S. Loch-Ahring, F. Decker, S. Robbert and J. T. Andersson

Chill-haze – Identification and Determination of Haze-active Constituents by HPLC and Mass Spectrometry

Part I: The role of polyphenols and the astonishing impact of hop components on chill haze formation

The formation of haze is a serious problem of bright beers because it limits the storage life of the bottled or barrelled product. The problem is particularly evident in the bottle. Haze (chill haze) is said to be caused by different substances and mechanisms. The most commonly held opinion makes a protein polyphenol interaction responsible for its formation. Therefore efforts of the brewing industry are targeted towards a minimization of one or both of these components by filtration through PVPP (polyvinylpolypyrrolidone) and diatomite (Celite). The objective of this study was to trace the influence of polyphenols as well as other phenolic substances on the colloidal stability of beer and to lay the foundation for tracing them throughout the brewing process. In regard to chill haze performance and brewing stabilisation, the repeated reference to polyphenols led us to examine the polyphenol content of isolated chill haze in detail. Based on an existing method normally used to determine the polyphenol composition of beer and wort, a modified approach was developed to examine polyphenols in chill haze. To examine the possible influence of polyphenols on chemo-physical endurance, beer samples taken throughout the brewing process, isolated chill haze and permanent beer turbidity were determined by HPLC-DAD-QTOF-MS, UPLC-DAD-QTOF-MS and nano ESI chip-QTOF-MS. This is the first part of our work and only discusses the polyphenol analysis in wort, beer and isolated haze performed by HPLC-DAD-QTOF-MS and nano ESI chip-QTOF-MS. Haze properties were not studied using kinetics but analytical methods with the aim to identify single components. The influence of some polyphenols on the formation of haze clouding was observed. The strong influence of hop substances on the colloidal stability could be demonstrated

Descriptors: chill haze, colloidal stability, polyphenols, catechin, xanthohumol, hop components, PVPP stabilisation

1 Introduction

Hazes frequently appear throughout the beer brewing process, especially chill-haze and permanent beer turbidity resulting from it. They are of great importance since they have an influence on the product shelf life. Furthermore, consumers judge the quality of a beer from its immaculate visual appearance that must be guaranteed until the expiration date.

To ensure a high quality of both the fresh and the stored product, breweries perform a large-scale stabilisation treatment with silica gel and PVPP (polyvinylpolypyrollidone). In theory, the former selectively removes proteins which may include haze-active (HA) proteins, whereas the latter is utilized for the removal of HA polyphenols. A great disadvantage of the application of those stabilisers (mainly PVPP) is the high costs connected with it. Since both, an improved quality as well as a reduction of costs, are common aims of brewers, it is of particular importance to better understand the basic reactions leading to beer haze. A detailed knowledge of the mechanism of haze formation may be helpful in optimising the stabilisation treatment and a brewery-dependent adaption to increase the quality of the beer.

Authors: Stefan Loch-Ahring, Fabienne Decker, Sascha Robbert, Brewery C. & A. Veltins, D-59872 Meschede-Grevenstein, Germany; Jan T. Andersson, Institut für Anorganische und Analytische Chemie, Universität Münster, Corrensstrasse 30, 48149 Münster, Germany.

Tables and figures see Appendix

Information on the mechanism of haze formation is still sparse even though investigations have been conducted to discover major triggering components. Different models specifying the main haze precursors have been put forward; most of those that were developed in the last several years concentrate on reactions that occur between HA polyphenols and HA proteins (or their fragments). One characteristic of the HA proteins seems to be their high levels of proline [4,5,6,8]. The polyphenols are thought to interact with binding sites of these HA proteins to form intermolecular bridges [4–8]. The most popular and most cited mechanism are illustrated in the haze models of *Haslam, O´Rourke, Siebert, Beart, Gracey* and *Kaneda* [see 3–8,13,26].

Our schematic description of a possible haze model is shown in Figure 1. As the related chemical mechanism have not been fully defined yet, this model only includes visible and reproducible changes that a beer goes through (independent of the kind of beer brand analysed).

The protein-polyphenol composition of beer has been reviewed extensively in several studies [5–11] which highlight the influence of polyphenols on flavour and physico-chemical stability. Due to the ability of polyphenols to form haze with proteins in model reactions, these interactions have been assumed to be responsible for haze reactions in the beverage container, too. Despite theoretical considerations and model reactions, no haze formation mechanism has been proved yet. Furthermore, the elemental composition of isolated chill haze has not been analysed in detail

up to now. For prediction purposes, some standard methods have been developed by MEBAK [27] to predict the tendency of a beer to generate haze (e.g. the labour test (MEBAK 2.19.2.1), the test for sensitivity to chill according to Chapon (MEBAK 2.19.2.3), the analysis of the total polyphenol content (MEBAK 2.21.1) and the analysis of the anthocyanogen content (MEBAK 2.21.2)) but none of these analyses is useful or able to identify components in isolated chill haze. For these reasons, this paper only focuses on the determination of polyphenols and their influence on chill haze formation in particular.

Polyphenols are secondary plant metabolites and occur both in plants and in plant-derived products. Their influence on mouthfell, bitterness and astringency are determining characteristics [13]. Polyphenols include a great number of substance categories. Their structures are based on phenol or phenol ether monomers, whereas molecules with two or more phenolic groups are defined as "polyphenols" [12].

Phenolic components associated with beer are found in raw materials like malt and hops. Their behaviour during the beer production is dependent on a number of parameters, e.g. their origin, the malting technology process and the brewing process [13]. The diversity of polyphenols is high and several individual components might have an impact on colloidal turbidity. In this regard flavanoids (Fig. 2) like the flavan-3-ols catechine, epicatechine, gallocatechine and epigallocatechine, phenol carboxylic acids like gallic acid and vanillic acid, as well as prenylflavanoids like xanthohumol and isoxanthohumol are mentioned [11,14–17, 23, 24]. Polymeric polyphenols called proanthocyanidins normally occur as dimers, trimers and polymers of flavan-3-ols or flavanols [12]. The best known representatives of the latter in beer are the dimers procyanidin B_3 (catechin(4α-8)catechin) and prodelphinidine B_3 (gallocatechin(4α-8)catechin) [12, 23, 24]. Proanthocyanidins are considered to be the most important agents in haze formation [1, 4, 5, 10, 13].

Although no analyses on isolated haze material have been done, several substances are likely to have an influence on its formation, namely the flavan-3-ols (+)-catechine, (–)-epicatechine, (–)-epi-gallocatechine, (+)-gallocatechine, as well as oligomers like prodelphinidine B_3, procyanidin B_3, procyanidin C_2, and further unidentified dimers and trimers. Tetramers and pentamers which could be identified, too, were stated to be less relevant [21]. In addition, phenolic acids like vanillic acid, caffeic acid, syringic acid, ferulic acid and sinapic acid were found and their haze-performing ability was estimated [21].

The recent development of new analytical tools has opened up the possibility to study the composition of phenolic components in detail. Our research is based on two such techniques, HPLC-DAD-QTOF-MS and nano ESI chip-QTOF-MS. Independent of the chosen sample material, MS and additional MS/MS analysis allow us to identify polyphenols with a very high selectivity. Necessary for such analyses was the development of a method for chill haze preparation and polyphenol enrichment.

Estimating the different polyphenols in beer and wort as well as isolated haze is one of the main topics of this paper. Furthermore, we focus on the influence of polyphenols and hop components on beer turbidity.

2 Experimental

2.1 Chemicals

Methyl alcohol (MeOH) was from Baker (Mallinckrodt Baker B.V., NL- 7400 AA Deventer). Solvents such as ammonia, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethyl alcohol, isopropyl alcohol, butanol, acetonitrile, formic acid, acetic acid, ethyl acetate, ethyl hexanoate, acetone, diethyl ether, pentane, hexane, and heptane were from Merck (D-64293 Darmstadt). (+)-catechine, (–)-epicatechine, (–)-epigallocatechin and other polyphenol standards were from Sigma-Aldrich (D-89555 Steinheim). Pure water was produced by a Millipore synthesis A10 system. ROTH (D-76158 Karlsruhe) delivered the standard substances of (+)-catechin, (–)-epicatechin and (–)-epigallocatechin. The EBC standard (xanthohumol enriched extract) was ordered from NATE-CO_2 (D-85283 Wolnzach) and an ICE 2 (international calibration extract) was available from Labor Veritas (CH-8027 Zürich).

Samples

An extensive collection of samples was taken during the whole brewing process to monitor significant data during the brewing and to create reproducible data. In addition to the samples from the brewing process, chill haze was prepared from the stored sample material (0 °C, ambient temperature) and analysed. A large number of other materials were prepared including foreign beers, laboured (thermally induced haze formation) beer samples and material from other investigations.

2.2 Experimental methods

Solid Phase Extraction (SPE).

A polyamide sorbent was used to concentrate and purify beer polyphenols. Sample volumes of 15 mL were used and eluted with 2.5 mL dimethylformamide. This solution was directly used for the HPLC analysis.

Analytics:

Chromabond PA, cat. No. 730 127, Macherey-Nagel (Düren)

Conditioning	5 mL pure water
Sample	15 mL
Wash	5 mL 1 % acetic acid
Elute	2.5 mL 80 % DMF

Hop components were extracted on an octadecyl-modified silica solid phase extraction (SPE) cartridge for polar analytes and eluted with 90 % methanol. The solution was analyzed using HPLC.

Analytics:

Chromabond C18 Hydra, cat. No. 730 300, Macherey-Nagel (Düren)

Conditioning	5 mL methanol
Reconditioning	5 mL pure water
Sample	15 mL
Wash	5 mL 10 % methanol
Elute	2.5 mL 90 % methanol

Chill haze preparation.

Beer was stored for eight to ten weeks in a freezer at 0 °C. First an opalescence and later an amorphous chill haze was formed in the bottle. This turbidity was concentrated via centrifugation and washed with water. The procedure was repeated four times to clean up the material and wash out remaining beer components. Then the pellet was dissolved in dimethyl sulfoxide. The solution was frozen at –80 °C and then lyophilized.

The lyophilized samples could be dissolved in dimethyl sulfoxide, dimethylformamide and ammonia/methanol. The material was not soluble in water, pentane, hexane, heptane, methanol, ethanol, ethyl acetate, ethyl hexanoate, diethyl ether, acetone, acetonitrile, acetic acid or formic acid.

For analysis, the chill haze was dissolved in dimethyl sulfoxide again and injected into the HPLC.

The second analytical approach used the Triversa Nanomate in the infusion mode. The samples were dissolved in dimethyl sulfoxide and cleaned up by solid phase extraction.

Cleaning up procedure for chill haze samples.

The ZipTip technique was used to clean up chill haze samples. NuTips with an octadecyl-modified silica sorbent (Sunchrom, Friedrichshain) were conditioned with methanol and reconditioned with water. The chill haze samples in dimethyl sulfoxide were flushed three times and washed with water four times. Depending on the sample concentration, this procedure was repeated five to ten times. The sample was eluted with methanol into a 96-well plate and analysed by the Triversa Nanomate in the infusion mode.

HPLC Analysis

HPLC analysis was performed on a Waters (Eschborn) Alliance system 2695 with a photodiode array detector 2996 and Micromass (Waters) QTOF micro mass spectrometer.

