D. Dixius, S. Hanke, G. Stettner and A. Lagemann

Transfer of flonicamid and its metabolites (TFNA, TFNG and TFNA-AM) from hops into beer during wort boiling and during dry hopping

Flonicamid is a systemic aphicide that is selectively effective against aphids. In this study the substance transfer of flonicamid and its metabolites was investigated during hop addition at the beginning of boiling and during dry-hopping with a real flonicamid treated hop sample. For the transfer from hops into wort, wort-hopped beer, and dry-hopped beer, transfer rates of 96 %, 103 %, and 88 % were evaluated respectively. From these results, the processing factors for flonicamid and its metabolites TFNA and TFNG (calculated as flonicamid) were defined as 0.001. These results showed that flonicamid, TFNA, and TFNG are fully soluble in wort and are stable during wort boiling and fermentation. In a second investigation, we analyzed 16 commercial Pilsner type beer samples of different brands in a market survey. As a result, flonicamid could not be detected in any of the samples, whereas the degradation products TFNA and TFNG were detected.

Descriptors: LC-MS/MS, hops, pesticide, flonicamid, metabolites, processing factor

1 Introduction

Based on the German purity law, beer in Germany has to be produced only with water, malt, hops (*Humulus Lupulus*), and yeast. Even if a low amount of hops is added to the beer, the hopping has a significant impact of the later beer characteristics. Hops are responsible for the characteristic bitter taste of the final beer. It prolongs the beer's shelf life because of its bacteriostatic effect and it improves foam stability and body [1]. Depending on the hop variety, growing region, climate, quality and the hopping conditions, hops can also give the beer an unique taste. To date, several hundred substances have been identified that contribute to the hop aroma. For example, it is undisputed that linalool, geraniol, β-damascenone, β-citronellol, various esters (e.g. ethyl 4-methylpentanoate and (Z)-4-decenoate) and organic acids (e.g. 2 and 3-methylbutanoic acid) contribute significantly to the hop aroma in beer [2]. The large number of possible flavor combinations along with the synergetic and masking effects [3] illustrate the complexity of the hop aroma.

Crop protection agents are used in modern hop growing in order to preserve the valuable properties of the hop and to minimize crop losses. They should preserve the health of crops and preventively protect them from diseases and pest infestation [4, 5]. Discovered in 1992 by the Japanese chemical company Ishihara Sangyo Kaisha, ltd., the active ingredient flonicamid (N-cyanomethyl-4-trifluoromethylnicotinamide) is a systemic aphicide that is selectively effective against aphids and some other species of sucking insects [6, 7]. Negative influences on beneficial insects and mites have not been reported up to now and it seems that the probability for developing cross-resistances to conventional insecticides is minimal [6, 8, 9]. In addition, flonicamid is characterized by its long-lasting effect. Even after 3 – 4 weeks aphids are still effectively controlled in a concentration of 55 ppm [7, 8]. Flonicamid acts as a feeding and contact poison in insecticides [8]. After absorption, the salivary excretion is inhibited [9], so that the aphids quickly stop their sucking activity [6, 8, 9, 10]. This ultimately leads to the death of the pests [9]. A short time after dietary intake also the honeydew production is stopped [9]. Therefore, aphids can cause no further damage [8]. The mode of action of Flonicamid differs from that of conventional insecticides [7]. It is not effective at the sites of action of neonicotinoids, organophosphates, carbamates and pyrethroids [8]. Instead a selective inhibition of the potassium ion channels takes place and this already appears at nanoconcentrations [9]. The degradation of flonicamid seems to be comparable in plants (wheat, peaches, peppers, potatoes) [11]. The main metabolites are **TFNG** (N-(4-trifluoromethylnicotinoyl)glycine) and **TFNA** (4-trifluoromethyl nicotinic acid). For risk assessment in plants the sum of flonicamid, TFNA and TFNG expressed as flonicamid is calculated. Other metabolites such as **TFNG-AM** (N-(4-trifluoromethylnicotinoyl) glycinamide) and **TFNA-AM** (4-trifluoromethylnicotinamide) are not expected in any significant amount and are therefore ignored [11]. This residue definition also applies to processed products/ food of plant origin [12].

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Plant protection products that are used in agriculture can get into the environment and thus also in food. Food residues can be a health risk for the consumer, depending on the mode of action and their concentration [13]. According to Regulation (EC) No 1107/2009, the active substance and its relevant degradation products must be considered for the evaluation and authorization of a plant protection product, although it is well known that the resulting metabolites may have the same or similar toxicological properties as the active substance itself [14]. To improve food safety, the European Food Safety Authority (EFSA) assesses the risk of pesticides. Following the EFSA's assessment of the residue behavior of the pesticides and possible consumer health risks from residues in food, the European Commission (EC) sets maximum residue levels (MRL) [13, 15]. Their control requires suitable analytical methods. Therefore, a simple, fast and robust LC-MS/MS method was developed for the analysis of flonicamid and its metabolites TFNG, TFNA and TFNA-AM in hops, wort and beer.

