves were determined and are exemplified in **Figure 5** for some iso-α-acids. These data clearly demonstrate that all functions are within a range of ±10%. To obtain the best fit for all calibration curves, second-order polynomial equations were calculated. To avoid negative or exaggerated estimates at the low end of the concentration ranges, the functions were forced through zero. This approach leads to correlation coefficients of >0.99 for all standard substances.

To check the accuracy of the analytical method, recovery experiments were performed for selected bitter compounds. To achieve this, hop-free "zero beer" was spiked with defined amounts of isoxanthohumol, xanthohumol, *trans*-isocohumulone, cohumulone, and colupulone in three different concentrations prior to quantitative analysis, and the amounts determined after spiking were compared with those found in the zero beer (control). The recovery rates, calculated on the basis of the content of each bitter compound added to the zero beer prior to analysis, were found to be 88% for isoxanthohumol, 94% for xanthohumol, 102% for *trans*-isocohumulone, 104% for *trans*-isohumulone, 98% for *trans*-isoadhumulone, 94% for cohumulone, and 100% for colupulone (**Table 6**). These data demonstrate the developed ECHO technique to be a reliable tool enabling a rapid and accurate quantitative determination of hop-derived bitter compounds in beer.

Concentrations of Bitter Compounds in Beer Samples. To gain a first insight into the concentrations of all 26 bitter compounds in beer, these were quantitatively determined in 10 commercially available beers samples (I−X in **Table 7**). Among the bitter prenylflavanoids, the concentrations of isoxanthohumol (**1**) and xanthohumol (**2**), respectively, were found to vary between 0.17 and 6.14 μmol/L or between 0.008 and 0.387 μmol/L, which is in the range of data reported earlier (*1*, *20*, *21*). The Pilsner-type beer samples I−IX exhibited a xanthohumol to isoxanthohumol ratio between 0.01 and 0.03, being well in line with mean values found for American lager beers (*1*). Interestingly, the wheat beer sample X showed a higher xanthohumol to isoxanthohumol ratio of 0.1, which might be due to the late adding of hops to the wort. As carbon dioxide extracts of hops contain only very low amounts of xanthohumol (*1*), the extremely low levels of **1** and **2** in sample II may indicate the use of a carbon dioxide extract for wort processing instead of hop cones, hop pellets, or ethanolic hop extracts.

The bitter-tasting *cis*- and *trans*-iso-α-acids were confirmed as the quantitatively predominating group of hop-derived constituents in all of the beer samples. The total iso-α-acid content found for the Pilsner-type beer samples I−IX was between 80 and 130 μmol/L, whereas the wheat beer sample X contained iso-α-acid only in concentrations of 32 μmol/L (**Table 7**). In agreement with the literature (*20*, *22*), the *trans*/*cis* ratio was found to be around 0.4 for the beer samples. All of the beer samples investigated show rather similar ratios between the co-, n-, and ad-congeners of the iso-α-acids. The *cis*/*trans*-isohumulone (**7**, **8**) and *cis*/*trans*-isocohumulone (**5**, **6**) are the quantitatively predominating congeners in all of the beer samples and account for about 43 and 39% of the total iso-α-acid content, followed by *cis*/*trans*-isoadhumulone (**9**, **10**) with a somewhat lower content of 16.5% (**Table 7**). In comparison, the group of iso-α-acids contains only about 2% of the post- (**3**, **4**), pre- (**11**, **12**), and adpre-congeners (**13**, **14**).