5 µL of the sample was injected onto a reversed phase column (Phenomenex, Synergi hydro-RP, 4 µm, 80 Å, 150 mm x 2.00 mm) and eluted in 90 minutes with a flowrate of 0.3 mL/min using a gradient from 100 % solvent A (water with 1 % acetic acid) to 100 % solvent B (acetonitrile with 1 % acetic acid).

The column was thermostated at 40 °C.

Time	A %	B %	flow	curve
0.00	100.0	0.0	0.3	linear
50.00	60.0	40.0	0.3	linear
70.00	40.0	60.0	0.3	linear
80.00	0.0	100.0	0.3	linear
83.00	0.0	100.0	0.3	linear
85.00	100.0	0.0	0.3	linear
90.00	100.0	0.0	0.3	linear

Elution was monitored with a photodiode array detector (PDA) in the scan mode from 190 nm to 600 nm and extracted wavelengths of 280, 300 and 330 nm.

A QTOF mass spectrometer was serially connected to the PDA.

QTOF micro mass spectrometer.

Mass data acquisition for HPLC chromatograms was performed on a Micromass QTOF micro. It is a bench top quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer that enables routine exact mass measurements in both MS and MS/MS modes.

With a resolution above 5000 FWHM (full width, half height), the system works with a mass measurement accuracy better than 5 ppm. To create this resolution, the system can be used with an automatic lockspray dual electrospray source in HPLC mode. This source enables automated exact mass measurement from a second sprayer, eliminating the need for T-plumbing and potential ionisation interferences between analytes and standard.

HPLC – Mass spectrometry

Ionisation was performed in the negative mode with the standard ion source under the following conditions:

Capillary voltage 2800 V, sample cone voltage 48 V, desolvation temperature 220 °C, source temperature 120 °C (Fig. 3).

Phosphoric acid was used for instrument calibration in a mass range from 100 to 2000 Da. The reference leucine/enkephaline was used for equipment tuning on the one hand and to generate a lockspray which guaranteed exact mass data acquisition on the other. Lockspray was performed by external signaling. Depending on the sample material and signal intensity a syringe rate of between 5.0 and 10 µL was used.

LC-DAD-MS tests included a reference scan frequency of 6.6 s for which a 5–10 µL/min volume flow was applied. The reference cone voltage was adjusted to 55.0 V. The chosen reference scan ratio was 6 to 1.

Mass spectrometry with Nanomate.

The quadrupole and TOF parameters were the same as in the HPLC-QTOF-MS mode. Only the source conditions were adapted to the soft ionisation of the Triversa Nanomate.

1.7 kV were applied to the electrospray nozzle and the sample cone voltage was 30 V. The source temperature was limited to 100 °C and the desolvation temperature was kept at ambient temperature. The calibration parameters were the same as in the normal electrospray mode.

Chip-based nano electrospray source and infusion.

The Triversa Nanomate is the latest achievement in chip-based technology by Advion (Advion Bioscience, Ithaca, NY) and combines the strength of LC, fraction collection and chip-based infusion in one integrated system. Samples or fractions in a 96 or 384 well plate were infused into the mass spectrometer by a nano chip with a nozzle diameter of 0.5 µm. This technique yields more reproducible, sensitive and less discriminated information than a HPLC run. [22]

MS/MS fragmentation.

The Q-Tof micro features Data Directed Analysis (DDA) for the discovery of precursor ions. The present software tool enables the instrument to perform DDA, switching from MS to MS/MS mode and then returning to MS mode using data-dependent criteria. The system allows for the possibility of sample measurement by online LC-MS/MS. This includes a precursor selection in real time. The system continuously records MS survey spectra throughout a chromatographic run and dynamically discovers candidates for full-product ion analysis. When a component of interest elutes, the m/z value is determined and the QTOF switches to transmit the discovered precursor m/z only. This is followed by the run of a collision energy profile fitting the targeted precursor m/z, and simultaneously MS/MS spectra are recorded. Afterwards the system returns to MS survey to trace the next component. Nevertheless, samples could also be analysed by a classical procedure which involves a sample analysis in the MS mode to identify precursor ions and a re-run of the sample material in the MS/MS mode to acquire MS/MS data from each of the precursor ions. Both methods were pursued when working with chill haze.

In the case of online LC-MS/MS, the fragmentation was created as continuum data with the standard ion source under the following conditions: capillary voltage 2.200 V, sample cone voltage 48 V, desolvation temperature 250 °C, source temperature 120 °C, mass range from 100 Da to 2.000 Da. The scan parameters were automatically adapted to the precursor ions.

Classical analyses performed with the Triversa Nanomate were adopted individually to single samples and their behaviour during the pre- and survey scans.

3 Results

A new method for the detection of polyphenols in wort, beer and isolated haze by HPLC-DAD-QTOF-MS and nano ESI chip-QTOF-MS has been established. Both analytical devices will be examined individually and in more detail because they have characteristic advantages as well as disadvantages and show differences concerning their selectivity and results.

In breweries, HPLC-MS analysis of polyphenols involve an examination of wort and beer samples as well as a pursuit of their appearance throughout the brewing process. As these methods offer a well-established basis for the investigation of polyphenols, we decided to adapt them to haze analyses

The Triversa Nanomate works in three modes: LC/MS, fraction collection and chip-based infusion. With this kind of sample delivery neither pre-scans nor single-ion mode analysis was done under internal calibration parameters because the Nanomate does not allow lockspray performance in parallel.

3.1 HPLC-DAD-QTOF-MS analysis of beer and wort

A determining characteristic of beer, being a natural product, is its high diversity. Liquid chromatograms of wort and beer provide an insight into the sample complexity, illustrated in the chromatograms in Figure 4. The high diversity makes a determination of single components difficult. To separate, concentrate and detect polyphenols, a sample pre-treatment and preparation based on polyamide solid phase extraction (PA-SPE) was used which led to adequate polyphenol enrichment and reproducibility.

The selection of an HPLC column was a second important fact to consider due to the polar character of polyphenols. Different stationary phases (Phenomenex: Aqua, Synergi Hydro RP, MAX RP and Gemini C18; Macherey-Nagel: Nucleodur C18 and Nucleosil C18) were tested. As the Synergi Hydro RP column showed the best peak separation during the whole chromatographic run, it was chosen for the analysis. The polar endcapping of the C18 column material provides a good retention of both polar and hydrophobic compounds and enhances the polar resolution in a 100 % aqueous mobile phase. These characteristics enabled the selective detection of polyphenols from all the liquid sample materials used.

During the first analyses, which were performed on a stand-alone HPLC system, variations in retention time occured due to variations in temperature and sample type (Table 1). HPLC data were irreproducible and this made difficult the realisation of MS and MS/MS coupling.

Acetic acid was added to the HPLC eluent to increase the yield of ions in the LC-MS experiments. Contrary to theory, an accumulation of phenolate or acetate, resulting in complex or interfering ion spectra [23], could not be observed. MS analyses were performed in the negative ion mode ($[M–H]^-$) since this kind of detection yielded more sensitive and selective data. The chemical structure (polar property) and the weak acidic character of polyphenols allow proton loss rather than proton addition. Mass spectrometry cannot be used to distinguish between isomers (identical elemental composition but different structures), like catechine and epicatechine or gallocatechine and epigallocatechine. Isomers can be chromatographically separated so that the combination HPLC-MS allows the analysis of isomers to be made. HPLC-MS turned out to be a powerful tool to gain additional information on polyphenolic isomers.

Standard substances of the monomers catechine, epicatechine and epigallocatechine were analysed. Based on these results, catechine, epicatechine and epigallocatechine could be unambiguously identified through their retention times in beer samples. Gallocatechin was not available as a standard substance, but based on the fragmentation behaviour of its isomer, epigallocatechine, it could also be identified. Dimeric and trimeric polyphenols could be distinguished by their predicted mass traces (calculated with the MassLynx software tool) extracted from HPLC-MS chromatograms, too. The decisive peaks in the extracted nominal mass traces could be recognised without difficulty and the appropriate chromatograms and MS spectra always showed identical shape when performing an overlay.

The established LC-MS method allowed us to follow single polyphenolic substances throughout the brewing process. The observed retention times varied slightly throughout the large number of samples since these could not be analysed continuously. The trend of catechine throughout the brewing process could be observed and it mirrored well-known facts: during mashing polyphenols are released from the barley grains. Thus their concentration increases slowly, whereas it rises strongly during finning and concentration of the wort until the maximum is achieved. Dilution with clean water leads to a strong decrease. Hot haze is formed during the boiling process and the wort can be separated before the cleared fluid is transferred to the fermentation tank. The brewing process results in the unfiltered product; on filtering, the second decrease of catechine is observed.

Retrospectively, the combination of polyamide (PA) solid phase extraction and the subsequent HPLC- DAD-QTOF-MS analysis was the appropriate technique to analyse the polyphenol content of wort and beer samples. Extracted chromatograms of a beer sample showed a broad differentiation and allocation of monomeric, dimeric and trimeric polyphenols (Fig. 5). In the case of monomeric and dimeric polyphenols, accurate masses could be estimated within a range of 5 ppm. Components of a molecular weight greater than 600 Da (e.g. trimeric polyphenols) showed variations up to 10 ppm (Table 2) because of their increasing masses and the resulting signal complexity which made precise determinations difficult.

Isomeric structures could be successfully separated. Due to the good resolution property of the selected column with a 100 % aqueous mobile phase, polyphenolic components eluted during the first third of the 90 min HPLC runs. Similar results were observed with wort samples. As the experience with beer and wort was promising, the same analytical conditions should be useful for the identification of chill haze components.

3.2 Preparation and analysis of isolated chill haze

Different batches of stored beer were prepared and chill haze was isolated and lyophilised. A lack of solubility after lyophilisation was the determining characteristic of chill haze. As a successful dissolution of it was the precondition for further analyses, a large solubility test series was performed. An ultrasonic bath treatment was always included to facilitate the dissolution. Alkanes (pentane, hexane, heptane) did not dissolve the haze, nor did pure water, buffered water (ammonium carbonate, pH 3), acetonitrile, buffered acetonitrile (ammonium carbonate, pH 3), esters (ethyl acetate, ethyl hexanoate) or diethyl ether. A gradually increased solubility could be observed from dimethylformamide (80 %) and methyl alcohol with ammonia (pH 10) to dimethylformamide

(100 %). The aprotic solvent dimethyl sulfoxide (high dielectric coefficient and high dipole moment) showed the best solubility results and was therefore used to dissolve lyophilised chill haze. The decisive chemical property which allows haze dissolution is unknown but HPLC-QTOF-MS chromatograms (see below) show that dissolution with DMSO is a successful method.

To draw a comparison between PA-extracted beer, PA-extracted wort and different chill haze samples, the latter were injected into the HPLC-QTOF-MS system under identical chromatographic conditions. Whereas the appearance of proanthocyanidines in chill haze was expected and predicted by several theories, the chromatograms did not show any polyphenolic substances or their fragments. Even small peaks would have been seen after a careful search in the relevant retention time window between 10 and 30 min. There was no evidence of polyphenol-related signals in chill haze HPLC-MS chromatograms. Amplified peaks only reflected background noise. The high mass accuracy and the technical possibilities of the MS system combined with the use of standard substances guaranteed valid results and left no room for doubt concerning low molecular and low polymeric (n = 2–3) polyphenols. Table 3 summarises relevant results and compares the composition of chill haze and PA-SPE treated wort and beer samples. An identification of monomeric, dimeric or trimeric polyphenols from chill haze was not possible. This, however, does not preclude the existence of flavon-3-ol in general. Higher organized complexes which may not be identified by this kind of technique are possible.

