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Analysis of iso-α-acids and reduced iso-α-acids in beer by direct injection and liquid chromatography with ultraviolet absorbance detection or with mass spectrometry

G. Vanhoenacker [a,b], D. De Keukeleire [b], P. Sandra [a,c,*]

[a] *Research Institute for Chromatography, Kennedypark 20, B-8500 Kortrijk, Belgium*
[b] *Laboratory of Phytochemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Gent, Belgium*
[c] *Laboratory of Separation Sciences, Faculty of Sciences, Ghent University, Krijgslaan 281 (S4), B-9000 Gent, Belgium*

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Abstract

A liquid chromatography (LC) method is described for the simultaneous analysis of iso-α-acids and reduced iso-α-acids in beer. Volatile mobile phase additives were selected to enable hyphenation to mass spectrometric (MS) operated in the atmospheric pressure chemical ionization (APCI) mode. Contrary to other recent LC optimization procedures for the same compounds, an alkaline pH was selected hereby improving peak shape and selectivity. Both UV and MS detection are sensitive enough to analyze beers without sample pre-concentration. All major bitter acids are separated within 65 min with exception of *cis*-dihydroisoadhumulone, which co-elutes with *trans*-isocohumulone. Due to the selectivity of the MS, these compounds could be differentiated according to their *m*/*z* value. The performance in terms of quantification of bitter acids by LC–UV and LC–MS are compared for standard solutions and a selection of 14 beers.

Keywords: Injection methods; Beer; Direct injection; Iso-α-acids

1. Introduction

The bitter taste of beer is derived from hops (*Humulus lupulus* L.) or hop extracts added to the wort during brewing [1]. In the boiling process, the hop α-acids or humulones, which are almost tasteless, are isomerized into the bitter-tasting iso-α-acids or isohumulones. These products are not only responsible for the bitter taste of beer, but they also exhibit bacteriostatic properties and furthermore play an essential role in enhancing the foam stability of beer as well as in the formation of off-flavors like the lightstruck flavor [2–4]. Reduced iso-α-acids such as dihydroiso-α-acids (dihydroisohumulones, also known as rho-isohumulones) and tetrahydroiso-α-acids (tetrahydroisohumulones) are often used in the brewing process to enhance both the light and the foam stability of beer. Addition of non-natural reduced forms of iso-α-acids is not allowed in beers for the German market due to the "Reinheitsgebot" stating that only natural hop compounds may be used in the brewing process. Analytical methods for detection and quantitation of both iso-α-acids and reduced iso-α-acids are, therefore, of utmost importance.

Liquid chromatography (LC) is intensively used for the analysis of α- and β-acids in hops [5,6] and iso-α-acids in beer [2,7]. Problems related with interaction of the solutes with trace metals in the chromatographic system resulting in poor resolution and quantitation, have been reported [2,7]. Many LC methods use involatile buffer additives such as phosphate and citrate for the separation of iso-α-acids and reduced iso-α-acids [8,9]. These mobile phases are not compatible with mass spectrometric (MS) detection. Only few reports have been published on the LC–MS analysis of hop acids and no literature is available on the analysis of iso-α-acids and reduced iso-α-acids by LC–MS. Using ammonium acetate or acetic acid as mobile phase additives and electrospray ionization (ESI) in the negative mode, hop acids were analyzed in beer after direct injection [10]. Humulones (α-acids) were found to be present in beer at concentration levels of 150–200 μg/l, while lupulones (β-acids) were absent. Iso-α-acids and reduced iso-α-acids were not

* Corresponding author. Tel.: +32-9-2644462; fax: +32-9-2644998.
E-mail address: pat.sandra@richrom.com (P. Sandra).

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analyzed. Another report focused on the quantitation of 8-prenylnaringenin, a potent phytoestrogen, in hops, hop products, and beers. Reversed phase LC was carried out on a C18 column using formic acid as a buffer additive and ESI MS in the positive mode for detection [11]. No quantitation was performed for the hop and beer bitter acids.

For the analysis of the iso-α-acids in beer, a pre-concentration step is often performed. Enrichment is commonly carried out by liquid–liquid extraction or by solid phase extraction (SPE) on reversed phase material [8,9,12–14]. Recently, the application of a new solventless extraction method named stir bar sorptive extraction (SBSE) has been described [15]. SBSE combined with liquid desorption (LD) was successfully applied for the analysis of bitter compounds in beer and wort by LC [16], and in beer by micellar electrokinetic chromatography (MEKC) [17].

In this contribution, an LC method is described that enables simultaneous analysis of iso-α-acids and reduced iso-α-acids in beer without sample preconcentration. Beer is only degassed and filtered prior to injection. An alkaline mobile phase is used for reversed phase LC. Quantitative data obtained with LC–UV and LC–MS is compared.

2. Experimental

2.1. Chemicals

Ammonium acetate (p.a.) was from Merck (Darmstadt, Germany) and ammonia (p.a., 28–30 wt.% solution) from Acros (Geel, Belgium). Methanol, ethanol, acetonitrile and water were all gradient grade for LC from Merck (Darmstadt, Germany).

2.2. Samples and standards

Beers were bought at local stores, kept at room temperature, and freshly opened prior to analysis. Degassing was done by ultrasonication. Due to possible interaction of iso-α-acids with the foam and, consequently, loss of iso-α-acids, ultrasonication was continued until all foam was disappeared. A portion of the degassed beer was filtered through a syringe filter (0.2 μm, PTFE) from Alltech Associates (Lokeren, Belgium) prior to injection.