In literature, several analytical methods for the analysis of just flonicamid [16, 17, 18, 19, 20] and for the analysis of flonicamid and its metabolites [21, 22, 23, 24, 25, 26, 27] have already been described. In many cases, these applications relate to residue analysis in fruits and vegetables [17, 18, 23, 25, 26, 27]; whereas hops is a complex matrix that is difficult to analyze [21, 28]. Therefore significantly fewer methods are available for the analysis of flonicamid in hops [16, 19, 29] and beer [19, 20] as well as for the analysis of flonicamid and its metabolites in hops [21, 22].

The maximum residue level set by the European Commission (EC) in January 2016 for the sum of flonicamid and its metabolites TFNA and TFNG in hops is 3 ppm [30]. Many agricultural products are processed before being consumed. Depending on their production conditions and the physical and chemical properties of the active compound, pesticide residues in the product can be reduced or elevated [31]. The ratio between the amount of residue in the processed product and the amount of residue in the unprocessed product is defined as the processing factor [32] and is valid for the given processing properties [31]. The processing factor of a given product indicates whether the MRL is within the limits or not. Therefore, it helps to assess the consumer's risk [32]. To date and to the best of our knowledge, no processing factors for flonicamid from hops to beer are available [33]. The aim of this work was, therefore, to determine the processing factors for two Pilsner type beers during the brewing process. The transfer at the hop addition at the beginning of boiling and during dry hopping was investigated. For this a real flonicamid treated hop sample was used and no spiked hop sample.

2 Materials and methods

2.1 Reagents

All chemicals were obtained from the named commercial sources: acetonitrile and methanol hypergrade for LC-MS (Merck KGaA, Darmstadt, Germany); formic acid 98 – 100 % (Merck KGaA, Darmstadt, Germany); flonicamid (Dr. Ehrenstorfer GmbH, Augsburg, Germany); TFNA-AM (BePharm, Shanghai, China); TFNA (Alfa Aesar, Thermo Fisher Scientific, Karlsruhe, Germany); TFNG (Toronto Research Chemicals, North York, Canada); ultrapure water was produced with a Milli-Q IQ7000 (Merck KGaA, Darmstadt, Germany).

Table 1 Residue results of the used hop HMG

Compound	Residue
Flonicamid	0.2 ppm
TFNA*	1.7 ppm
TFNG*	1.1 ppm
∑ Flonicamid, TFNA*, TFNG*	**3.0 ppm**

* expressed as flonicamid

2.2 Samples

For the brewing trials, a hop lot with the maximum tolerable residue level of flonicamid and its metabolites of the variety Hallertauer Magnum (HMG, pellets type 90, 13.3 % alpha, harvest 2017) from the Hallertau region was used. The total concentration of flonicamid and degradation products TFNA and TFNG was 3.0 ppm expressed as flonicamid (Table 1).

For the market survey, 16 commercial Pilsner type beer samples from German breweries were purchased (Table 4, see at page 114). The examined beers were sourced in November 2017 from a local beverage store.

2.3 Brewing trial

The brewing trials were carried out in the 20 hl pilot brewery of the Bitburger Braugruppe GmbH (Bitburg, Germany).

For all trials 100 % pale Pilsen malt was mashed in a two-step decoction mashing regime followed by a wort separation in a lauter tun. The wort was collected in the kettle and boiled with a low pressure boiling process for 85 min. After trub separation in a whirlpool, the wort was cooled down to 9.5 °C pitching temperature. For fermentation, a standard lager yeast strain was used. The maximum fermentation temperature was 11 °C. After the main fermentation a diacetyl rest was performed at 11 °C until it reached a threshold below 0.1 ppm. The green beer was cooled down to –1.5 °C and stored for two weeks prior to filtration. All beers were filtered separately with kieselguhr (100 g/hl body feed) and bottled with pre-evacuation. All brews were made in duplicate.

For evaluation of the transfer during dry hopping, an unhopped brew was carried out according to the described procedure. The brew was divided into two fermenters and dry hopping was performed after maturation at –1.5 °C for 7 days in a static way. The dry hop rate was 150 g/hl. After seven more days of cold storage, the remaining hops were removed and then the beers were separately filtered and bottled.

The evaluation of the transfer during wort boiling was performed by brewing according the explained procedure. The hopping rate was equivalent to 9.1 g alpha/hl (68.1 g hops/hl). It was dosed only at the beginning of wort boiling. No dry hopping was made in these trials.