Instead of proanthocyanidins previously identified in beer and wort, the datasets of chill haze showed two characteristic peaks of unknown substances with m/z $[M–H]^-$ 549 and m/z $[M–H]^-$ 579 during the first 40 minutes in the chromatogram. These "unknown" substances were not found in PA-extracted samples. Further analysis of chill hazes (different storage, treatment, laboured or not) always showed the appearance of these two signals. Other masses found are m/z $[M–H]^-$ 353 from the isomeric hop components xanthohumol/ isoxanthohumol and an unknown component with the striking mass m/z $[M–H]^-$ 329. In contrast to the previously mentioned unknown components this one could also be found in PA-SPE treated samples.

Haze chromatograms also showed a high complexity but the peak assignment differed from beer chromatograms (Fig. 6). Keeping in mind that several other substances deriving from the sources hop and malt could have an influence on chill haze formation, we decided to analyse further potential triggering components. With the help of standard substances, we could identify α- and β-acids comprising cohumulone, adcohumulone, humulone, colupulone and adcolupulone and lupulone as characteristic chill haze constituents. Many peaks still remained unidentified, but their frequent appearance with sizeable count numbers makes a documentation of them necessary. Retention times, accurate masses and fragment peaks of all identified substances and the unknown substances are listed in Table 4. Altogether five unidentified substances (m/z $[M–H]^-$ 417, 431, 549, 579 and 761) belong to the most intense components causing chill haze formation.

The spectra of the major hop components of masses m/z $[M–H]^-$ 353, m/z $[M–H]^-$ 347, m/z $[M–H]^-$ 361, m/z $[M–vH]^-$ 339, m/z $[M–H]^-$ 399, m/z $[M–H]^-$ 413 and of the unknown substance ($[M–H]^-$ 579), are extracted from the total chromatogram (Fig. 6) and reproduced in Figure 7.

The comparison of PA treated samples and chill haze revealed that an identification of hop α- and β-acids, as well as some of the unknown substances from beer and wort was not possible. Judging from their chemical properties, it is not expected that these components would partition into the PA-SPE material and thus they are lost during sample preparation.

In conclusion it is safe to say that the sample preparation involving PA extraction is unsuitable for the analysis of the wide range of relevant chill haze substances in beer.

3.3 Sample preparation of beer and wort by C18-solid phase extraction

To avoid the described loss of compounds, the sample pre-treatment was changed. The poor retention of hop α- and β-acids and prenylflavanoids on the PA solid phase required an octadecyl-modified silica solid phase extraction. Pre-treatment was again performed with beer and wort samples and subsequently analysed under the same chromatographic conditions as above. In Figure 8 the PA-SPE beer sample is compared with a C18-SPE treated beer and a chill haze sample and clearly shows major differences in sample composition depending on the sample preparation method.

The chromatography of the C18 sample shows all substances with a higher polarity and was developed to reveal compounds like phenols, pharmaceuticals or phenoxycarboxylic acids. The stationary phase of the chosen HPLC Synergi Hydro RP column enhanced the retention of polar compounds e.g. polyphenols and allowed their separation, even when they differ only slightly in hydrophobicity. This resulted in compounds eluting with a mobile phase of a higher percentage of organic solvent. They are observable in a more complex signal cluster at the end of the C18 chromatograms. Again the complexity of beer samples remains considerable. An added advantage of the higher percentage of organic solvent is the better MS sensitivity compared to aqueous eluates.

Chill haze analysis showed longer retention times for substances derived from hops and so did C18 SPE treated samples. Prenylflavanoids eluted with about 40 % organic solvent at a retention time of ca 48 minutes and the α- and β-acids left the column during the last 10 minutes of the ninety-minute HPLC run.

Single mass traces of xanthohumol/isoxanthohumol ($[M–H]^-$ 353) and the β-acids, lupulone/adlupulone ($[M–H]^-$ 413) and colupulone ($[M–H]^-$ 399) are illustrated in Figure 9. The results of all performed sample preparations are compared in Table 4. The two unknown masses which could not be identified in PA-SPE treated samples were found in C18-SPE treated samples too but are still not identified. Whereas MS/MS fragmentation experiments performed with their monoisotopic mass traces of m/z $[M–H]^-$ 549 and m/z $[M–H]^-$ 579 hinted at similar chemical structures, they did not reveal a polyphenolic background.

Monomeric, dimeric or trimeric flavan-3-ols could not be seen. As mentioned before, haze samples were not anticipated to show such compounds but they were expected from the liquid samples because of their specific C18-pretreatment.

The results in Table 4 make clear that the C18-SPE extraction showed a better agreement with chill haze with respect to the detection of hop-derived substances.

The question remains whether flavan-3-ols can really be regarded as important chill haze relevant substances. Both methods showed that there was no relationship between these polyphenols and beer turbidity. The results described until now rather suggest an involvement of hop-derived components, including prenylflavanoids, α- and β-acids as well as additional unknown substances. Whereas the substances could be identified by LC-MS experiments, MS/MS structure elucidation was not possible because of the low

concentrations of the substances. Therefore, a different analytical method had to be applied to obtain further information on isolated chill haze (Fig. 10).

We decided to go deeper into the analysis using a nano ESI-chip application in form of a Nanomate system. In contrast to LC-MS, ESI-QTOF-MS allowed a stable spraying which resulted in a higher reproducibility. In addition, the performance of MS/MS experiments was possible because the technique yields chromatograms with sufficient peak count numbers despite the low concentration in the sample material.

3.4 Quantitative analyses of isolated chill haze by LC-ESI-QTOF-MS and LC-PDA

The quantitative analyses of xanthohumol, isoxanthohumol and the α- and β-acids of hops demanded the preparation of standard calibration curves. For this purpose an EBC- and an ICE 2 standard calibration extract were chosen which contain a defined mass of the single substances. Analysis and evaluation were performed based on the UV traces (xanthohumol: m/z = 353, UV = 370 nm; isoxanthohumol: m/z = 353, UV = 290 nm; humulone/adhumulone: m/z = 361, UV = 320 nm; cohumulone: m/z =347, UV = 320 nm; lupulone/adlupulone: m/z = 413, UV = 340 nm and colupulone: m/z = 399, UV = 340 nm). In each case a linear calibration was successful and R^2 (correlation coefficient) ranged from 0.993 to 0.999. Chill haze was isolated from 1 L of a standard beer through storage for 15 months at 0 °C. In addition, differerently treated beers (stabilisation dosage, KZE-treatment, hop typ, hop amount) were prepared. In case of the standard beer, 5.2 mg chill haze could be isolated. The other beers showed amounts of 1 up to 10 mg/L chill haze. In the following only the standard beer will be reviewed extensively whereas the other test beers will be examined in a following publication. 1.11 ppm isoxanthohumol and 0.24 ppm xanthohumol could be determined. Isoxanthohumol was the most abundant component. The concentration of the α-acids humulone/adhumulone was 0.4 ppm and 0.04 ppm was found for cohumulone. The β-acids appeared as minor components with an lupulone/adlupulone amount of 0.06 ppm and colupulone amount of 0.08 ppm.

3.5 Analysis of isolated chill haze and EBC hop standard by nano ESI-QTOF-MS and MS/MS

An EBC hop standard was analysed to reveal characteristic fragment ion series of hop components. MS survey scan spectra and the corresponding MS/MS fragment ion spectra are exemplarily illustrated in Figure 11. The survey spectrum shows the relevant precursor ions of the four hop substances already mentioned (see Section 3). The BPI count ranged from 1500 in the lowest peak to 6000 in the highest (12 a).The MS/MS spectra showed typical fragment ion patterns (12 b) which occured due to elimination reactions from the basic molecular structures.

Chill haze samples, redissolved in DMSO, were cleaned up and desalted for further MS/MS experiments using the ZipTip technique. Then the samples were diluted with methanol to support ionisation in the chip-based nano electrospray source. The nano ESI chip-QTOF-MS allowed an efficient ionisation and detection of major and minor compounds and demonstrated a stable as well as constant spray. The most abundant ions were submitted to an MS/MS investigation.

A complete set of fragment ions of high signal-to-noise ratio could be obtained after a few minutes of acquisition. For ions of low abundance the spray stability under the optimized MS conditions allowed a longer acquisition time so that enough fragment ions could be collected for a reliable assignment of the molecular structure. Table 5 shows all MS/MS fragments of assumed hop substances in the EBC hop standard and chill haze which could be identified under defined nano ESI chip-QTOF-MS conditions with a fair signal-to-noise ratio.

The MS/MS spectra showed reproducible results. In case of the hop bitter acid humulone/adhumulone (Fig. 12) single α-, β- and allyl cleavages (loss of 57, 43 and 69 Da fragments, resp.) from the basic molecule led to fragment ions of m/z 303, 317 and 291. In addition, combinations of the different cleavages, e.g. allyl- and β-cleavage or double allyl cleavage, occured, causing signals at m/z 248 and 220, resp. With the help of the MassLynx software the signals could be allocated to hop α- and β-acids.

Similar results were obtained with haze prepared from Pilsener. Survey scans also showed the important precursor ions belonging to hop components. Fragmentation patterns with identical masses could be found. The results obtained with the EBC hop standard together with the software tools helped identify the α- and β-acids (Fig. 13).

Nano ESI chip-QTOF-MS analysis of isolated chill haze focused on the substance class of hop components and on prenylflavanoids and polyphenols. Prenyflavanoids undergo a cleavage called Retro-Diels-Alder reaction. The mechanism is illustrated schematically using isoxanthohumol (Fig. 14) whose six-membered ring is cleaved into a diene (233 Da fragment) and a substituted alkene (120 Da), also known as a dienophile. This reaction is typical of polymeric polyphenols and is expected to occur during soft ionization in pre-scans as well as in single-ion mode (SIM) monitoring.

While prenylflavanoids could be easily identified, contrary to expectations the detection of flavan-3-ols or polymeric polyphenols by nano ESI chip-QTOF-MS was unsuccessful and they could not be identified in beer haze. The conclusion is that no relationship between polyphenols and beer turbidity may exist.

To further substantiate these results and to study their precision, 10 differently treated beers and 6 branded beers were stored to generate haze. Different brewing pre-treatments included reduced PVPP stabilisation, labouring and KZE treatment, while the beers were stored at ambient temperature or at 0 °C. Once the beer samples were stored long enough, the haze and the chill haze were prepared, lyophilised and the dissolved samples (diluted in DMSO) were analysed.

In no samples could flavan-3-ols or polymeric polyphenols deriving from them be identified. On the other hand, the prenylflavanoids xanthohumol and isoxanthohumol, the α-acids humulone/adhumulone and cohumulone and the β-acids lupulone/adlupulone and colupulone were verified. In addition, the "unknown" but conspicuously dominant component signals could also be seen. These unknown substances are not found in the EBC hop standard but this does not bar hop as its source nor a polyphenolic character in general.

To relate the analytical data to technological parameters, some samples were stored at 0 °C while the others rested at ambient temperature. There was a different turbidity performance during storage dependent on the temperature (chill-stored beer showed a faster turbidity formation) but no distinction was possible based on their worked-up turbidity composition.