The calibration extract containing hop α- and β-acids (ICE 1) was from 'Versuchsstation Schweizerischer Brauereien' (Zurich, Switzerland). The pre-isomerized hop extract containing iso-α-acids was obtained from Hopstabil GmbH (Wolnzach, Germany). The dihydroiso-α-acids standard (all-*cis*, DCHA-Rho, ICS-R1) was from Labor Veritas (Zurich, Switzerland) and the tetrahydroiso-α-acids standard from Kalsec (Kalamazoo, MI, USA). The standards were dissolved separately in methanol to a concentration of 1 mg/ml. These stock solutions were stored in the freezer and were used to prepare standard solutions.

2.3. Instrumental

Analyses were performed on an Agilent 1100 LC equipped with a binary pump, vacuum degasser, autosampler, column thermostat, and diode array detector (Agilent Technologies, Waldbronn, Germany). Two Zorbax Extend C18 columns, 150 mm length × 4.6 mm i.d., packed with 5 μm particles, were coupled in series (Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of 5 mM ammonium acetate in 20% (v/v) ethanol adjusted to an apparent pH of 9.95 with ammonia (solvent A) and acetonitrile/ethanol 60/40 (v/v) (solvent B). The flow-rate was set at 1 ml/min and gradient elution was performed. The gradient was the following: 0–3 min: 0% B isocratic, 3–4 min: 0–16% B, 4–54 min: 16–40% B, 54–57 min: 40–95% B, 57–65 min: 95% B isocratic. The column temperature was maintained at 35 °C. One hundred microliters of degassed and filtered beer samples was injected. UV detection was performed at 256 nm (iso-α-acids and reduced iso-α-acids) and 330 nm (α- and β-acids).

The mass spectrometer was an Agilent 1100 Series Trap version SL with an atmospheric pressure chemical ionization (APCI) source (Agilent Technologies, Waldbronn, Germany). MS detection was started 8 min after injection. Negative ionization was performed in the scan mode (150–600 *m*/*z*). The target mass was set at 350 *m*/*z* and maximum accumulation time at 300 ms. Interface settings were: N_2 drying gas temperature 330 °C, N_2 drying gas flow 5 l/min, APCI vaporizer temperature 450 °C, nebulizer 60 psi, capillary voltage 4000 V. For experiments on the same mass spectrometer operated in the ESI mode, the interface parameters were: N_2 drying gas temperature 300 °C, N_2 drying gas flow 12 l/min, nebulizer: 50 psi, capillary voltage 3000 V.

3. Results and discussion

3.1. Method development

The structures of the main hop acids, the beer iso-α-acids, and the reduced iso-α-acids (tetrahydro- and dihydroiso-α-acids) are shown in Fig. 1.

During the brewing process, nearly all (relatively insoluble) β-acids are removed or oxidized, while each α-acid is transformed into its corresponding mixture of epimeric iso-α-acids (*cis* and *trans* isomers). As a result, six iso-α-acids originate from the three main hop α-acids. Tetrahydroiso-α-acids also exist as *cis* and *trans* isomeric pairs totaling six stereoisomers. Reduction of iso-α-acids to dihydroiso-α-acids introduces an additional chiral center, leading to two epimeric reaction products for each iso-α-acid. The group of dihydroiso-α-acids, thus, consists of twelve stereomeric members. Chromatographic separation of all twenty-four structurally similar compounds in a single analysis represents a daunting task. LC of iso-α-acids and reduced iso-α-acids is commonly carried out in the

