Behavior of [^{14}C]Atrazine, [^{14}C]Terbutylazine, and Their Major Metabolites in the Brewing Process

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The behavior of some *s*-triazine herbicides and metabolites ([^{14}C]atrazine, [^{14}C]deethylatrazine, [^{14}C]-deisopropylatrazine, [^{14}C]hydroxyatrazine, [^{14}C]terbutylazine, and deethylterbutylazine) during beer production was studied. In the mashing process reduction of the triazine concentration in brewing liquor is caused by adsorption on spent grains. Adsorption affinity depends on the polarity of the triazine component: the more polar the triazine, the lower the adsorbed amount. Terbutylazine was found almost completely in the spent grains, whereas the major part of the dealkylated atrazine metabolites was detected in the wort. Adsorption on spent hops in the cooking process and on kieselguhr in the filtration step was insignificant. During fermentation hydroxylated triazines were formed. Top-fermenting yeasts (*Saccharomyces cerevisiae*) were able to convert atrazine into its hydroxylated analogue with first-order kinetics and a half-life of 98 days. The reactivity of bottom-fermenting yeasts (*Saccharomyces carlsbergensis*) was negligible. Isolation of hydroxyatrazine and hydroxyterbutylazine in beer required several cleanup steps before determination by HPLC with UV/DAD detection and identification by GC−MS.

Keywords: *s-Triazines; atrazine; terbutylazine; metabolites; beer*

INTRODUCTION

s-Triazine herbicides are used in large quantities throughout the world for pre- and post-emergent weed control (Buser, 1990). In the United States atrazine has been the most heavily used herbicide over the past 30 years (annual dispersion: 80 million pounds according to EPA) (Newman, 1995). Residues of triazines are frequently found in environmental samples of soil and water owing to their persistence and relatively high solubility in water. Highest concentrations are often above EPA's maximum contaminant level for drinking water of 3 ppb, particularly in the corn belt areas (Pionke, 1990; Belluck et al., 1991; Newman, 1995).

Until its application ban in Germany (April, 1991) greatest atrazine concentrations in ground water were also in the ppb range (Friesel, 1986; Herzel and Schmidt, 1987; Müller-Wegener and Milde, 1989; Otteneder and Pütz, 1992). The highest concentrations occurred mainly in maize plantations in spring and summer following rainfall and seasonal applications and when application restrictions were not obeyed. The amount of atrazine is decreasing steadily in the past few years, but not rarely, the maximum permissible level of 0.1 ppb (EEC Drinking Water Guideline 80/779/EEC) is still surpassed (Greb, 1995; Pietsch et al., 1995). Sometimes atrazine degradation products, especially deethylatrazine, are found in higher concentrations than their parent compounds (Stark and Zullei-Seibert, 1994). Monitoring of these polar degradation products is of increasing importance. Like atrazine, they are classified as possible human carcinogens (Belluck et al., 1991; Newman, 1995).

Since the prohibition of atrazine in Germany, the related terbutylazine was used as a substitute. The application of this compound seems to be less harmful because after several years no remarkable quantities could be detected in drinking water (Herzel and Schmidt, 1987).

In this research we studied the behavior of atrazine (ATR), deethylatrazine (DEA), deisopropylatrazine (DIA), hydroxyatrazine (OHA), terbutylazine (TER), and deethylterbutylazine (DET) during beer production. They may enter the brewing process via contaminated brewing liquor. Several investigations have been made for pesticide residues in food and their behavior in food processing. There are some dealing with their comportment during beer production (Nitz et al, 1984; Farris et al., 1992). Donhauser et al. (1992) examined the fate of atrazine, but considering the complex matrix statements about metabolism and their exact quantitation can hardly be made without using radiolabeled compounds.