2.4 Standard preparation for quantification

5.0 mg flonicamid, TFNG, TFNA and TFNA-AM were weighed into a 50 ml volumetric flask and dissolved in acetonitrile. This stock solution was then diluted with matrix. For the analysis of the hop pellets, a flonicamid-free hop extract should be used if possible. The stock solution was diluted with hop extract to the following target concentrations: 1 ppb, 5 ppb, 25 ppb, 50 ppb and 100 ppb. For the analysis of the Pilsner type beer samples a flonicamid-free matrix beer was used. The stock solution was diluted with matrix beer to the following target concentrations: 0.5 ppb, 1 ppb, 5 ppb, 10 ppb and 25 ppb. Hop and beer matrix were processed analogously to the samples described in chapter 2.5. Possible matrix residues were included in the calculation of the standard concentrations.

2.5 Sample preparation for hop pellets, wort and beer samples

4.0 g of milled hop pellets were weighed into a 50 ml centrifuge tube and diluted with 50 ml extraction solution (0.1 % formic acid in ultrapure water/methanol; 50/50 v/v). First the extraction was done on a shaker (15 min, 200 min^{-1}). Subsequently, the sample was heated in a water bath (60 min, 70 °C) and sonicated for 5 min. Then the sample was centrifuged (10 min, 14.000 min^{-1}) filtered through a folded filter (MN 614 ¼) and finally microfiltered (0.2 µm) into a vial.

Worts and beers were decarbonated by manual shaking and treatment in an ultrasonic bath, then filtered through a folded filter (MN 614 ¼) and microfiltered (0.2 µm) into a vial.

2.6 Liquid chromatography tandem mass spectrometry

The 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, California) consisting of degasser (G4225A), binary pump (G1312B), autosampler (G1367E), column oven (G1316A), thermostat (G1330B) and controller (G4208A) has been coupled with a TripleTOF 5600+ mass spectrometer (AB Sciex, Toronto, Canada) equipped with a DuoSpray ion source. The chromatographic separation was done with a Synergi Fusion-RP 80 A, 250 x 4.6 mm, 4 µm (Phenomenex, Aschaffenburg, Germany).

The mobile phase A was 0.1 % formic acid in ultrapure water and the mobile phase B was 0.1 % formic acid in methanol. The temperature of the column oven was set to 40 °C. For the analysis of hop pellets, the injection volume was 10 µl with the following binary gradient and a flow rate of 0.5 ml/min: 0 – 1 min 20 % B, 1 – 12 min 100 % B, 12 – 22 min 100 % B, 22 25 min 20 % B, 25 – 30 min 20 % B. For the analysis of wort and beer, the injection volume was 20 µL with the following binary gradient and a flow rate of 0.5 ml/min: 0 – 1 min 20 % B, 1 – 12 min 100 % B, 12 – 17 min 100 % B, 17 – 18 min 20 % B, 18 – 25 min 20 % B.

The electrospray ionization was carried out in positive mode (ESI+) at a temperature of 480 °C, an ionspray voltage of 5500 V and gas pressures of 40 psi and 50 psi for gas 1 and gas 2, respectively. Curtain gas was set to 50 psi. Mass transitions, declustering potentials and collision energies are shown in table 2. Nitrogen was used as collision gas. Multiple reaction monitoring (MRM) was used for data acquisition.

Table 2 List of specific mass transitions and MS/MS parameters

Compound	Precursor ion [m/z]	Product ions [m/z]	DP[a] [V]	CE[b] [V]
Flonicamid	230.1	203.06	20	30
		183.05		
		176.05		
		174.03		
		148.05		
		146.04		
TFNA-AM	191.1	148.05	30	30
		98.05		
		79.05		
TFNA	192.1	148.05	30	30
		146.04		
		98.05		
		79.05		
TFNG	249.1	203.06	30	36
		183.05		
		176.05		
		174.03		
		148.05		

[a] Declustering potential
[b] Collision energy

2.7 Quantification

The quantification was made with the external calibration function via the software MultiQuant 3.0.2. For hop pellets, a dilution factor of 12.5 was used. For wort and beer samples, no dilution factor had to be considered. For quantification, the sum of all product ions of the respective parent compound was formed. The degradation products TFNA and TFNG were converted into flonicamid equivalents so that the result could be given as the sum of flonicamid, TFNA and TFNG (expressed as flonicamid) [34]. In the following, all TFNA and TFNG residues are understood as the "flonicamid calculated" residue. Each LC-MS/MS analysis was performed in duplicate. Due to the expected low levels of TFNA-AM and to ensure comparability with other laboratories, TFNA-AM was evaluated only qualitatively. However, quantification is possible.