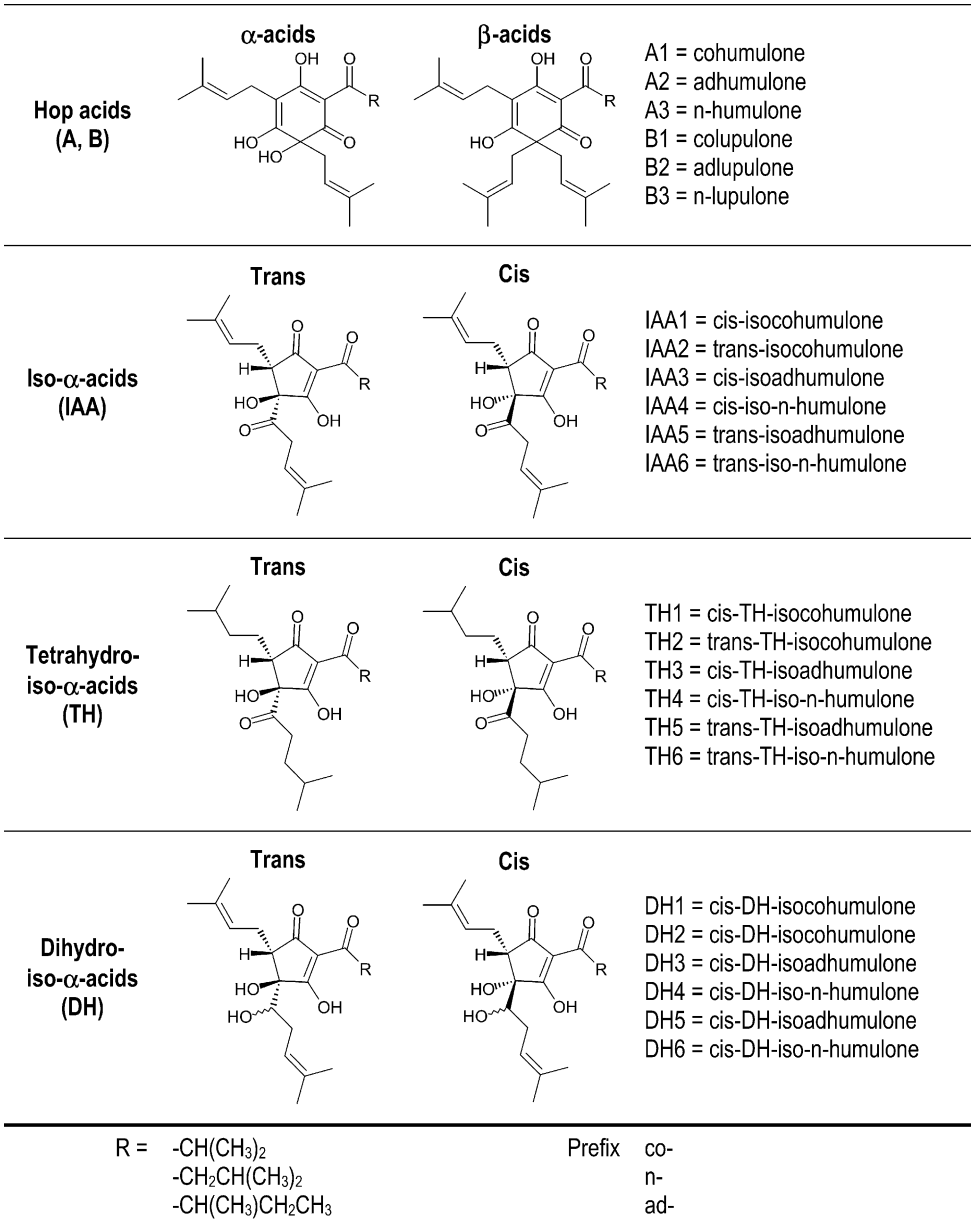

Fig. 1. Structures and peak codes for hop acids, iso-α-acids and reduced iso-α-acids.

reversed phase mode with a C18 or C8 stationary phase. Buffers are added to the mobile phase to improve separation and reproducibility. The buffer is usually acidic (phosphate) or neutral (citrate). In this work, a high pH mobile phase was opted for, because this enhanced peak shape and efficiency. As an example, the resolution for the iso-α-acids IAA3, IAA4, IAA5, and IAA6, the most critical part of the separation, improves significantly, when the pH of the mobile phase is raised from neutral to alkaline. This is caused by the increased efficiency and the enhanced selectivity for these acidic solutes at elevated pH. It was the aim to develop a method compatible with MS detection and a volatile mobile phase additive was therefore chosen. Ammonium acetate was selected and the appropriate pH was attained using ammonia. The optimum apparent pH (in 20% (v/v) ethanol) was found to be 9.95. Care should be taken to accurately control the pH, since small variations cause significant changes in chromatographic selectivity. The high pH mandates selection of a stationary phase that is stable in basic media. The columns used (Zorbax Extend) are stable up to pH 11.5. A combination of ethanol and acetonitrile in the mobile phase was preferred over methanol, acetonitrile or mixtures of both, because this consistently provided a better separation.

Preliminary experiments were carried out on various types of mass spectrometers. Although all instruments generally gave the required sensitivity, some differences were noted. On an LCQ Ion Trap MS (ThermoFinnigan, San

Jose, CA, USA) in both the ESI and APCI mode significant, post-separative polymerization of the compounds occurred for all compounds, except for the hop β-acids. Adducts, dimers, and trimers were formed by a combination of the acids with sodium, ammonium and acetate ions. The same phenomenon was observed using a quadrupole MS (1100 Series MSD version SL from Agilent Technologies, Waldbronn, Germany) in the ESI mode. The oligomers were less abundant in the Agilent 1100 Series Trap. These oligomers appear to be created during the ionization process and are more abundant at higher concentrations. Since the ionization sources are the same for both the Agilent MSD and Trap, one could expect similar mass spectral profiles. However, the oligomers are less abundant in the Trap. Since a target mass set-point has to be entered for the Trap (set for the molecular ions of the compounds), some additional selectivity is obtained compared to the quadrupole MSD. As a result, a more efficient detection of the molecular ions was obtained compared to the oligomers.

The 1100 Series Trap provided the best mass spectral data with adequate sensitivity. Therefore, this detector was selected for further analyses. Standard solutions containing 20 mg/l α- and β-acids, and of each group of iso-α-acids and reduced iso-α-acids were analyzed by LC–MS with ESI and APCI. The latter performed significantly better in terms of peak shapes, resolution, and sensitivity due to more efficient evaporation of the mobile phase in the APCI interface compared to ESI and the volatility and medium polar nature of the investigated compounds.

The mass spectra of the co-homologues, obtained by LC–MS in the negative APCI ionization mode, are shown in Fig. 2. Fragmentation in APCI is more pronounced compared to ESI. A distinctive fragmentation pattern is observed for each group of acids. Iso-α-acids and reduced iso-α-acids fragment similarly via dehydration and loss of a side chain. For the iso-α-acids and the tetrahydroiso-α-acids, dehydration is followed by loss of the 4-methyl-3-pentenoyl or the 4-methylpentanoyl side chain, respectively. Dihydroiso-α-acids are characterized by cleavage of the 1-hydroxy-4-methyl-2-pentenyl side chain followed by dehydration. Splitting of water from α-acids sets the stage for ring opening and loss of a ring carbon together with a hydroxyl group and the 3-methyl-2-butenyl side chain. Reclosure furnishes a five-membered ring. β-Acids are marked by loss of the 3-methyl-2-butenyl side chain only.