MATERIALS AND METHODS

Reagents. Triazine standards (purity >98%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany): 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine (atrazine, ATR); 2-chloro-4-amino-6-(isopropylamino)-*s*-triazine (deethylatrazine, DEA); 2-chloro-4-(ethylamino)-6-amino-*s*-triazine (deisopropylatrazine, DIA); 2-(ethylamino)-4-hydroxy-6-(isopropylamino)-*s*-triazine (hydroxyatrazine, OHA); 2-chloro-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine (terbutylazine, TER); 2-(ethylamino)-4-hydroxy-6-(*tert*-butylamino)-*s*-triazine (hydroxyterbutylazine, OHT); 2-chloro-4-amino-6-(*tert*-butylamino)-*s*-triazine (deethylterbutylazine, DET); 2-methoxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine (atratone); 2-methoxy-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine (terbumetone). A standard solution was prepared by dissolving 100 mg/L of each herbicide in methanol. This solution was further diluted to obtain a working standard solution of 1 mg/L. Uniformly ring-labeled [^{14}C]atrazine (specific activity: 440 MBq/mmol) was received from Sigma Chemical Company (St. Louis, MO), and [^{14}C]terbutylazine (specific activity: 829 MBq/mmol) was from DuPont (Boston, MA). Aliquots of both [^{14}C]standards were

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purified by HPLC. During this procedure the main degradation products [^{14}C]deethylatrazine and [^{14}C]deisopropylatrazine could be isolated as pollutants of their parent standards. Thus [^{14}C]triazine standard purities were >99%. [^{14}C]Hydroxyatrazine was obtained after acid hydrolysis of [^{14}C]atrazine (Delley et al., 1967). For assaying ^{14}C a universal liquid scintillation cocktail (Ultima Gold XR) from Packard (Canberra-Packard GmbH, Frankfurt, Germany) was used. Methanol, acetonitrile, and hexane were of HPLC-grade, ethyl acetate, 2-propanol of pesticide residue quality, and all other reagents of analytical grade (all reagents from E. Merck, Darmstadt, Germany). *N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald), used for preparation of diazomethane, was obtained from Sigma-Aldrich (Germany). Deionized water was glass-distilled prior to use.

Apparatus. For HPLC analyses a high-precision pump model 480 GT (Gynkotek, Germering, Germany), capable of ternary gradient separations, was used with degasser (Shodex DEGAS KT series), UV detector (Gynkotek SP 6, wavelength set to 225 nm), and radioactivity detector (Berthold HPLC Radoactivity Monitor LB 506 C 1 equipped with solid substance cell. YG-150 and Berthold HPLC program software LB 506 C 1, version 1.3, from Berthold, Wildbad, Germany) connected in series.

Chromatography was performed on a LiChrospher 60 RP-select B-column, 5 μm, 250 × 4 mm following a LiChrosorb RP-18 guard column (5 μm, 4 × 4 mm; Merck). Gradient elution was carried out at a flow rate of 0.9 mL/min beginning with 15% acetonitrile and 85% buffer (0.5 mmol of sodium acetate), isocratic for 5 min, followed by a linear gradient to 60% acetonitrile within 30 min and finally to 100% acetonitrile (5 min). After that, the initial conditions were reached within 3 min and the system equilibrated for another 7 min. The injection volume was 100 μL.

Analyte identity in beer samples was confirmed by HPLC-DAD and GC−MS. For diode-array detection a UV/vis detector model UVD 340 S (Gynkotek) with Gynkosoft Chromatography data system, version 5.42A, was employed. GC−MS analysis was performed on a Carlo-Erba/HRGC−MS system with a 5970 mass-selective detector (MSD) from Hewlett-Packard (Palo Alto, CA). Operating conditions were as follows: ionization voltage, 70 eV; ion source temperature, 250 °C; electron multiplier, 2200 V. Separation of the herbicides was accomplished with a fused silica capillary column (DB 5 from J&W Scientific, Folsom, CA); film thickness, 0.25 μm; 30 m × 0.25 mm i.d. Helium was used as a carrier gas at a flow rate of 1 mL/min. The column temperature was held at 80 °C for 5 min and then ramped at 3 °C/min to 280 °C where it was held for 20 min. The injector temperature was 280 °C.

Diazomethane generation was performed in a diazomethane development apparatus (Cohen, 1984). Diazald (1 g), diethylene glycol monoethyl ether (3 mL), and diethyl ether (2 mL) were placed in the reaction tube (20 mL). The apparatus was purged by a gentle stream of nitrogen. Diazomethane was started by adding 2 mL of 40% aqueous potassium hydroxide solution (Klaffenbach et al., 1993).