Another interesting experiment reveals the comparison of KZE-treated beer and less extensively PVPP stabilised beer to beer brewed under standard conditions. A short time heating (KZE)

is used to advance the stability during storage. PVPP is normally used to attain a high physical stability by partially removing the polyphenols from beer via adsorption [11, 13, 18–20]. As a change of only two out of many brewing parameters can only define some of the variations that can happen during the brewing process, an exact examination of further variables will be examined in a following publication. In addition a larger sample number will be observed to achieve a statistical significance.

Heat treatment (KZE) during the brewing process leads to a product of higher stability during cold storage with a greatly reduced haze production. KZE is one of the possibilities to exert a direct influence on the product´s shelf life. An explanation for this may be the β-glucosidase denaturation during boiling.

The results summarised and all the experiments described here cast suspicion on previous theoretical approaches. Polyphenols are often cited as chill haze inducing substances but this study could not support that view. It is important to differentiate between the polyphenols investigated (monomeric, dimeric and trimeric flavan-3-ols) and the higher ones. Potentially it is possible that polyphenols are disguised as members of larger entities (proanthocyanidins) which cannot be recognised by this kind of analysis. However, as a consequence of naturally occurring fragmentation via the "Retro-Diels-Alder" mechanism [see ref. 23, 25 for the detailed mechanism], the typical fragmentation pattern of these higher polyphenols should be visible in MS experiments. Depending on which fragment carries the negative charge after fragmentation, typical fragment ion signals should appear. There is disagreement on where such charges are located in dimeric and polymeric flavanols [25]. In addition to this fragmentation, the literature also refers to a disruption of inter-flavanoid linkages. Throughout the whole test series, no adequate fragmentation spectra could be observed that would hint at the presence of these higher polyphenols. The existence of such species, however, could not be ruled out completely because characteristic signals could potentially be hidden by other signals in the complex spectra. The positive identification of hop components gives a hint to the influence of hop components and/or other high molecular species resulting in haze formation.

The astonishing impact of hop components and therefore the prenylflavanoids xanthohumol and isoxanthohumol illustrates another aspect that the food industry may pay regard to. The physiological influence of these substances include anti-carcinogenic and anti-oxidative properties [13, 14, 15]. This should be kept in mind when thinking of a prevention or attenuation of haze formation in the end product by the use of carbon dioxide extracted hops, polyphenol-free extract hops or anthocyanogen-free barley.

4 Conclusions and perspectives

Investigations of wort, beer and especially beer turbidity have been carried out by HPLC-DAD-QTOF-MS and nano ESI chip-QTOF-MS for the detection of their polyphenolic composition. It is shown that in the case of beer and wort, sample pre-treatment with PA-SPE makes it possible to detect polyphenolic components, whereas a work-up involving C18-SPE reveals hop components such as prenylflavanoids as well as α- and β-acids. A work-up procedure for beer turbidity was successfully established. In case of beer turbidity neither of the detection techniques gave a hint of polyphenols nor a hint of a relationship between polyphenols and beer haze. Neither monomeric polyphenols like catechine or epicatechine nor their polymeric derivatives like procyanidin B_3 or prodelphinidin B_3 could be detected, even though they are classically mentioned as haze causing constituents. Hop prenylflavanoids and α- and β-acids, on the other hand, were successfully identified. Indeed the quantification of these hop substances hinted at their occurrence as minor components in chill haze, and their astonishing significance and influence on chill haze formation cannot be excluded and will be highlighted in a later publication. Changes in brewing parameters, hop type, polyphenol or α- and β-acid content mean that many influencing parameters have to be investigated. Both polyphenol-protein interactions as well as protein identifications in isolated chill haze offer a huge field of research in their own right.

However, the results of this study have led us to think critically about the use of PVPP in brewery processes. The beer turbidity composition did not reveal any influence of the PVPP dosage on the polyphenol content or give a relationship between beer turbidity and polyphenols. Therefore the question arises if a highly dosed PVPP filtration results in a more efficient removal of polyphenols. Is it really necessary to perform such an operation with the intent to achieve a better haze stability since appreciable costs are directly connected to PVPP dosage?

5 References

1. Bamforth, C.W.: (1999) "Beer Haze"; J. Am. Soc. Brew.Chem. **57** (3); 81-90.
2. Batchvarov, V. and Chapon, L.: (1985) "Vorausbestimmung der kolloidalen Bierhaltbarkeit (Teil 1)"; Monatsschrift für Brauwissenschaft. **38** (8); 331-342.
3. O´Rourke, T.: (2002) "Colliodal stabilisation of beer"; The Brewer international – Technical Summary January 2002.
4. Asano, K. et al.: (1982) "Characterization of Haze-Forming Proteins of Beer and Their Role in Chill Haze Formation"; J. Am. Soc. Brew. Chem. **40** (4); 147-154.
5. Siebert, K. J., et al.: (1996) "Nature of polyphenol-protein interactions"; J. Agric. Food. Chem. **44**; 80-85.
6. Siebert, K. J., et al.: (2003) „Effects of Alcohol and pH on Protein-Polyphenol Haze Intensity and Particle Size"; J. Am. Soc. Brew. Chem. **61** (2); 88-98.
7. Siebert, K. J., et al.: (1997) "Effects of Protein-Polyphenol Ratio on the Size of Haze Particles"; J. Am. Soc. Brew. Chem. **58** (3); 117-123.
8. Siebert, K. J., et al.: (1997) "Mechanism of Beer Colloidal Stabilisation"; J. Am. Soc. Brew. Chem. **55** (2); 73-78.
9. Robinson, L.H., et al.: (2005) "Improving beer haze stability by investigating the interaction between malt protein quality and brewing conditions"; 30th EBC-Congress Prag; Poster No.64.
10. Mikyska, A., et al.: (2002) "The role of Malt and Hop Polyphenols in Beer Quality, Flavour and Haze Stability"; J. Inst. Brew. **108** (1); 78-85.
11. Kusche, M.: (2005) „Kolloidale Trübungen in untergärigen Bieren – Entstehung, Vorhersage und Stabilisierungsmaßnahmen"; Dissertation an der Universität München.
12. Harborne, J. B.: (1994) "The flavanoids – Advances in Research since 1986"; Chapman&Hall.
13. Narziß, L. and Back, W.: (2005) „Abriss der Bierbrauerei"; WILEY-VCH, 7. aktualisierte und erweiterte Auflage.
14. Bellmer, H.G., Galensa, R. and Gromus, J.: (1995) „Bedeutung der Polyphenole für die Bierherstellung – Teil 1"; Brauwelt **135** (28/29); 1372-1379.
15. Bellmer, H.G., Galensa, R. and Gromus, J.: (1995) „Bedeutung der Polyphenole für die Bierherstellung – Teil 2"; Brauwelt **135** (30); 1477-1483.

16. Roeder, A., Lam, T.M.L. and Galensa, R.: (1995) „Thermospray-LCMS-Untersuchungen über Proanthocyanidine und andere Polyphenole im Malz, Bier und Hopfen"; Monatsschrift für Brauwissenschaft **48** (11/12); 390-396.

17. Whittle, N., Eldridge, H. and Bartley, J.: (1999) "Identification of the Polyphenols in Barley and Beer by HPLC/MS and HPLC/Electrochemical Detection"; J. Inst. Brew. **105** (2); 89-99.

18. Leiper, K.A., et al.: (2005) "Optimising Beer Stabilisation by the Selective Removal of Tannoids and Sensitive Proteins"; J. Inst. Brew. **111** (2); 118-127.

19. Mitchell, A.E., et al.: (2005) "A Comparison of Polyvinylpolypyrollidone (PVPP), Silica Xerogel and a Plyvinylpyrollidone (PVP)-Silica Co-Product for Their Ability to Remove Poyphenols from Beer"; J. Inst. Brew. **111** (1); 20-25.

20. Peterson, S.M.: (2003) "Filtration and use of Silica Hydro Gel and Polyvinylpolypyrrolidone for Removal of Haze-Active Proteins and Polyphenols in Beer"; Thesis for Master of Science Degree, Graduate School University of Wisconsin-Stout.

21. Whittle, N., Eldridge, H. and Bartley, J.: (1999) "Identification of the Polyphenols in Barley and Beer by HPLC/MS and HPLC/Electrochemical Detection" J. Inst. Brew. **105** (2); 89-99.

22. Firmenhompage von Advion: www.advion.com

23. Friedrich, W.: (2000) „Proanthocyanidine in den Braurohstoffen Gerste (Hordoum vulgare L.) und Gerstenmalz: HPLC-Bestimmung mit UV- und Elektrodenarray-Detektion"; Dissertation an der Rheinischen Friedrich-Wilhelms Universität Bonn.

23. Forster, A.: (1998) „Hopfen – mehr als nur ein α-Säurenträger"; www.nateco2.de

24. Wollersen, H.: (2004) „Bestimmung und Identifizierung von Flavonoiden in Gerste mit HPLC-DAD-MS/MS"; Dissertation an der Fakultät für Naturwissenschaften der Universität Paderborn.

25. Hausen, T.: (2000) „Bedeutung phenolischer Substanzen für die Stabilität von Bier"; Diplomarbeit an der Rheinischen Friedrich-Wilhelms Universität Bonn.

26. Pfenninger, H. :(Hrs.) (1993) „Brautechnische Analysemethoden Band 2"; Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK).

Received 18 January, 2008, accepted 10 March, 2008

Appendix

Table 1 **Retention times of substances identified in wort, beer and chill haze. (Chromatographic conditions; see experimental)**

substances	abbreviation	retention time [min] (+/– Δt)
catechine [a)]	C	13.40 (+/– 1)
epicatechine [a)]	C	17.70 (+/– 1)
gallocatechine	G	6.83 (+/– 1)
epigallocatechine [a)]	G	13.45 (+/– 1)
polymeric	CC	12.443 (+/– 1)
	GC	21.07 (+/- 1.5)
polyphenols	CG	21.07 (+/– 1.5)
	GG	18.73 (+/- 1)
	CCC	13.37 (+/– 1)
(e.g.	CCG	10.66 (+/- 1.5)
prodelphinidine,	CGG	10.69 (+/– 1)
procyanidine)	GGG	29.77 (+/- 1)
xanthohumole [b)]	xan	47.82 (+/– 1.5)
isoxanthohumole [b)]	isoxan	68.00 (+/– 1)
humulone [c)]	hum	78.55 (+/– 1)
adhumulone [c)]	adhum	77.84 (+/– 0.5)
cohumulone [c)]	cohum	76.03 (+/– 1)
lupulone [c)]	lup	82.20 (+/– 0.5)
adlupulone [c)]	adlup	82.69 (+/– 1)
colupulone [c)]	colup	81.41 (+/– 0.5)

a) single standard substance (ROTH)
b) EBC standard = xanthohumol enriched standard (NATECO2)
c) ICE 2 standard = international calibration extract (Labor Veritas)

Table 2 **Comparison of polyamide-extracted beer- and wort-derived substances and those from isolated chill haze (+ : found, – : not found)**

substance	monoisotopic mass $[M-H]^-$	mass acc. [ppm]	PA-SPE	chill haze
catechine	289.0712	0.3	+	–
epicatechine	289.0712	4.8	+	–
gallocatechine	305.0661	2.0	+	–
epigallocatechine	305.0661	0.0	+	–
proanthocyanidine		5.0		–
dimere,	577.1346			
	609.1244	3.3	+	–
procyanidine,	593.1295		+	
	593.1295	1.5	+	–
prodelphinidine,			+	
		1.5		–
		3.1		–
	865.1980	3.1	+	
	881.1929		+	–
proantho trimere	897.1878	6.2	+	
	913.1827		+	
		8.0		–
xanthohumole	353.1389	1.4	+	+
isoxanthohumole	353.1389	1.8	+	+
unknown	549.501	5.0	–	+
unknown	579.520	5.0	–	+
unknown	339.271	5.0	+	+