Fig. 3 shows a LC–DAD–MS analysis of a standard solution containing ca. 10 mg/l of each group of acids. Peak identification was based on the detected MS ions and on literature data [8,9]. For UV detection, the iso-α-acids and reduced iso-α-acids are monitored at 256 nm and the α- and β-acids at 330 nm. The iso-α-acids are quantified at 256 nm. In UV detection, co-elution occurs between a *cis*-dihydroisoadhumulone epimer (DH5) and *trans*-isocohumulone (IAA2). The MS traces are extracted ion chromatograms (EIC) composed of the molecular ions and most important fragments. The selected ions were also used for quantitative analyses (Table 1). Iso-α-acids and dihydroiso-α-acids share an identical fragment ion (*m*/*z* 233 for co-forms and *m*/*z* 247 for *n*- and ad-forms). Also, isocohumulones and tetrahydroisohumulones and tetrahydroisoadhumulones have a common ion (*m*/*z* 347). However, these compounds are chromatographically sufficiently separated to avoid interference. The co-elution of DH5 and IAA2 is not problematic for MS detection, as the *m*/*z* values differ. MS selectivity, thus, enables to resolve both compounds and to quantify them correctly.

Table 1
Molecular ions and fragment ions used for constructing LC–MS extracted ion chromatogram (EIC) for the various groups of compounds

Compound	MW	Ions (negative ionization)
IAA		
co	348	233, 329, 347
n/ad	362	247, 343, 361
DH		
co	350	233, 251, 349
n/ad	364	247, 265, 363
TH		
co	352	235, 333, 351
n/ad	366	249, 347, 365
α		
co	348	233, 277, 278, 329, 347
n/ad	362	247, 291, 292, 343, 361
β		
co	400	331, 399
n/ad	414	345, 413

IAA, iso-α-acids; TH, tetrahydro-iso-α-acids; DH, dihydro-iso-α-acids; α, α-acids; β, β-acids.

3.2. Method evaluation

Four point calibration graphs in the range of 5–50 mg/l were constructed by analyses of standard solutions (prepared in 10% (v/v) methanol in water). Because of co-elution of DH5 and IAA2 in UV detection, two separate calibration series were made, the first series containing the reduced iso-α-acids and the second series the iso-α-acids and α- and β-acids. Repeatability of injection was performed by six consecutive injections of the calibration solution of both series at a concentration of ca. 10 mg/l for each group of acids. The obtained R.S.D.s for the iso-α-acids and reduced iso-α-acids were all below 0.3% for UV detection, while these values varied between 5 and 9% for MS detection. Since UV and MS are performed on-line, the difference in repeatability is only caused by the detection. It is known that MS performs inferior in this respect compared to UV or DAD [18] and the rather high R.S.D.s for MS detection are not unusual for an ion trap MS. Poor results for the α- and β-acids are due to instability of those sensitive solutes in the injection vial. Also, adsorption of these hop acids at the wall of the glass vials must be taken into account, in particular, when trace amounts are present. Their impact on the bitterness of beer, however, is not important; hence,