3 Results and discussion

3.1 Validation of the method

The calibration curves were built by plotting the peak areas over the analyte concentrations. They show a very good correlation of an average r = 99.7 %, with an average coefficient of determination of R^2 = 99.4 %. The recoveries for all parameters ranged between 99 – 102 %. This complies with the SANCO (2017) requirements

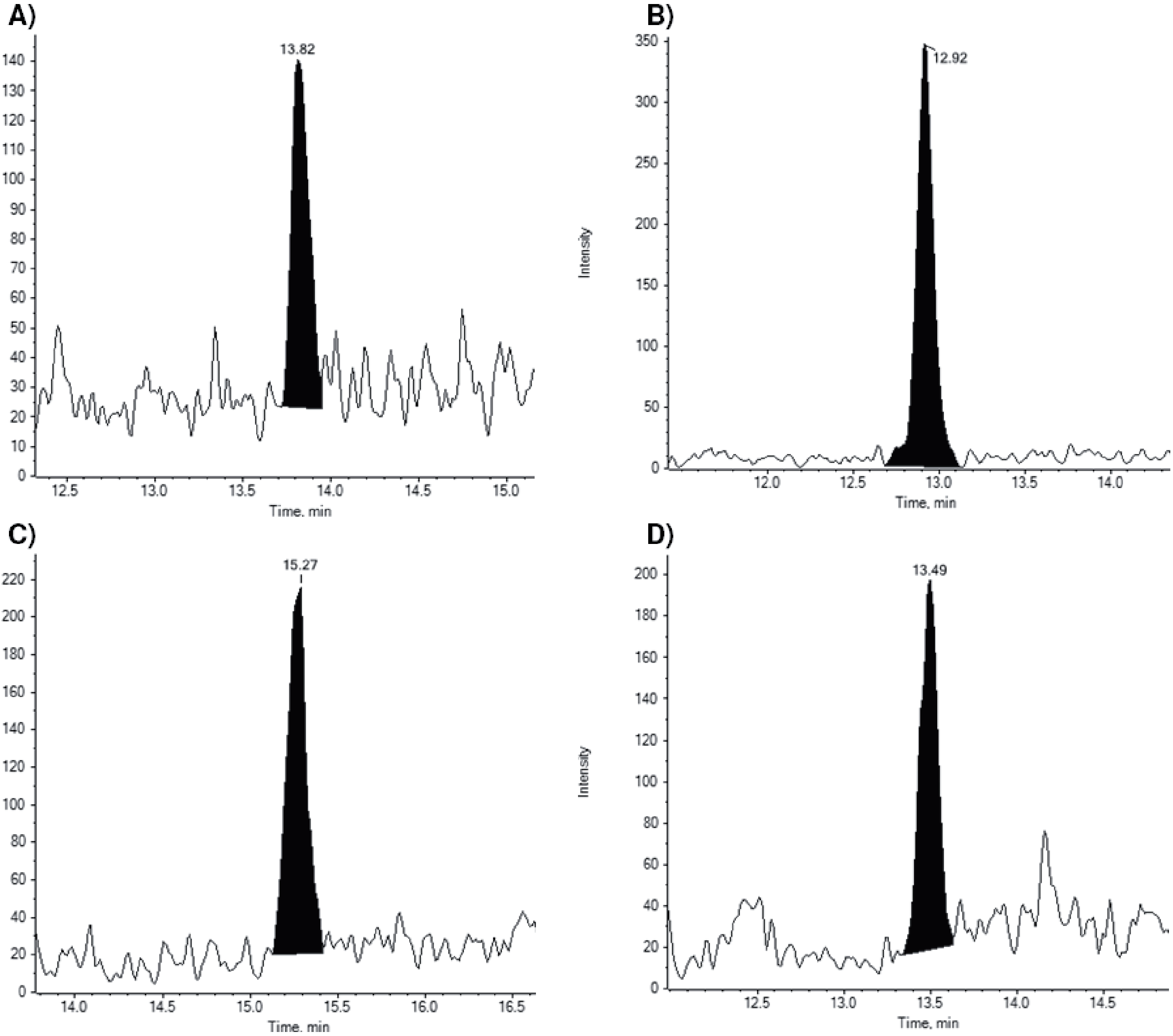

Fig. 1 MRM chromatograms at 0.2 ppb flonicamid (A), 0.2 ppb TFNA (C) and 0.3 ppb TFNG (D) in beer samples and 0.5 ppb TFNA-AM (B) in the calibration solution