Compared to the iso-α-acids, only low concentrations were determined for the α-acids and, in particular, for the β-acids in beer. The total amount of α-acids was found to be 1.1−10 μmol/L, whereas the total amount of β-acids was about 50 times lower, ranging between 0.05 and 0.17 μmol/L. The major α- and β-acids were humulone (**17**) and colupulone (**22**), respectively, present in concentrations of 0.65−4.93 and 0.03−0.07 μmol/L (**Table 7**). Calculating the ratio of a *cis*/*trans*-iso-α-acid to its corresponding α-acid for the different congeners revealed values of 7 for the postderivatives **3**/**4**, 20 for co- (**5**/**6**) and n-derivatives (**7**/**8**), and even 150 for the pre- (**11**/**12**) and adpre-derivatives (**13**/**14**). This change in the ratio of the congeners might result not only from different isomerization rates of the congeners but also from a structure-dependent adsorption of the congeners to macromolecules, filter materials, or yeast cells during beer manufacturing. To explain this phenomenon on a molecular level, more extensive quantitative studies on intermediary products of the beer manufacturing process are needed in the future.

Distinct differences in the ratio of *cis*/*trans*-iso-α-acids to α-acids were observed between the single beer samples (**Table 7**). For example, sample I was found to contain 94 times more iso-α-acids than α-acids, whereas sample II showed just a factor of 13 between both groups of bitter compounds, thus indicating differences in the efficacy of the wort-boiling process.

Influence of Storage on the Bitter Compounds in Beer Samples. To gain more detailed insights into the influence of storage on the concentrations of hop-derived bitter compounds in beer, the closed beer samples I, II, and III were aged under accelerated conditions for 8 months at 28 °C in the dark and selected bitter compounds were quantitatively determined by means of LC-MS/MS using the ECHO technique (**Table 8**). In agreement with previous findings (*20*, *22*), the *trans*/*cis* ratio was found to be around 0.4 for the fresh beer samples and decreased to a mean value of 0.16 after storage. In contradiction to the literature (*20*), the ratio of the individual congeners was not significantly affected, thus indicating that the degradation of the *trans*-iso-α-acids is independent from the nature of the variable alkanoyl side chain.

During the aging period of 8 months, the total amount of iso-α-acids decreased by about 27%, whereas the loss of the *trans*-isomers accounted for about 66% of the iso-α-acid degradation (**Table 8**). These data confirm previous reports showing losses of 10−15% total iso-α-acids and 70% of *trans*-iso-α-acids during the storage of beer for 9 months at 22 °C (*22*). Among the samples analyzed, beer sample I showed the strongest degradation of iso-α-acids by about 30%. Although the other beer samples showed somewhat less degradation of 24−27% of the total iso-α-acids, the ratios between the isomers did not differ significantly, thus indicating a similar degradation mechanism for all of the beers samples investigated.

Interestingly, the concentrations of α- and β-acids were differently influenced by storage when compared to the iso-α-acids. Among the bitter compounds investigated, the α-acids were found to be most prone to degradation; for example, 81 and 96% of the cohumulone detectable in the fresh beer samples I and III were lost after a storage period of 8 months (**Table 8**). This observation further strengthens recently published data on the instability of α-acids in beer samples (*23*). As beer sample II, containing 10-fold higher amounts of α-acids than samples I and III, showed a similar decrease of about 92% of the α-acids, it can be concluded that the rate of degradation seems to be independent from concentration. In contrast to the α-acids, the concentrations of the β-acids **22**−**24** as well as of the prenylflavonoids **1** and **2** did significantly increase upon storage of all beer samples investigated. This unexpected phenomenon might be explained by a slow release of β-acids and prenylflavonoids either from unknown, hop-derived precursor molecules or from macromolecule-bound complexes upon storage and needs further investigation on a molecular level in the future.

In conclusion, HPLC-MS/MS operating in the MRM mode allowed for the first time a simultaneous detection of all known congeners of α-acids, β-acids, and *cis*/*trans*-iso-α-acids as well as the prenylflavonoids xanthohumol and isoxanthohumol in authentic beer samples without any sample cleanup steps. By application of the ECHO technique, all individual bitter compounds were quantitatively determined in various beer samples. These studies were able to demonstrate a remarkable instability of α-acids and *trans*-iso-α-acids during beer storage, which was found to be independent from the nature of the alkanoyl side chain of the congeners, as well as an increase of the concentrations of β-acids and prenylflavonoids during the storage of beer. The chemical mechanisms underlying these phenomena on a molecular level are currently under investigation.

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