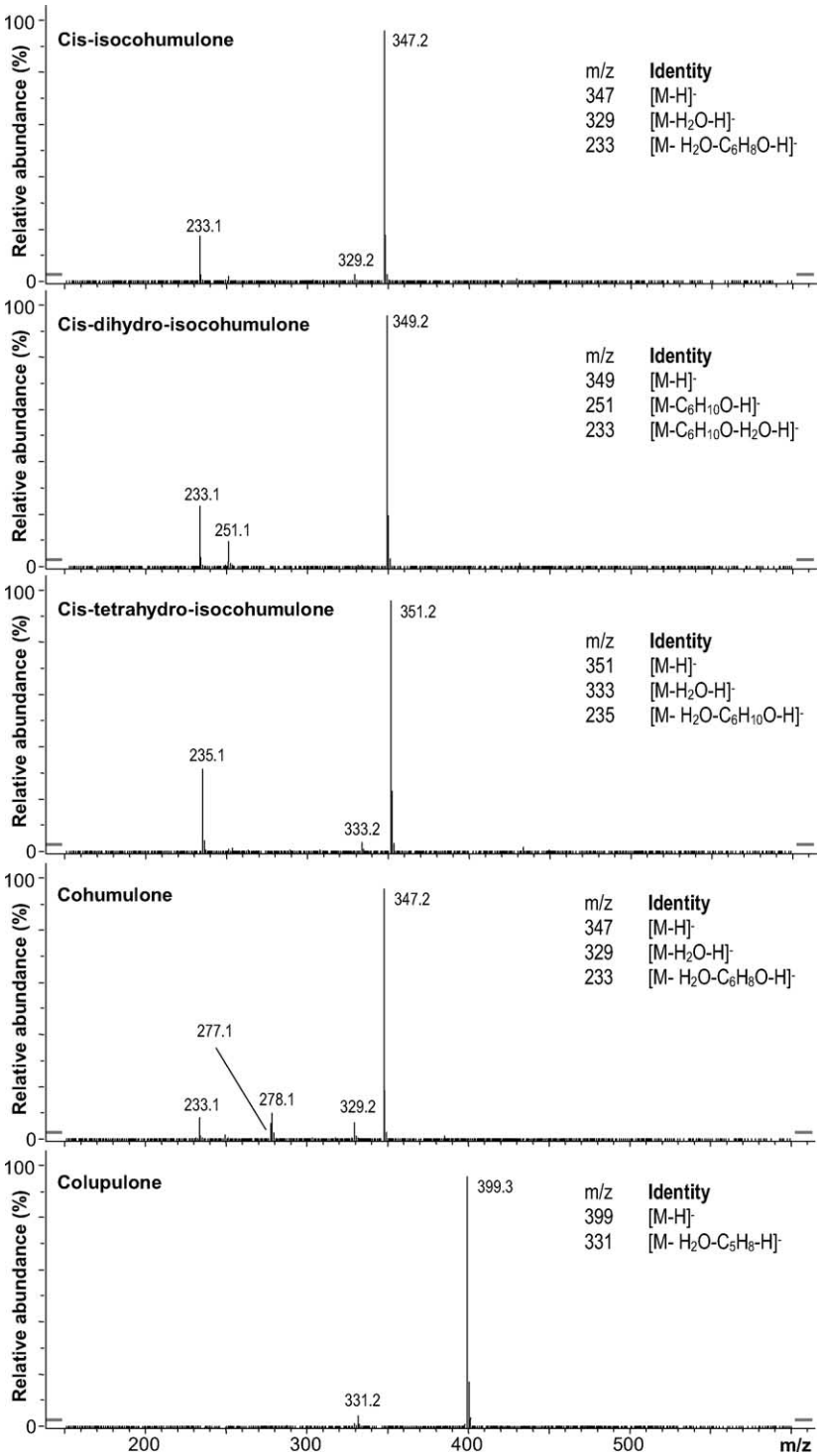

Fig. 2. Mass spectra of the co-homologues of iso-α-acids, reduced iso-α-acids and hop α- and β-acids in the APCI negative ionization mode.

they were not determined. The results for calibration and repeatability are summarized in Table 2.

No loss in performance of the method or the column due to or matrix contamination (e.g. sugars, proteins) was observed during this study. Also, the use of the alkaline mobile phase did not have adverse effects on the long-term stability of the method.

3.3. Comparison of various beers

A selection of fourteen beers was analyzed by LC–UV–MS and the iso-α-acids and reduced iso-α-acids contents were quantified (Table 3). For iso-α-acids, the ratio of co-, *n*-, and ad-isohumulones and the *cis–trans* ratios were calculated for each beer. The quality of the bitterness differs among the