(70 – 120 %) [34]. The detection limits (LOD) and quantification limits (LOQ) for flonicamid and its metabolites TFNA and TFNG in hops are 0.003 ppm (LOD) and 0.01 ppm (LOQ), respectively. Therefore the quantification limits are clearly below the required MRL of the EC of 3 ppm [30] and comply with the EFSA requirements (LOQ = 0.03 ppm, sum of flonicamid, TFNG and TFNA) for an adequate analytical method [28]. Hence the method described is well suited to assess the risk of flonicamid in regards to the food safety of hops. The detection and quantification limits for flonicamid and its metabolites TFNA and TFNG in beer are 0.06 ppb (LOD) and 0.2 ppb (LOQ), respectively. Examples of chromatograms near the quantification limit are shown in figure 1. These low limits allow a very good evaluation of the safety of Pilsner type beers with regard to flonicamid residues. All in all, a simple, robust and fast LC-MS/MS method was developed for the quantification of flonicamid, TFNA and TFNG. The straightforward sample preparation saves time and money. To the best of our knowledge it is the first LC-MS/MS method for the analysis of flonicamid in beer including the metabolites TFNA and TFNG.

3.2 Transfer rates and processing factors

The total residue concentration of flonicamid and its metabolites TFNA and TFNG in the analyzed hop pellets was 3.0 ppm. The permitted maximum residue level set by the EC for the sum of flonicamid and its metabolites TFNA and TFNG in hops is 3 ppm [30]. Consequently, the used hops are still be marketable according to the current EC guidelines.

The total flonicamid residue in the used hops is composed as follows: 0.2 ppm flonicamid, 1.7 ppm TFNA and 1.1 ppm TFNG (Table 1). This result is consistent with the literature. According to literature, flonicamid degrades within a few days or weeks after application [17, 26, 27, 35]. In literature, TFNG is often described as the metabolite that is formed in significant quantities during degradation [26, 27]. However, this observation refers to water-rich plants, such as peppers [26] and oranges [27]. And the higher TFNG content in these products could be due to the possibility that the metabolization to TFNG begins before the metabolization to TFNA

[23]. Maybe the TFNA residue might increase depending on a longer storage time. In addition, the ratio of TFNG to TFNA may also differ significantly depending on the plant [11]. All in all, it is not contradictory that significant amounts of TFNA and TFNG were determined in a complex matrix such as the HMG hop. The TFNA concentration was 1.7 ppm and therefore higher than the TFNG concentration of 1.1 ppm. This result coincides with *Hengel* and *Miller* [21], who also found more TFNA than TFNG in flonicamid treated hops after harvesting and drying. But how the degradation processes of flonicamid in hops after harvest and during processing into pellets works was not the subject of this investigations.

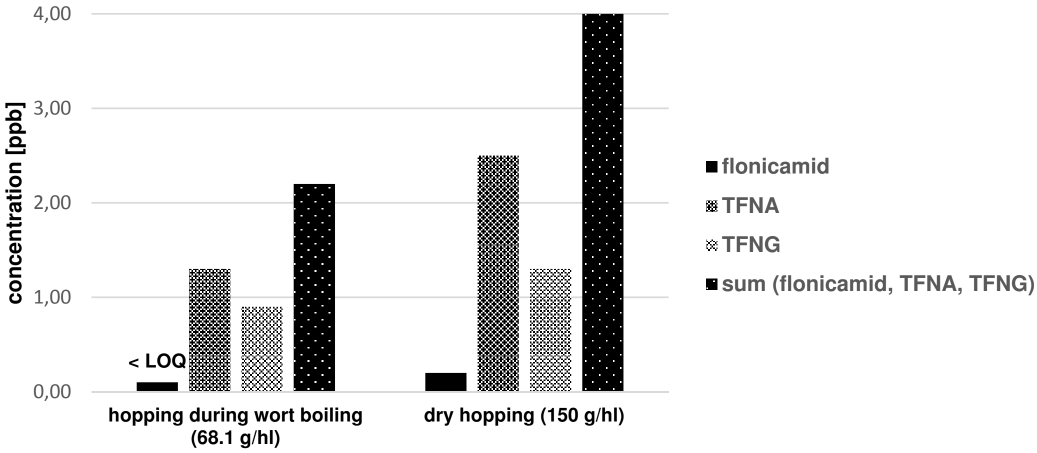

Fig. 2 **Concentration of flonicamid, TFNA and TFNG and its sum after hopping during wort boiling (68.1 g/hl) or rather after dry hopping (150 g/hl). Taking into account the technological variations in the brewhouse and analytical errors, the measurement uncertainty is on average < 5 % for hop addition at the beginning of boiling and on average < 15 % during dry hopping respectively**