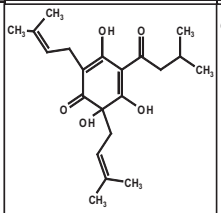
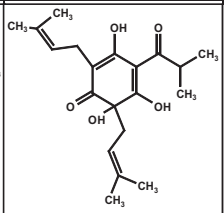
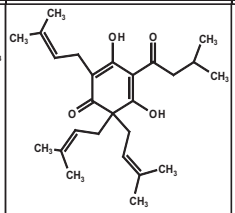
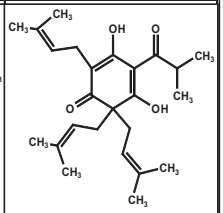
Table 3 **General overview of characteristic haze peaks. Peaks of identified substances and the most intense peaks of unidentified substances (highest TIC) are bold**

ret.-time [min]	main peak $[M-H]^-$	charge state $[M-nH]^{-n}$	acc. mass $[M-H]^-$	fragment peaks $[M-H]^-$	substance
1,6	761	1	761.2146	599,761, 923,1085, 1247,1409 1571	
19,8	595	1	595.3010		
20,6	595	1	595.3054		
21,6	579	1	549.2989	423	
21,9	549	1	579.3084	393	
22,4	609	1	609.3202		
26,7	463	1	463.0964	301	
28,2	233	1	233.0801	263	
29,4	447	1	233.0761	285	
37,0	233	1	233.0761	263	
43,4	329	1	329.0723	329,314, 299	
43,8	329	1	329.0733	517,495, 329,314	
43,9	608	1	608.4515	652,608	
44,3	329	1	329.0723	547,525, 329,314	
44,9	845	1	845.2876	867,845	
45,2	329	1	329.0723	517,495, 329,314	
45,8	329	1	329.0723	547,525, 329,314	
49,8	**353**	1	353.1436	729,451, 391,375, 353,328, 233,119	isoxan/ xan
57,8	1272	3	3817.2		
58,1	339	1	339.1271	339,313, 219	
62,1	1343	3	4031.3		
63,8	339	1	339.1317	477,339, 313,219	
65,7	299	1	299.1680	299,277	
68,0	**353**	1	353.1437	353,295, 233,119	xan
76,9	**347**	1		278,235	cohum
77,5	299	1	299.1680		
78,5	**361**	1		292,249, 221	adhum/ hum
79,7	**417**	1	417.2692		
80,8	**431**	1	431.2857		
81,9	**399**	1	399.2576	287	colup
82,7	**413**	1	413.2733	301	ad/lup

Table 4 **Comparison between PA-SPE and C18-SPE beer-derived substances and those isolated from chill haze (+ : found, - : not found)**

substance	monoisotopic mass $[M-H]^-$	PA-SPE	C18-SPE	chill haze
catechine	289.0712	+	–	–
epicatechine	289.0712	+	–	–
gallocatechine	305.0661	+	–	–
epigallo-catechine	305.0661	+	–	–
				–
			–	
	577.1346	+	–	–
procyanidine,	609.1244	+		
prodelphinidine,	593.1295	+	–	–
proantho dimere	593.1295	+		
			–	
				–
				–
			–	
	865.1980	+	–	–
proantho trimere	881.1924	+		–
	897.1878	+	–	
	913.1827	+		
			–	
				–
xanthohumole	353.1389	+	+	+
isoxanthohumole	353.1389	+	+	+
humulone	361.2015	–	+	+
adhumulone	361.2015	–	+	+
cohumulone	347.1858	–	+	+
lupulone	413.2692	–	+	+
adlupulone	413.2692	–	+	+
colupulone	399.2554	–	+	+
unknown	549.501	–	+	+
unknown	579.520	–	+	+
unknown	339.271	+	+	+

Table 5 **Comparison between MS/MS fragmentation from beer haze and hop standard α- and β-acids including their chemical structures**

	substances	Humolone /Ad	Cohumulone	Lupulone/ Ad	Colupulone
	formula	$C_{21}H_{30}O_5$	$C_{20}H_{28}O_5$	$C_{26}H_{38}O_4$	$C_{25}H_{36}O_4$
	molecular mass	362.47	348.44	414.59	400.56
sample description	structure				
EBC-hops-standard	m/z $[M-H]^-$	360.8	346.8	412.9	398.9
	MS/MS-fragmentation	291.9/ 248.8/ 220.9	277.9	300.9	286.9
chill haze	m/z $[M-H]^-$	360.9	346.9	412.9	398.9
	MS/MS-fragmentation	291.9/ 248.8/ 220.9	277.9/ 234.8	300.9	286.9

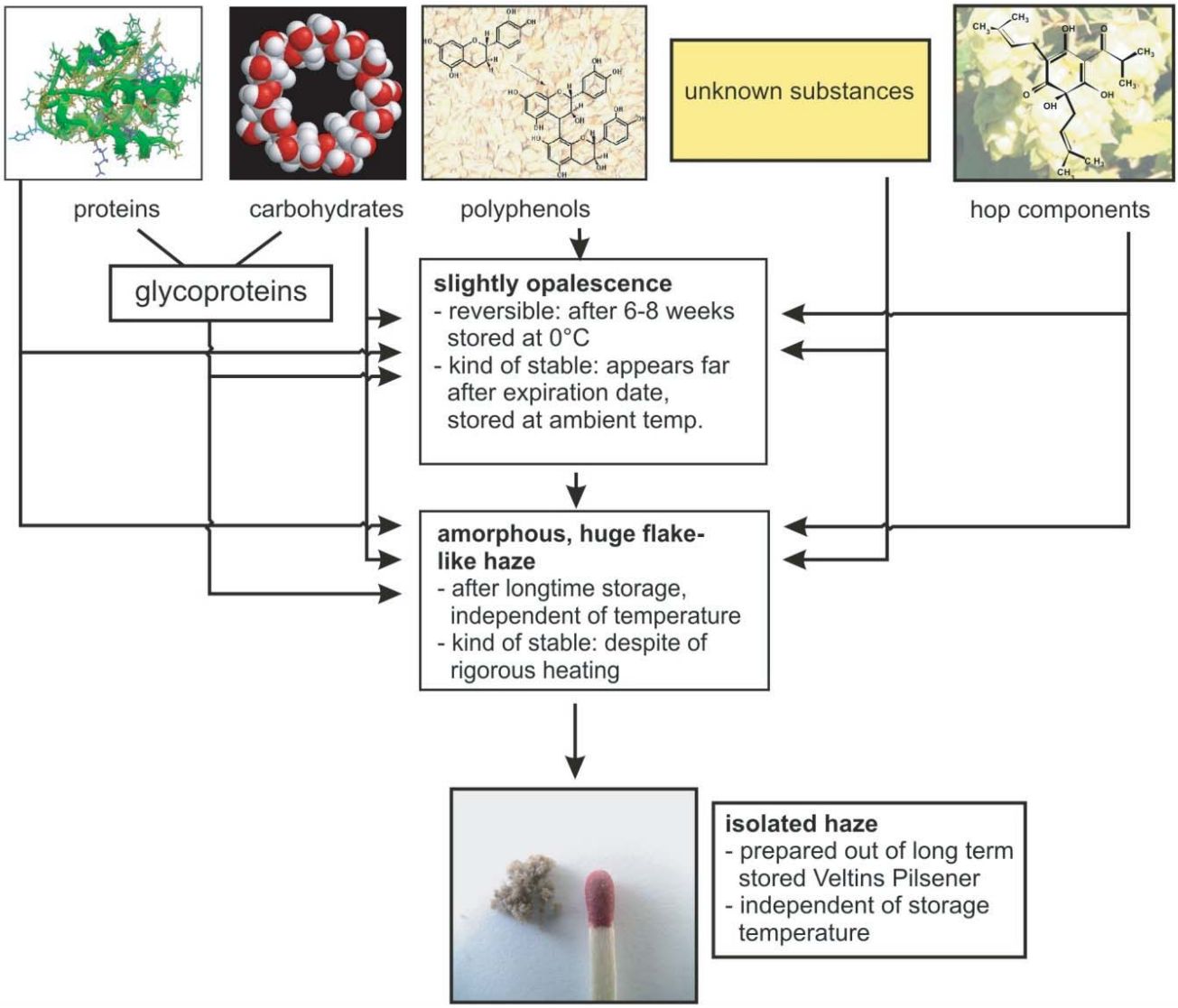


Figure 1 **Model of possible HA ingredients and their effect on the formation of chill haze**

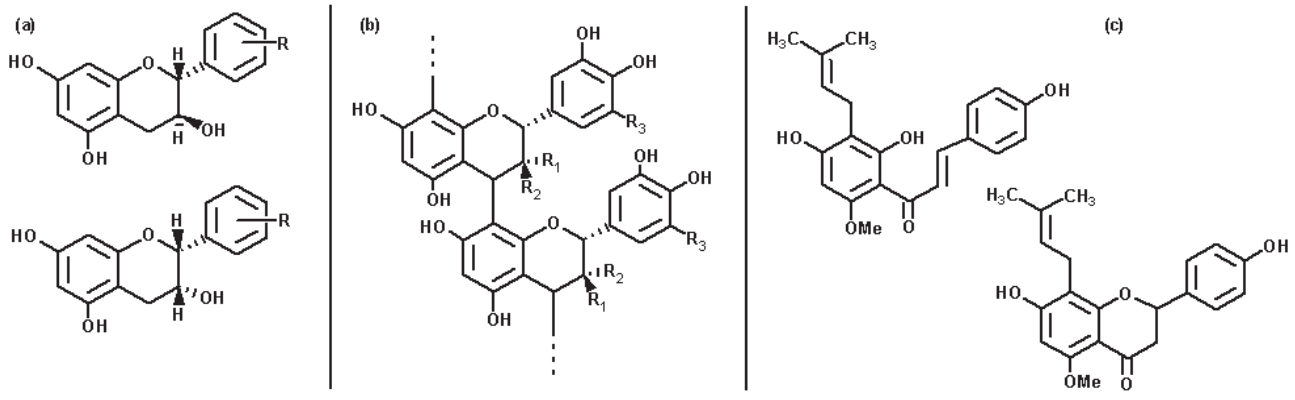


Figure 2 **Chemical structures of polyphenols (designed by ChemSketch). a) flavan-3-ols: (+)-(gallo)catechine (upper structure) and (–)-epi-(gallo)catechine, b) procyanidine where R_3=H: catechin (R_1=H, R_2=OH) and epicatechin (R_1=OH, R_2=H); prodelphinidine where R_3=OH: gallocatechin (R_1=H, R_2=OH) and epigallocatechin (R_1=OH, R_2=H), c) prenylflavanoids: xanthohumol and isoxanthohumol**