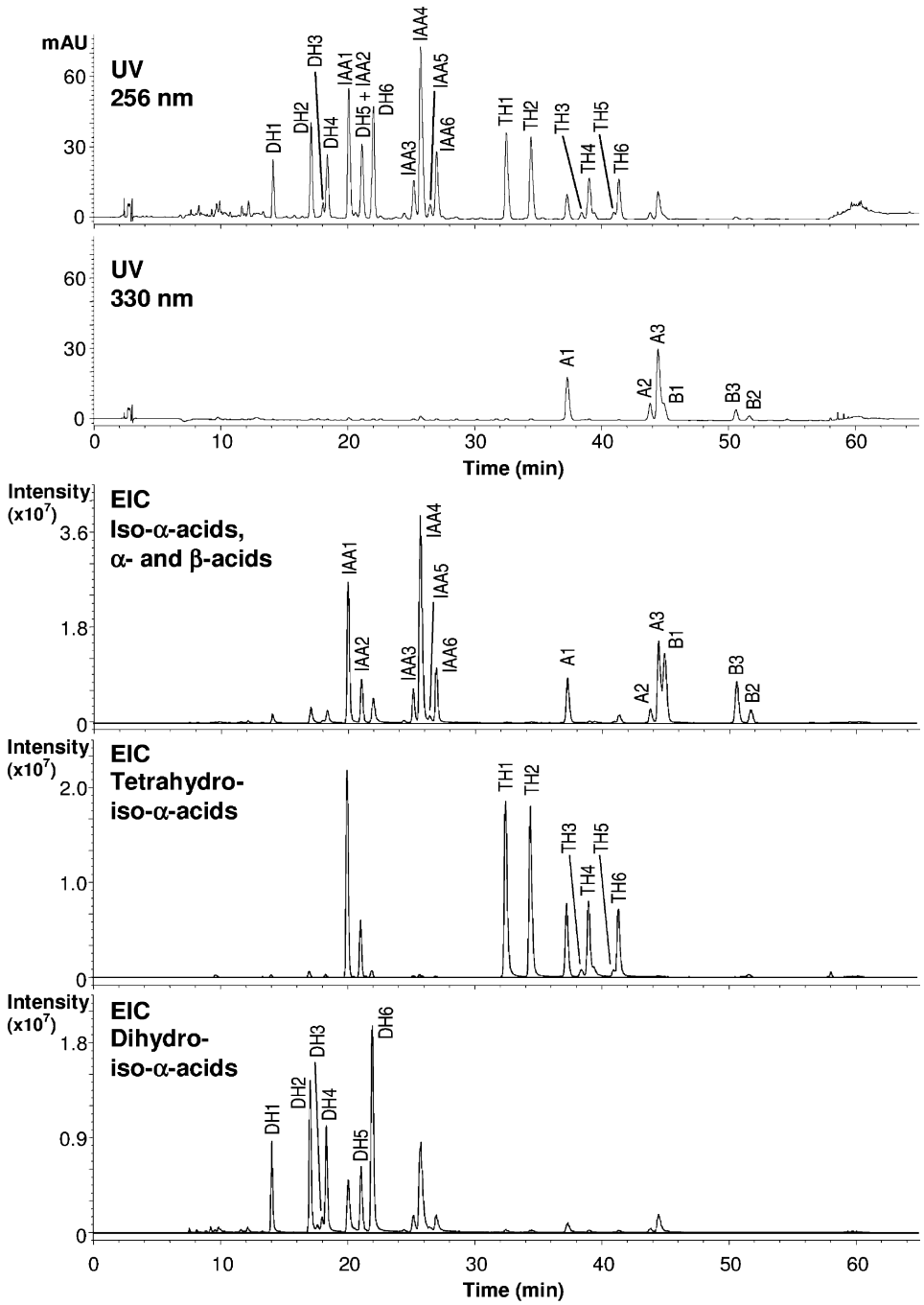

Fig. 3. LC–UV and LC–MS chromatograms for the analysis of a 10 mg/l standard mixture. EIC, extracted ion chromatogram (selected ions: see Table 1).

Table 2
Comparison of the calibration data and repeatability of injection for LC–UV and LC–MS (10 mg/l, $n = 6$)

	IAA (5.85, 11.70, 23.40, 58.50 mg/l)		TH (5.28, 10.55, 21.11, 52.76 mg/l)		DH (5.18, 10.35, 20.70, 51.75 mg/l)		α/β (4.33, 8.67, 17.34, 43.34 mg/l)	
	UV	MS	UV	MS	UV	MS	UV	MS
Calibration (R^2)	1.0000	0.9994	1.0000	0.9987	1.0000	0.9992	0.9994	0.9946
Repeatability R.S.D. (%)	0.3	8.9	0.2	6.4	0.2	7.1	4.5	22.7

All solutions were prepared in 10% (v/v) methanol in water. IAA, iso-α-acids; TH, tetrahydro-iso-α-acids; DH, dihydro-iso-α-acids; α/β, α- and β-acids.