The analysis of the beers brewed from the described hop pellets resulted in a total residue concentration of the final beers of 2.2 ppb (hop addition at the beginning of boiling 68.1 g/hl) and 4.0 ppb (dry hopping 150 g/hl) respectively. The individual values of flonicamid, TFNA and TFNG are present in table 3 as well as in figure 2. Keep in mind that the measurement uncertainty is on average < 5 % for hop addition at the beginning of boiling and on average < 15 % during dry hopping respectively. This results from the technological variations in the brewhouse and the analytical errors. In the first investigation (hop addition at the beginning of boiling), the residues in the green beer are slightly higher than in the wort. This could be due to the fact that trub substances are separated between these process steps, which results in a volume reduction. From *Dušek* et al. [19] it is known that flonicamid has a low tendency to adsorb to solids (trub, yeast) during beer production. From this, it can be assumed that flonicamid and its metabolites remain unchanged in the young beer, which leads to an increase in the concentration when the volume is reduced at the same time. Through the filtration process, the total residue concentration decreases slightly during our experiment. This could be due to the polar character of the filtration aid kieselguhr. Flonicamid also has a polar character (log P = 0.3, 29.8 °C) [36], so that a part of the flonicamid could be adsorbed by the kieselguhr and thus separated. For TFNA and TFNG a similarly polar behavior could be expected on the basis of the related chemical structure. The residue reduction during the filtration in the second series of experiments (dry hopping) confirms this (Table 3).

Looking at the ratio TFNG/TFNA, it is noticeable that the ratio shifts through the beer production. In the used hop pellets, the TFNG/TFNA ratio is 0.6. In the production of the wort hopped beer, it shifts in the direction of TFNG (TFNG/TFNA = 0.7) and in the production of the dry hopped beer, it shifts in the direction of TFNA (TFNG/TFNA = 0.5). This could be due to the different temperatures in the hot and cold brewing process, respectively. Whether higher temperatures promote the formation of TFNG and lower temperatures promote the formation of TFNA during beer

Table 3 **List of flonicamid, TFNA and TFNG residue and its transfer rates and processing factors during brewing (hop addition at the beginning of boiling 68.1 g hops/hl and dry hopping 150 g hops/hl respectively)**

Sample	Flonicamid[a] [ppb]	TFNA[a,b] [ppb]	TFNG[a,b] [ppb]	Sum[c] [ppb]	Transfer rate [%]	Processing factor P_f
Wort	< LOQ	1.3	0.7	2.0	96	0.001
Beer (unfiltered)	< LOQ	1.4	0.9	2.3	108	0.001
Beer (filtered)	< LOQ	1.3	0.9	2.2	103	0.001
Dry hopped beer (unfiltered)	0.2	2.7	1.4	4.3	93	0.001
Dry hopped beer (filtered)	0.2	2.5	1.3	4.0	88	0.001

[a] average brew 1 and brew 2 (rounded to one decimal place)
[b] expressed as flonicamid
[c] total concentration of flonicamid, TFNA and TFNG (expressed as flonicamid)

production remains the subject of further investigations. We already know from the literature that the temperature has an impact on the degradation behavior of flonicamid [17, 26, 27]. Consequently, flonicamid degrades faster at higher temperatures [26, 27].

Table 4 List of flonicamid, TFNA and TFNG residue in commercial Pilsner type beer samples from Germany

Sample	BBD[a]	IBU[b]	Flonicamid [ppb]	TFNA[c] [ppb]	TFNG[c] [ppb]	Sum[d] [ppb]
Beer 1	03/2018	27	< LOQ	1.2	1.4	2.6
Beer 2	06/2018	40	< LOQ	0.7	1.3	2.0
Beer 3	04/2018	29	< LOQ	1.0	0.8	1.8
Beer 4	06/2018	34	< LOQ	0.8	0.8	1.6
Beer 5	05/2018	32	< LOQ	0.6	0.7	1.3
Beer 6	02/2018	29	< LOQ	0.5	0.8	1.3
Beer 7	06/2018	34	< LOQ	0.7	0.5	1.2
Beer 8	03/2018	25	< LOQ	< LOQ	1.1	1.1
Beer 9	06/2018	33	< LOQ	0.5	0.5	1.0
Beer 10	06/2018	31	< LOQ	0.5	0.5	1.0
Beer 11	05/2018	30	< LOQ	0.2	0.3	0.5
Beer 12	04/2018	24	< LOQ	0.2	0.3	0.5
Beer 13	04/2018	22	< LOQ	< LOQ	0.3	0.3
Beer 14	05/2018	31	< LOQ	< LOQ	< LOQ	< LOQ
Beer 15	04/2018	24	< LOQ	< LOQ	< LOQ	< LOQ
Beer 16	04/2018	35	< LOQ	< LOQ	< LOQ	< LOQ