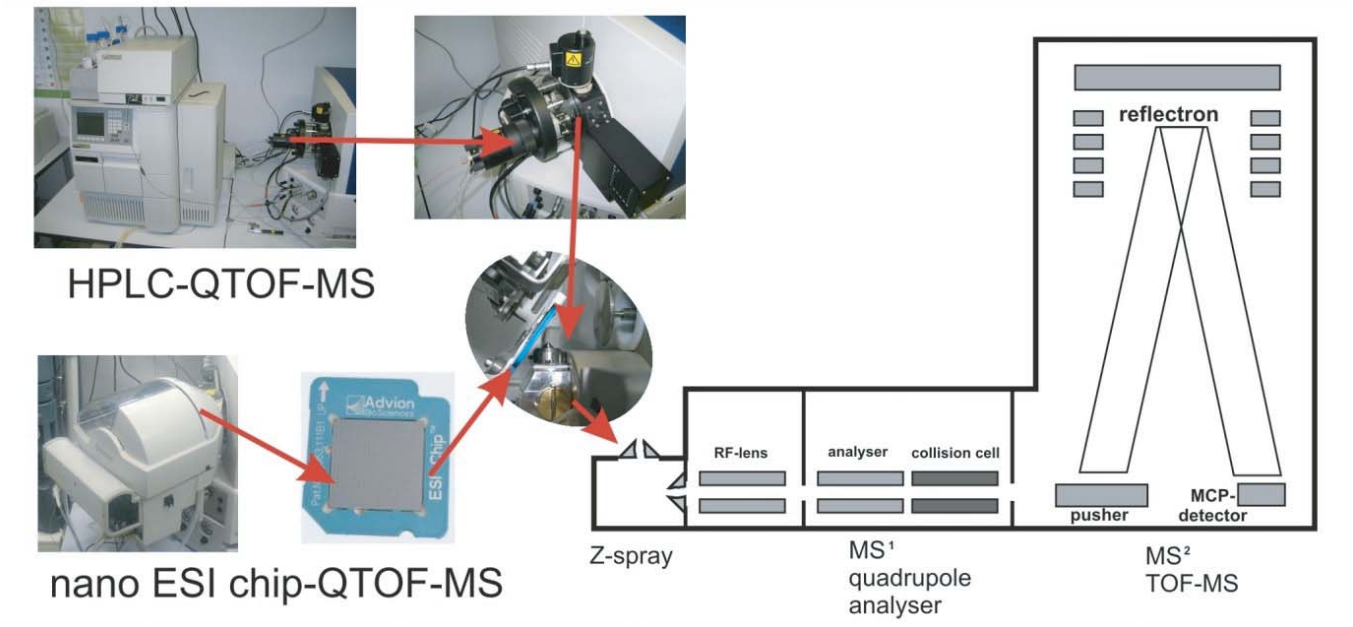


Figure 3 **Construction of the HPLC-DAD-QTOF-MS and the nano ESI chip-QTOF-MS used to analyse polyphenol standards and polyphenols in chill haze, beer and wort samples. LC/MS coupling produces a microspray whereas the high flow chip of the Nanomate application system enables a static and stable nanospray**

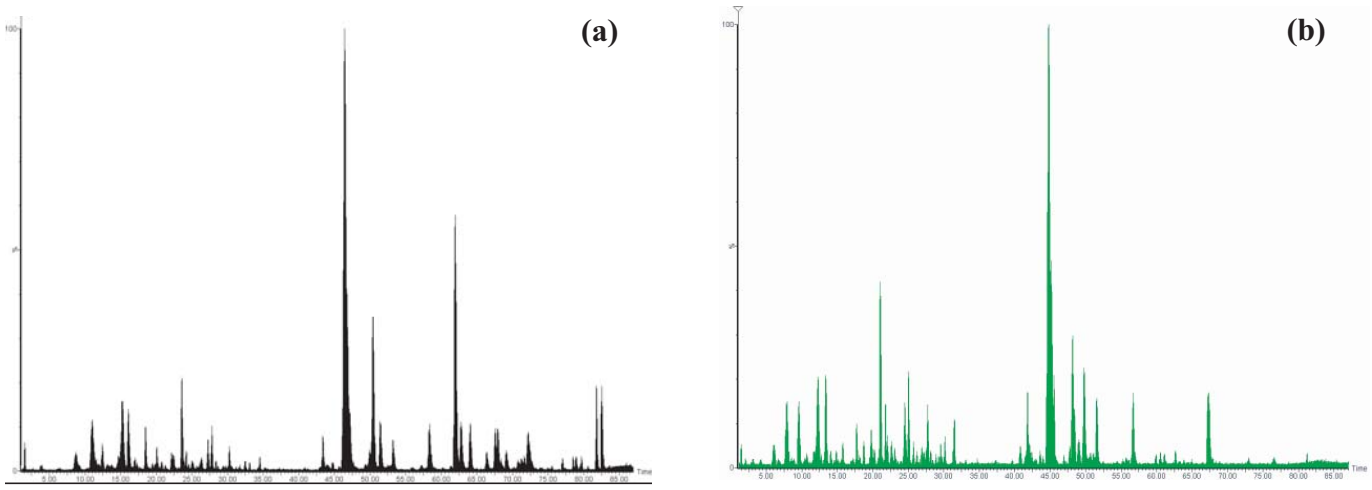


Figure 4 **HPLC-MS-chromatograms of a wort (a) and a beer sample (b). Pre-treated by PA-SPE**

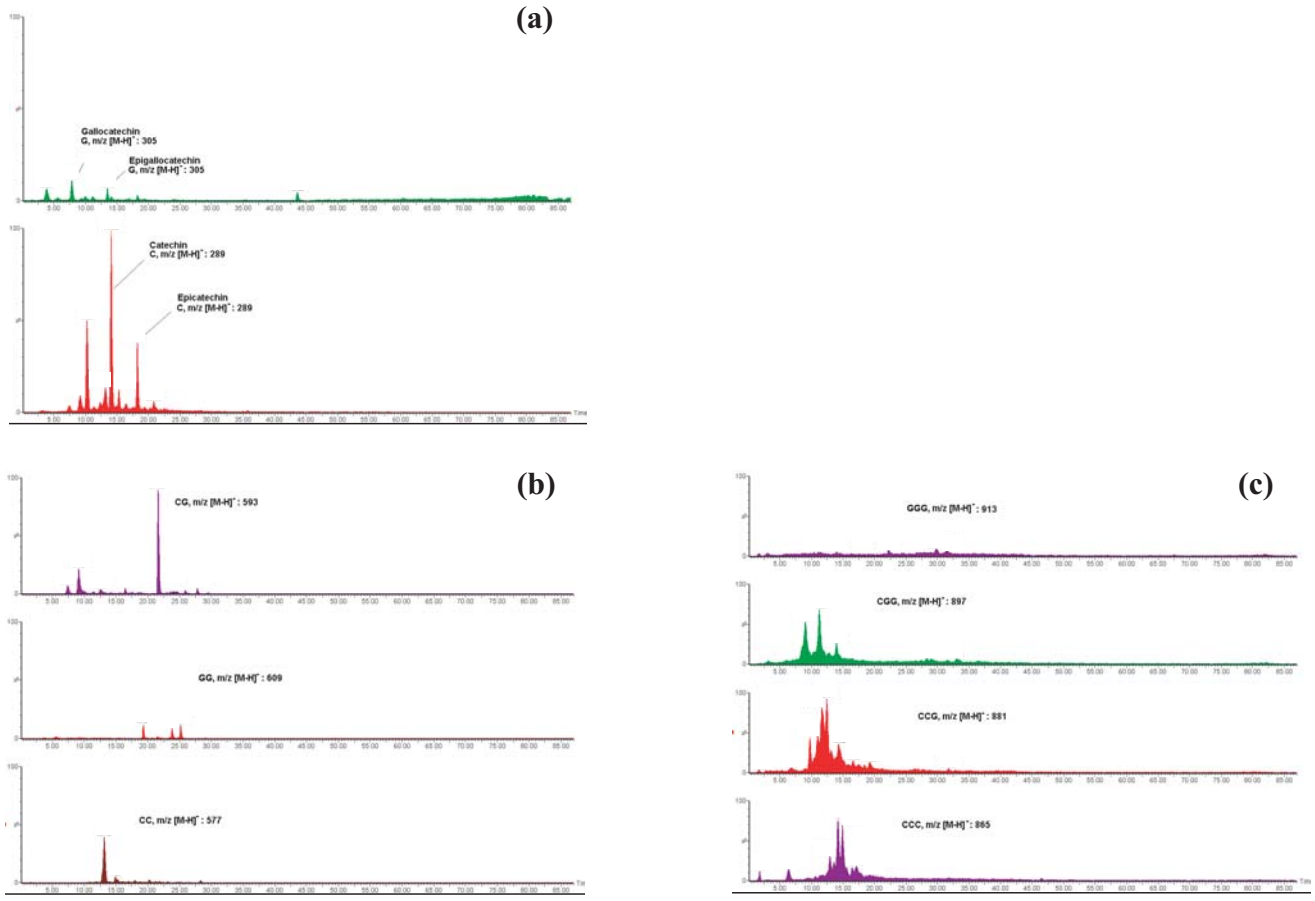


Figure 5 Extracted $[M–H]^-$ HPLC-MS chromatograms of polyphenols isolated from standard beer samples. Mass traces show the isomeric monomers catechine/epicatechine and gallocatechine/epigallocatechine (a), dimers (b) and trimers (c). Components are labelled by their nominal masses and standard abbreviations

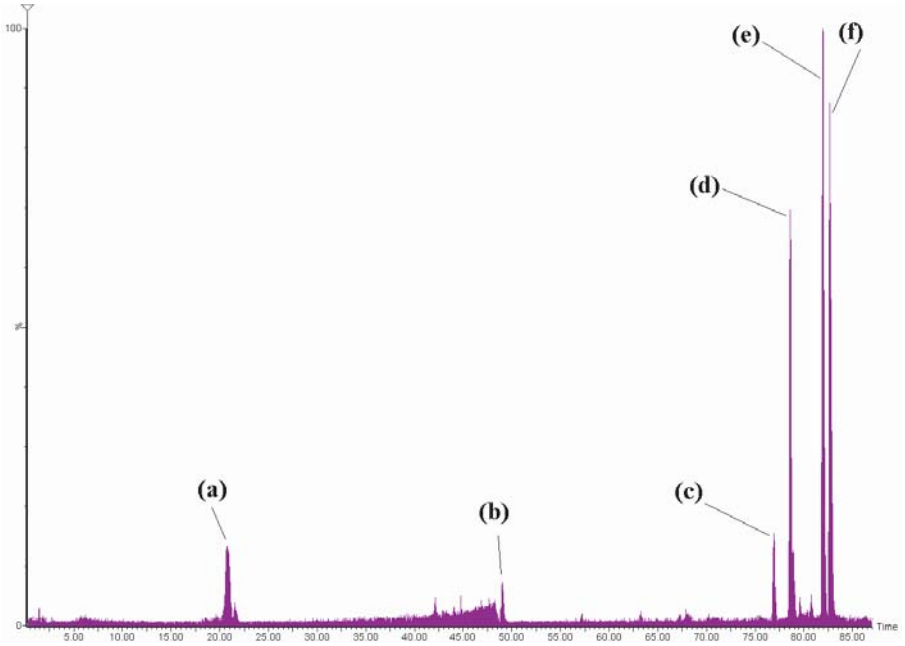


Figure 6 BPI-chromatogram of prepared haze of a beer dissolved in DMSO. Single specific peaks are amplified in Figure 7