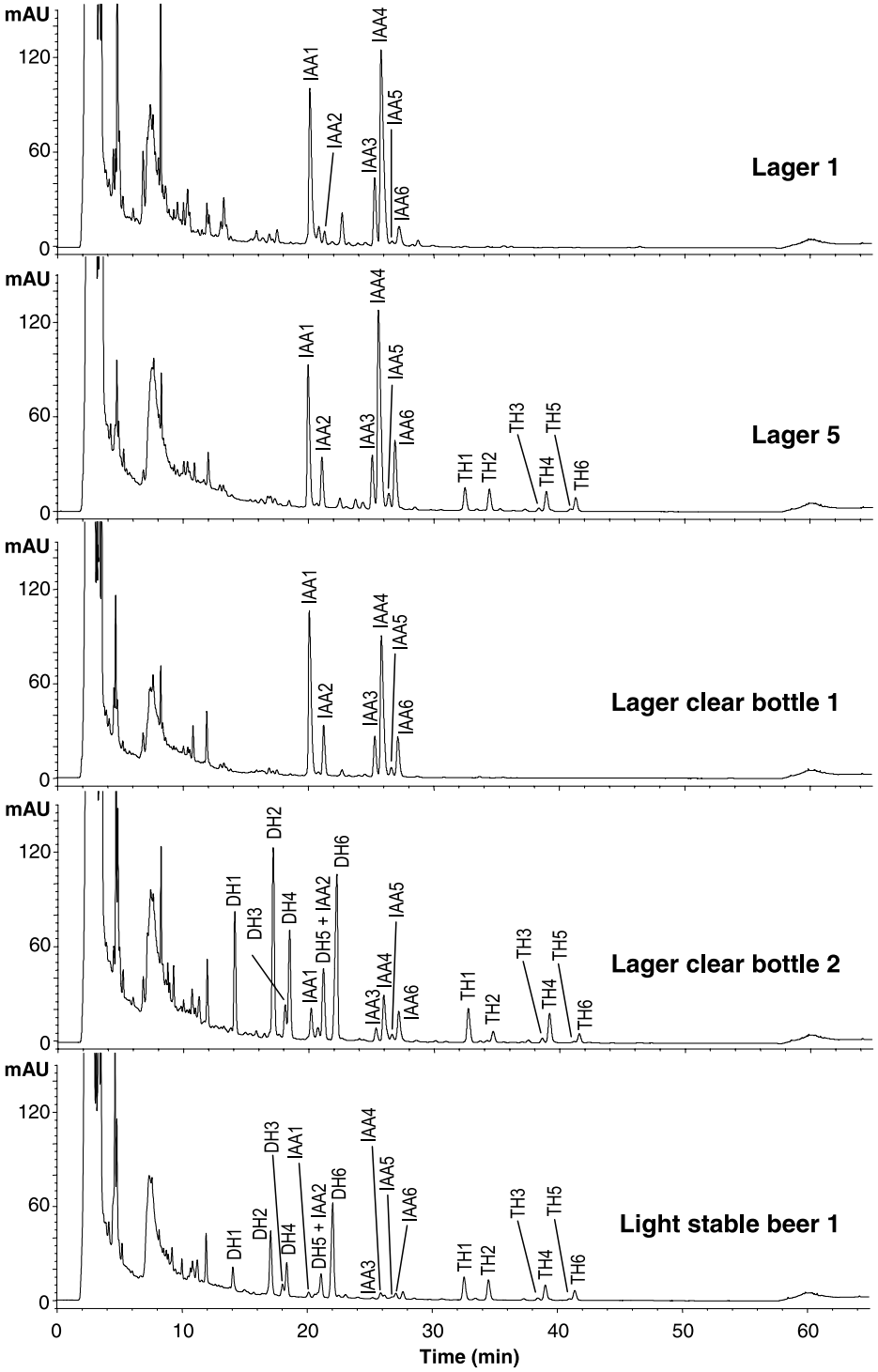

Fig. 4. LC–UV analyses of various beers after direct injection with detection at 256 nm.

individual isohumulones [19]. The co-derivatives show less desired organoleptic properties. There is also a longstanding issue about the unfavorable impact of co-α- and co-β-acids on sensory profiles. Beers that contain larger amounts of *n*- and ad-isohumulones are characterized by a more subtle bitterness, while the *cis–trans* ratios are relevant with respect to the flavor stability. Representative chromatograms of LC–UV analyses at 256 nm are shown in Fig. 4. Lager 1 is a German beer that is devoid of reduced iso-α-acids. The very high *cis–trans* ratio (>10) can be attributed to the use of advanced pre-isomerized hop products or to beer aging. As *trans*-compounds degrade faster than *cis*-compounds, the *cis–trans* ratio may increase on aging of the beer [20]. In the other beers, this ratio varies between 2 and 5. The profile of lager 5 is typical for a Western European pilsner-type beer. In current brewing practice beers (lagers 3–6) contain

Table 3
LC–UV and LC–MS data for the analysis of iso-α-acids and reduced iso-α-acids in beers after direct injection

Beer	IAA				TH				DH	
	Concentration (mg/l)		co/ad/*n* (%)		Ratio (*cis*/*trans*)		Concentration (mg/l)		Concentration (mg/l)	
	UV	MS	UV	MS	UV	MS	UV	MS	UV	MS
Lager 1	23.2	22.5	33.3/13.5/53.2	31.8/15.4/52.8	10.2	12.4	–	–	–	–
Lager 2	29.8	27.7	33.3/12.9/53.8	33.4/14.2/52.4	2.9	2.9	–	–	–	–
Lager 3	18.0	19.8	30.9/13.6/55.5	32.2/15.0/52.8	2.3	2.4	4.8	5.3	–	–
Lager 4	21.0	22.2	37.4/11.7/50.9	36.4/14.0/49.5	3.1	3.0	3.7	3.9	–	–
Lager 5	24.6	23.6	32.1/12.2/55.7	32.1/12.9/55.0	3.1	3.2	5.0	5.0	–	–
Lager 6	23.9	21.6	40.1/10.8/49.1	39.4/12.3/48.3	2.9	2.8	3.2	3.1	–	–
Non-alcoholic lager	33.6	27.1	30.6/12.5/56.9	31.0/13.6/55.4	3.4	3.2	–	–	–	–
Lager clear bottle 1	20.5	21.0	45.3/10.5/44.2	43.2/10.7/46.1	3.7	4.4	–	–	–	–
Lager clear bottle 2	6.2	6.2	21.7/15.0/63.3	38.3/12.5/49.2	2.2	1.5	5.4	4.8	5.4	5.5
Light stable beer 1	0.6	0.7	32.4/12.9/54.7	46.7/7.4/45.9	5.7	2.2	4.3	4.5	11.7	14.3
Light stable beer 2	–	–	–	–	–	–	0.8	0.7	23.4	27.9
Belgian trappist	24.1	23.3	40.9/12.5/46.6	39.6/14.6/45.8	5.0	5.0	–	–	–	–
Wheat beer 1	8.6	8.8	43.5/15.9/40.6	42.8/13.2/44.0	3.9	4.8	–	–	–	–
Wheat beer 2	11.9	12.3	37.7/12.5/49.8	35.3/14.2/50.5	3.1	3.1	–	–	–	–

IAA, iso-α-acids; TH, tetrahydro-iso-α-acids; DH, dihydro-iso-α-acids.