[a] best before date
[b] International Bitterness Units (Mebak)
[c] expressed as flonicamid
[d] total concentration of flonicamid, TFNA and TFNG (expressed as flonicamid)

Just like the measured total residue concentrations (the sum of flonicamid, TFNA and TFNG), the determined transfer rates also fluctuate between the different steps of beer production. The possible reasons have already been discussed above. For the transfer of flonicamid and its metabolites, TFNA and TFNG, from hop to wort, a transfer rate of 96 % was determined. Consequently flonicamid, TFNA and TFNG are almost completely soluble in wort. This is due to the high solubility of flonicamid in water (5200 mg/L; 20 °C). Due to the chemical structure, similarly good solution behavior is to be expected for TFNA and TFNG, which is confirmed by this experiment. This result coincides with *Miyake* et al. [37]. Accordingly, the transfer rates of pesticides into beer should be predicted using the octanol-water partition coefficient (log P). Thus, polar, water-soluble pesticides with a log P < 4 tend to carry over into the wort. By contrast, non-polar, water-insoluble pesticides with a log P > 4 tend to be adsorbed by the spent grains, the hops and the yeast. The lower the log P value, the sooner pesticides can carry over into the wort. Miyake et al. [37] had not determined the transfer of flonicamid. However, with the knowledge that flonicamid has a very low log P of 0.3 (29.8 °C) [36], Miyake et al. [37] also could have expected a high transfer into the wort of up to 100 %. But Miyake et al. [37] also reported that hop pesticides do not carry over into beer in significant quantities due to the small amount of hops and the physical and the chemical processes during beer production. This conclusion was drawn because they couldn't detect hop derived pesticides after the wort boiling [37]. With the help of the high-resolution MS/MS technique and our particularly low detection limits, this conclusion of Miyake et al. [37] was refuted. Hop pesticides can pass into the final beer. However, the detectable flonicamid, TFNA and TFNG residues are very low compared to the raw material due to the high dilution.

Our determined transfer rate of 96 % with a real flonicamid treated hop sample coincides with the result of Dušek et al. [19], who have determined a transfer rate of 103 % for flonicamid in wort with a spiked hop sample. It seems that Flonicamid is stable during wort boiling and a degradation of flonicamid during fermentation, as Dušek et al. [19] described it, however, could not be determined in our investigations. This was attributed to the adsorption on yeast cells during the first fermentation phase (4 weeks) and/or to the acid hydrolysis during the second fermentation phase (6 weeks) [19]. However, flonicamid was probably metabolized to TFNA and/or TFNG during this long fermentation and therefore could not be detected. This, in turn, shows that evaluating a pesticide is meaningful only when looking at its metabolites at the same time. For this reason existing pesticide multi-residue methods should be extended by the metabolites of their active compounds.

For the finished beers, a transfer rate of 103 % (hop addition at the beginning of boiling 68.1 g/hl) and a transfer rate of 88 % (dry hopping 150 g/hl) were determined during this work, respectively (Table 3). Although more than twice the amount of hops was used for dry hopping, the maximum solubility of flonicamid (5200 ppm in water, 20 °C) was not reached with a calculated residual amount of 4.5 ppb for flonicamid, TFNA and TFNG. The slightly higher transfer rate during the hop addition at the beginning of boiling compared to the transfer rate during dry hopping is probably due to the fact that extraction processes work better at higher temperatures. From a previous investigation, we already know that the extraction of pesticides at the high temperatures during the wort boiling and the whirlpool is more efficient [38]. As we also know from *Kippenberger* et al. [38], the transfer of pesticides into beer depends not only on the production conditions (e.g. temperature), but also on the concentration of residues in hops, the amount of hops and the pesticide solubility.

Based on the mass transitions, the processing factors for flonicamid were determined for the hop addition at the beginning of boiling (68.1 g hops/hl) and for dry hopping (150 g hops/hl). The processing factor for the sum of flonicamid, TFNA and TFNG from hops to final beer is 0.001 in both cases. In the future, these values may give brewers some indications as to whether or not they have complied with the applicable MRLs for beers brewed with a hopping rate between 68.1 – 150 g hops/hl.