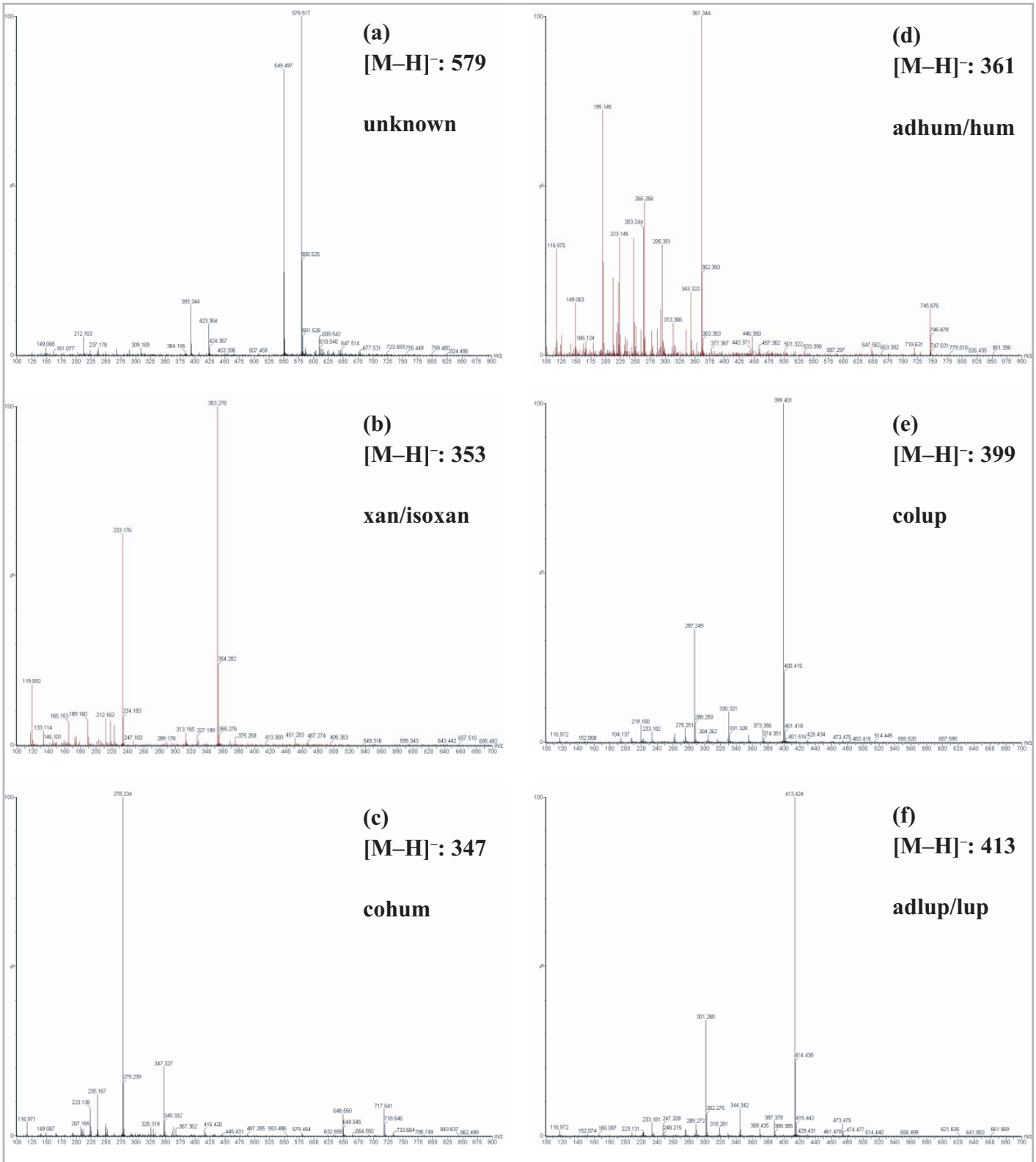


Figure 7 **Spectra of single haze components zoomed out of an overview chromatogram of beer derived chill haze (Fig. 6). Characteristic peaks are pictured: (a) one of the unknown substances, (b) xanthohumol/isoxanthohumol, the α-acids cohumulone (c) and humulone/adhumulone (d) and the β-acids colupulone (e) and lupulone/adlupulone (f)**

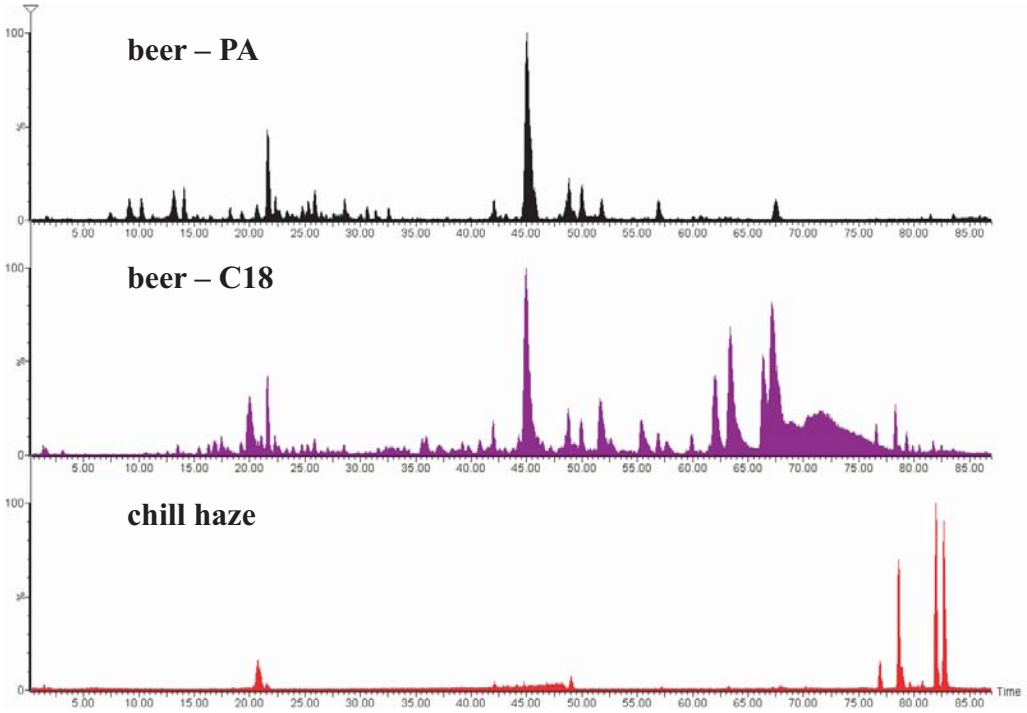


Figure 8 Comparison of a PA-SPE and a C18-SPE prepared standard beer sample with chill haze isolated from standard beer stored at 0 °C

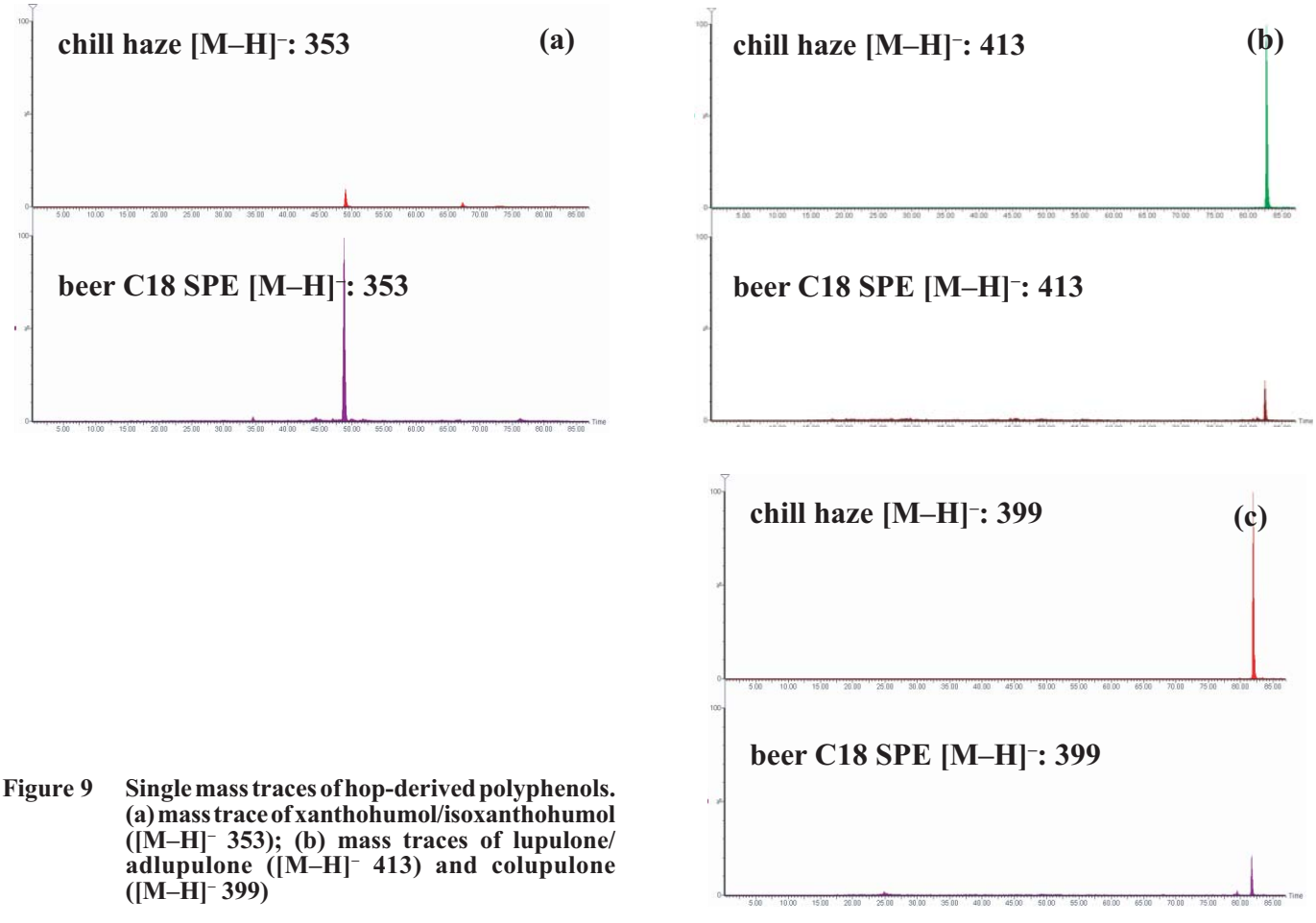


Figure 9 Single mass traces of hop-derived polyphenols. (a) mass trace of xanthohumol/isoxanthohumol ([M–H]⁻ 353); (b) mass traces of lupulone/adlupulone ([M–H]⁻ 413) and colupulone ([M–H]⁻ 399)