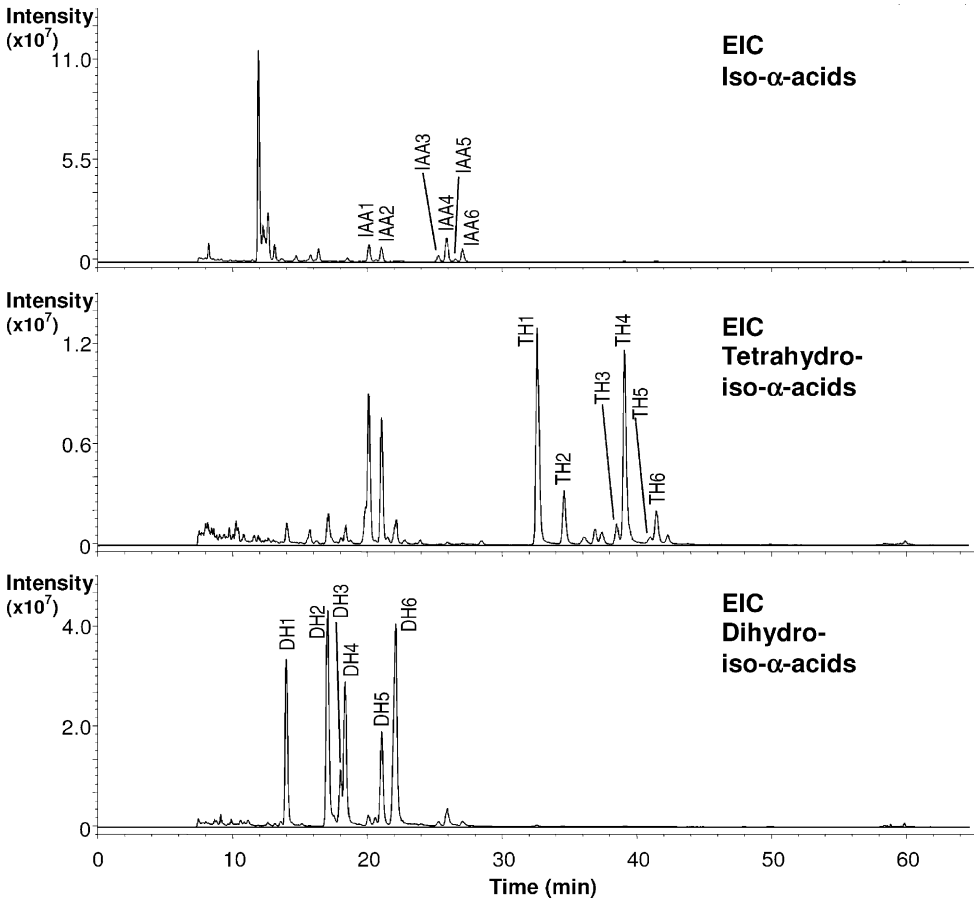

Fig. 5. LC–MS analysis of "lager in clear bottle 2" with APCI negative ionization after direct injection. EIC, extracted ion chromatogram (selected ions: see Table 1).

a small portion of tetrahydroiso-α-acids, mainly to improve foam stability. Smaller breweries often still use natural hop cones (lager 2, non-alcoholic lager, Belgian trappist). The difference between the two beers contained in clear bottles is striking. Because they are not protected from light, it could be expected that measures were taken to stabilize their flavor and bitterness. In lager clear bottle 2, dihydro- and tetrahydroiso-α-acids have been applied, resulting in a much lower concentration of iso-α-acids. Conversely, in lager clear bottle 1, only iso-α-acids are detected, while a high content of isocohumulone is found compared to iso-adhumulone and iso-*n*-humulone. Light stable beer 1 does only contain small amounts (below 1 mg/l) of iso-α-acids, the bitterness being delivered by dihydro- and tetrahydroiso-α-acids. The significant difference between the ratios of co-, *n*-, and ad-isohumulones in this beer is due to co-elution of a *cis*-dihydroisoadhumulone epimer and *trans*-isocohumulone. The peak area for *trans*-isocohumulone was not included in the calculations by LC–UV analysis. The peak was regarded as *cis*-dihydroisoadhumulone leading to inaccurate quantitation. Consequently, the isocohumulone content in LC–UV is significantly lower compared to LC–MS. No iso-α-acids were detected in Light stable beer 2. Wheat beers are much less bitter than lager beer, which is reflected by their low iso-α-acid contents.

The quantitative values obtained with LC–MS are close to those obtained with LC–UV. The only clear trend in the results is that the calculated recoveries of dihydroiso-α-acids is consistently higher in LC–MS compared to LC–UV. Other discrepancies for calculated recoveries and calculated ratios between LC–MS and LC–UV appear at random. The significant difference between the ratios of co-, *n*-, and ad-isohumulones in the second lager in a clear bottle again is due to co-elution of a *cis*-dihydroisoadhumulone epimer and *trans*-isocohumulone. The LC–MS chromatogram of this beer is shown in Fig. 5. Again, the peak for the co-eluting *cis*-dihydroisoadhumulone epimer and *trans*-isocohumulone in LC–UV was regarded as pure *cis*-dihydroisoadhumulone. Consequently, the iso-α-acid content is underestimated and the dihydroiso-α-acids are overestimated. This also explains for this particular beer why the isocohumulone content in LC–UV is significantly lower compared to LC–MS and why the difference between the recovered amounts of dihydroiso-α-acids is small. Thus, for this particular case, the result for the co-, *n*-, and ad-isohumulones ratios obtained with LC–MS are more reliable than those obtained with LC–DAD.

4. Conclusions

LC–UV and LC–MS were successfully applied for the simultaneous analysis of iso-α-acids and reduced iso-α-acids in beers by direct injection. Both methods provide adequate sensitivity. In LC–UV, the developed method results in co-elution of only a *cis*-dihydroisoadhumulone epimer with *trans*-isocohumulone. Thus, quantitation of iso-α-acids in the presence of dihydroiso-α-acids is questionable. LC–MS, however, is more selective enabling separation of all major compounds. This advantageous feature was proven by the analysis of a beer containing comparable concentrations of iso-α-acids and reduced iso-α-acids. The values obtained with LC–MS are similar to those obtained with LC–UV, except for the dihydroiso-α-acids. The calculated recoveries of dihydroiso-α-acids were consistently higher in LC–MS compared to LC–UV. MS detection proved superior to UV detection in view of enhanced sensitivity and selectivity, but R.S.D.s were higher.

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