3.3 Analysis of commercial beer samples

In a market survey, 16 commercial Pilsner type beer samples from German breweries were analyzed. The examined beers were sourced in November 2017 from a local beverage store. The used hop amount was not known. The determined flonicamid residues are given in table 4. Flonicamid residues could not be detected in any of the 16 samples. By contrast, TFNA and TFNG could be quantified. This could be due to the fact that beer is a matrix with a very high water content. High water contents enhance hydrolytic processes and thus the degradation of flonicamid and its metabolites [26]. From literature, we already know that flonicamid degrades during storage to TFNG and then to TFNA [23]. We suppose the same degradation path for beer. TFNA contents of 0.2 to 1.2 ppb were determined in 11 samples while TFNG contents of 0.3 to 1.4 ppb were determined in 13 samples. This results in a total of 0.3 to 2.6 ppb in 13 of the 16 samples. At this point, no conclusions to the flonicamid residue in the original hop can be drawn, since the hop addition of the individual beers is unknown. However, when looking at the ADI of flonicamid, it quickly becomes clear that no risk for human health arises from the total flonicamid residues found in beer. Flonicamid has an ADI of 0.025 mg/kg body weight [11] with low acute toxicity [39]. For example, an adult of 70 kg body weight can take 1.75 mg flonicamid per day. Hence, this person could ingest almost 673 L per day of the most heavily loaded sample "Beer 1". In general, the risk of a significant pesticide transfer from hops into beer seems to be quite low [40]. The majority of pesticides are reduced due to adsorption, hydrolysis and/or pyrolysis processes as well as volatilization during the brewing process [37, 40, 41]. After completing the entire brewing process, the risk of beer being contaminated with pesticides is significantly reduced. The few pesticides that can pass into beer are characterized by their low log P value [41].

Higher hop additions may result in higher pesticide levels. If there is a linear relationship between the amount of hops used for beer brewing and the total flonicamid residue in beer remains the subject of future work. For example, it is known from our previous study that the nitrate residue increases with the increasing hop addition [38]. This would be particularly important for stronger hopped beers.

4 Summary and conclusion

In this work, a simple, robust, highly selective and sensitive LC-MS/MS method was developed for the routine analysis of the insecticide flonicamid and its metabolites TFNA, TFNG and TFNA-AM in hops and beer. The LOQs for flonicamid, TFNA and TFNG are 0.01 ppm in hops and 0.2 ppb in beer and wort. Thus, the LOQs are well below the permitted MRL allowed by the EC of 3 ppm for hops [30] and meet the requirements of the EFSA (LOQ = 0.03 ppm for the sum of flonicamid, TFNG and TFNA) for a suitable quantification method [28]. In the future, these low limits will allow for a better assessment of the safety of beers with regard to flonicamid. Because of its similar chemical structure, the analysis of the short-lived degradation product TFNG-AM, which was first described by *López* et al. [27], is also possible. Nonetheless, the developed method could simply be extended to include these metabolites and other beer-related insecticides and pesticides.

With the developed method, the mass transfer of flonicamid, TFNA and TFNG was investigated during hop addition at the beginning of boiling and during dry hopping. Magnum hop pellets with a total flonicamid content of 3.0 ppm were used (cf. MRL is 3.0 ppm for the total flonicamid amount in hops [30]). The degradation products TFNA and TFNG made up a share of 93 %. This result confirms that flonicamid degrades already after a few days or weeks after application [17, 26, 27, 35]. For the transfer from hops to wort, a transfer rate of 96 % was determined. For the transfer from hops to the final beer, which was hopped at the beginning of boiling, a transfer rate of 103 % was determined. For the dry hopped beer a transfer rate of 88 % was found. These transfer rates result in processing factors for the sum of flonicamid, TFNA and TFNG from hops to the final beer of 0.001 for both Pilsner type beers and is valid for Pilsner type beers with a hopping rate between 68.1 – 150 g hops/hl. In the future, these values may give the brewer an indication whether the current MRL for the used hops during beer production has been complied with. To the best of our knowledge, the processing factor for flonicamid from hops to beer was determined for the first time. In addition, these results show that flonicamid, TFNA and TFNG are almost completely soluble in wort and behave stably during wort boiling and during the subsequent fermentation. Compared to the investigation of Dušek et al. [19] we used a real flonicamid treated hop sample and no spiked hop sample. The slightly higher transfer during wort boiling compared to the transfer during the cold dry hopping process is probably due to the fact that extraction processes take place more efficiently at high temperatures. Furthermore, it was shown that the process temperature could affect the ratio of TFNG to TFNA.

In a further investigation, we analyzed 16 Pilsner type beer samples. It was noticeable that flonicamid could not be detected in any of the samples, whereas the degradation products, TFNA and TFNG, were detected. This could be due to the fact that beer is a matrix with a very high water content and therefore, it enhances hydrolytic processes and thus finally the degradation of flonicamid to its metabolites. Additionally, these results show the need for the extension of existing pesticide multi-methods by the metabolites of their active compounds. Without the analysis of TFNA and TFNG, we would have detected no flonicamid residues in the measured beers.

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