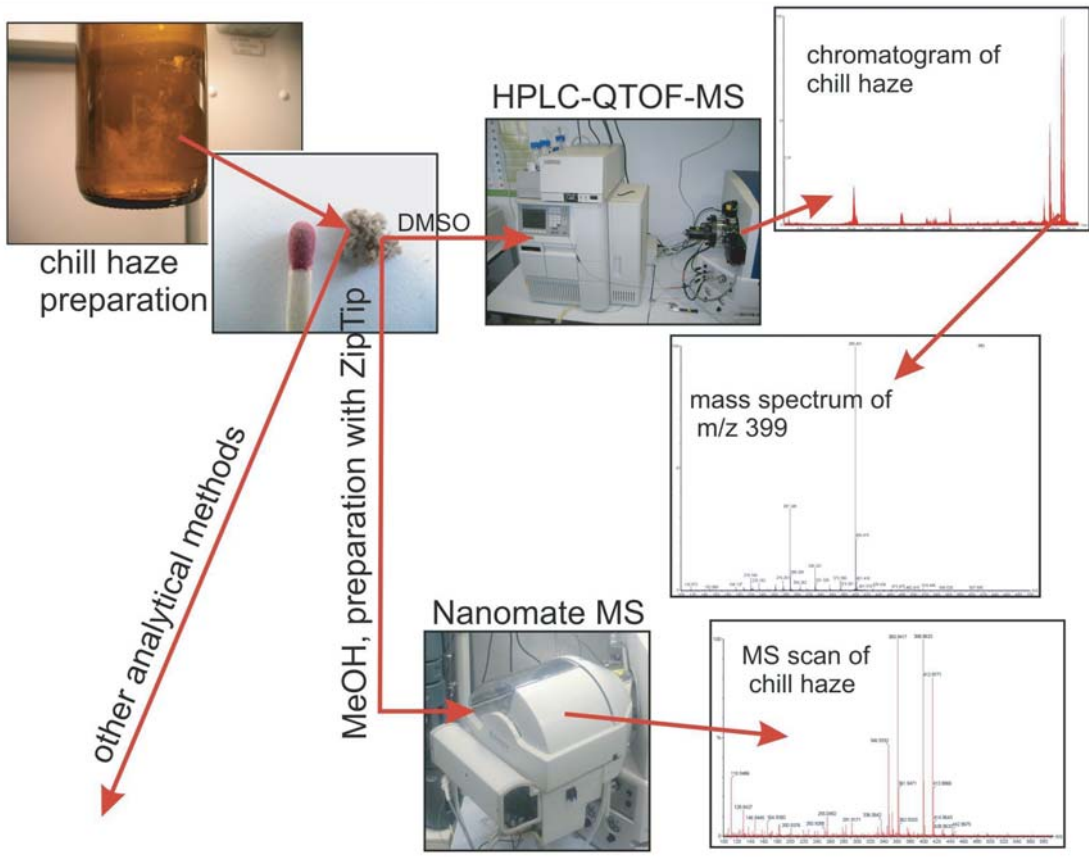


Figure 10 Schematic description of the different ways to analyse isolated chill haze or haze. Sample dilution in DMSO was followed by online LC-MS analysis during which the HPLC was directly coupled to the QTOF-MS. Identification of single components was guaranteed by comparison with standard substances. In contrast nano ESI chip-QTOF-MS was performed on sample material pretreated with ZipTip (to avoid interferences caused by salts) and dissolved in MeOH (to support the ionization). Subsequent sample analysis was performed with selected ion monitoring (SIM), following MS/MS fragmentation of characteristic precursor ions and the help of MassLynx software tools

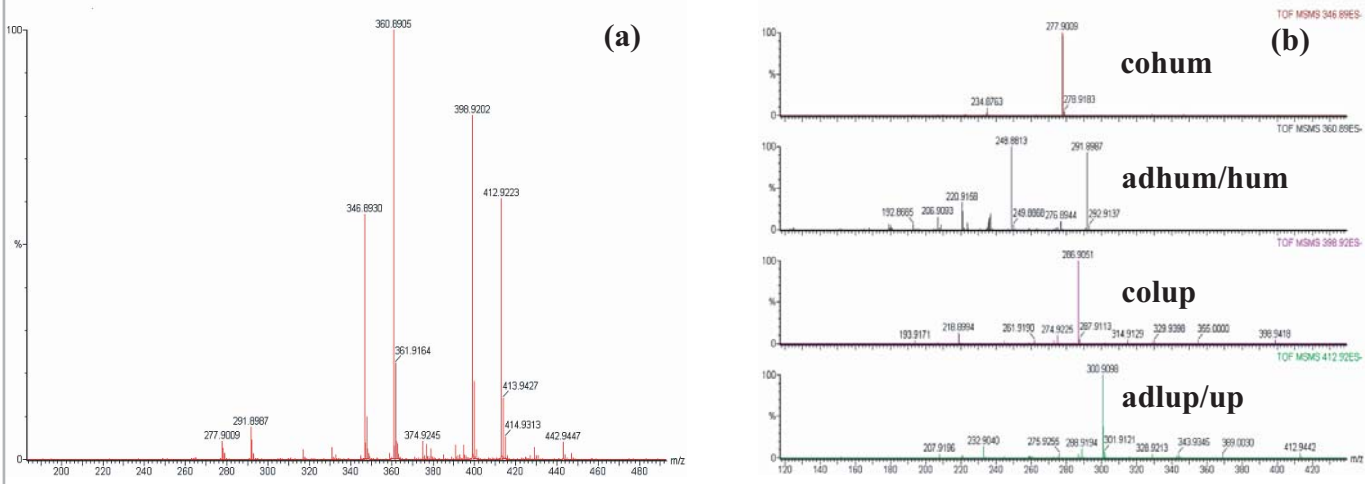


Figure 11 Nano ESI chip mass spectrum MS1 from hop standard operated in ESI(–)-mode (a): $[M–H]^-$ = 346.89, cohumulone; $[M–H]^-$ = 360.89, adhumulone/humulone; $[M–H]^-$ = 398.92, colupulone; $[M–H]^-$ = 412.92, adlupulone/lupulone. Nano ESI chip MS^2 experiments from hop standard in ESI(–)-mode (b)

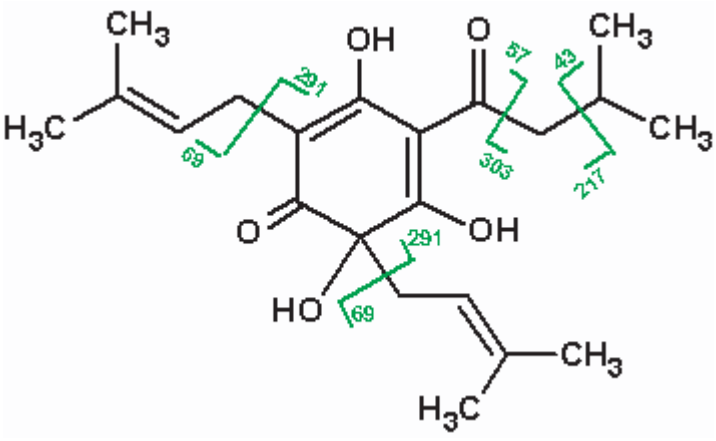


Figure 12 Fragmentation of the hop bitter acid humulone/adhumulone. Fragments occur out of α-cleavage (57 → 303), allyl cleavage (69 → 291) and β-cleavage (43 → 317)

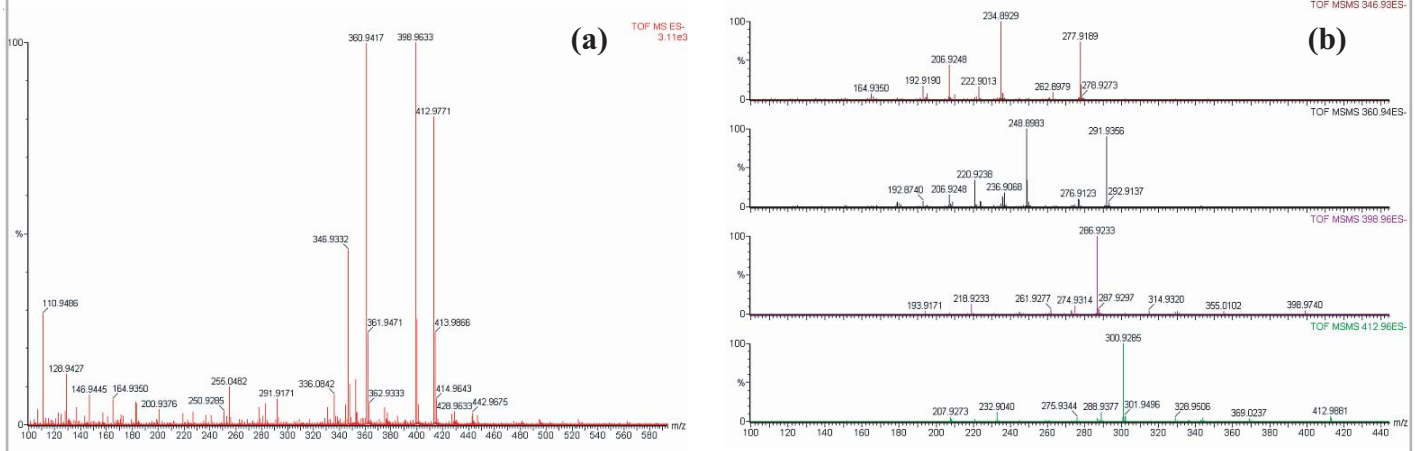
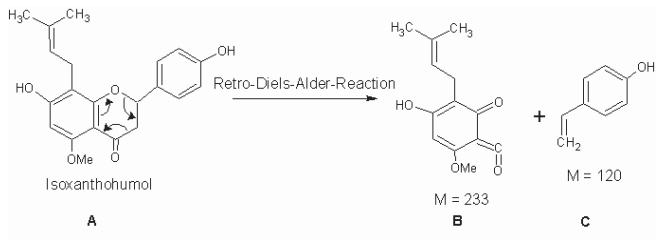


Figure 13 NanoESI chip mass spectrum MS1 from beer haze operated in ESI(–)-mode (a): $[M–H]^- = 346.89$, cohumulone; $[M–H]^- = 360.89$, adhumulone/humulone; $[M–H]^- = 398.92$, colupulone; $[M–H]^- = 412.92$, adlupulone/lupulone. Nano ESI chip MS^2 experiments from components found in beer haze in ESI-(–)-mode (b)



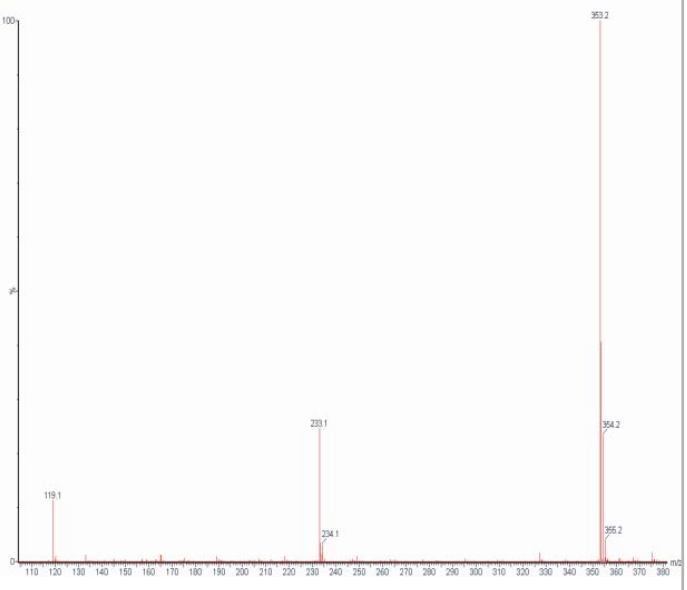


Figure 14 Fragmentation of isoxanthohumol via Retro-Diels-Alder-reaction in theory and practice