A Packard Tri-Carb Liquid Scintillation Analyzer (Model 1600 CA) was used to measure radioactivity. TLC plates were scanned for radioactive substances on an automatic TLC Analyzer (Tracemaster 20) supplied by Berthold (Wildbad, Germany). A Packard Sample Oxidizer, model 307, was employed for estimation of radioactivity in solid samples (spent grains, spent hops, and yeast). Carbosorb E and Permafluor E+ were used as carbon dioxide absorber and liquid scintillation counting cocktail, respectively.

Brewing Process. In Figure 1 a scheme of the brewing process is given. It was carried out both in a bench scale (two to seven replications per substance) and in a pilot plant (two replications per substance).

Laboratory Scale. On a MEBAK-mill (DLFU Bühler Miag) a 1/1 mixture of fine and sound grist was prepared. Deionized water was spiked with the radiolabeled triazine standard to obtain the desired concentration (0.1−2.5 ppb). For mashing in, a congress mashing procedure was carried out in a mash cooker (type MB3E) (Drawert, 1984). The first wort was carefully heated in a round-bottomed flask with boiling stones before adding hop pellets (80 mg of α-acid/L of wort) and cooking for 75 min. The steam was liquefied in a Liebig condenser and collected in a graduated cylinder. The amount of condensate was 10% of the total wort volume. After cooling and filtering the original wort was aerated and pitched in a small-scale fermenting equipment (fermentation tube, length, 1150 mm, 60 mm i.d., volume, 2.5 L) (Weinfurtner et al., 1961). Bottom fermentation was carried out at 7.5 °C (6 days), secondary fermentation at 2 °C (8 weeks). Top fermentation temperature was held at 18 °C following secondary fermentation for 1 week at 12 °C and 2 weeks at 5 °C.

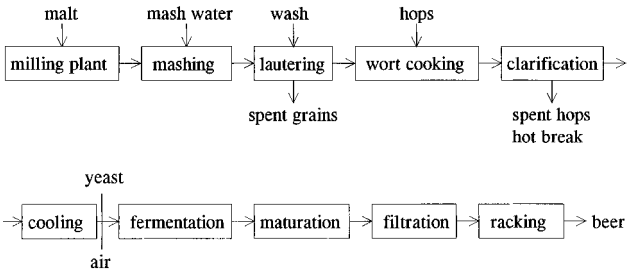

Figure 1. Scheme of the brewing process (Narziss, 1995).

Pilot Plant Scale. A 20 L brewery plant from A. Steinecker, Freising, was utilized with two replications per substance. 4 kg of EBC malt was milled on a Künzel milling plant (type 16/16, Kulmbach, Germany). In a mash tun the grist was mixed in portions with 20 L deionized water spiked with 5 μg/L of unlabeled triazine. The mashing-in temperature was 37 °C; protein rest was held at 52 °C and maltose rest at 65 °C. The final mash pumping temperature was 78 °C. Both the two-mash decoction method and the infusion mashing were applied according to Narziss (1980). After lautering, using further 15 L of spiked brewing liquor (78 °C), the wort was cooked for 75 min, adding hops as described above. On a coolship the final wort was cooled down to the pitching temperature of 8 °C. Primary fermentation was performed in an open fermenter. After reaching the final attenuation of 70%, the green beer was transferred to a cask for secondary fermentation (for conditions, see above).

Analysis. During the brewing process 5 mL each of water, first wort, finished wort, green beer, and beer was taken out and blended with 15 mL of scintillation cocktail to determine absolute radioactivity by liquid scintillation counting (LSC). Values were corrected automatically by a quench curve. Amounts of triazines included in the beer were also determined by HPLC with radio detection. Water was extracted by solid phase extraction with LiChrolut EN, a non-ionogenic, highly porous polystyrene−divinylbenzene polymer (from Merck, Darmstadt, Germany) and elution was performed with ethyl acetate. Triazines in wort and beer were isolated by means of Extrelut (diatomaceous earth, Merck) and ethyl acetate. The organic extract (wort or beer) was cleaned on a silica gel column, a strong cation exchange (SCX, benzenesulfonic acid), and an octadecyl-bonded silica cartridge before determination by HPLC (Hack et al., 1996). Unlabeled triazines were identified and determined quantitatively by radioisotopic dilution analysis. For metabolite determination sodium chloride was added to the concentrated beer sample before blending with Extrelut and elution with ethyl acetate/2-propanol (80/20). After solvent evaporation the residue was redissolved in methanol and transferred quantitatively to the origin of a TLC plate (0.5 mm, silica gel, Merck). The plate was first developed with ethyl acetate/hexane (60/40). A second development with toluol/acetic acid/water (50/50/3) improved the separation of the more polar compounds. [^{14}C]Triazines were located by the TLC analyzer, and the regions were scraped from the plate. In a 20 mL vial the silica gel fraction was mixed with scintillation cocktail; radioactivity was determined by LSC. Identification of the hydroxytriazines OHA and OHT in beer was accomplished after cleaning the Extrelut extract by cation exchange chromatography on a Dowex 50 W-X2 resin (Ramsteiner and Hörmann, 1979). The extract was further purified by preparative TLC as described above. Scraped off silica gel was percolated with ethyl acetate/methanol (1/1), evaporated to dryness, and redissolved in acetonitrile/acetic acid (1%). Finally, the SCX cartridge procedure, as described

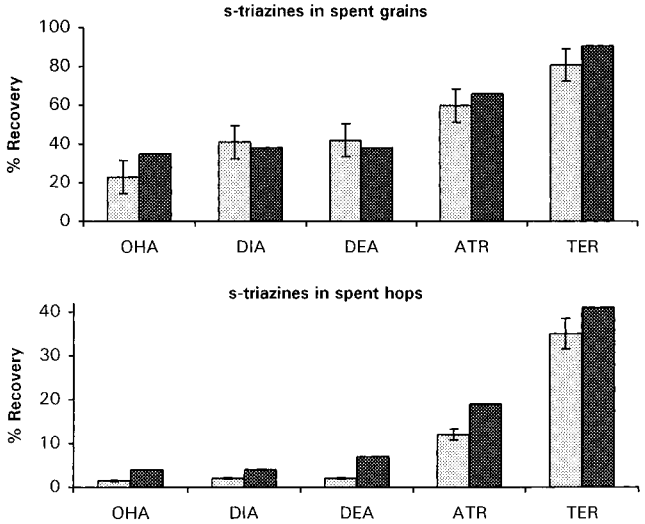

Figure 2. (Light columns) [^{14}C]-*s*-Triazine residues found in the spent grains (referring to the amount in the brewing liquor) and in the spent hops (referring to the amount in the first wort) with error bars as standard deviations (triplicate determinations). (Dark columns) Values derived from the triazine content in the wort.

above, was applied before determination by HPLC with UV/DAD and radio detection. Analyte identity was confirmed by GC−MS after methylation with diazomethane (Färber et al., 1994).

RESULTS AND DISCUSSION

Mashing and Cooking Process. As described in literature *s*-triazines and metabolites turned out to be very stable compounds. During mashing and cooking no metabolism was observed. Maximum temperatures of more than 100 °C caused no chemical reactions involving triazines. Figure 2 shows that a considerable amount of triazines adsorbed on spent grains and spent hops, while the rest was found in the wort. The amount of ^{14}C in the dried material was determined after combustion in the oxidizer and was generally lower than the amount derived from the triazine content in the wort. The quantity of adsorbed triazines depended on the polarity of the compound and on the amount of suspended material. While 23−42% (35−38%, respectively) of the main degradation products of atrazine were found in the spent grains, more than 80% (90%) of the total amount of terbutylazine adsorbed there. The same relative percent radioactivity was found when using different triazine concentrations (range from 0.1 to 5.0 ppb). Besides, the percentage of employed radionuclide was contingent upon the amount of malt grist used for mashing in. The higher the perfect primary of the wort, the higher the quantity of resulting spent grains and the higher the total amount of triazines in them. This was due not only to forces of adsorption but also to the higher water content in the spent grains after filtration.

During cooking the largest part of the triazine metabolites remained in the wort. Only a small amount was found in the distillate (1−2%) and on the spent hops [1.5−2% (4−7%)]. The parent triazines showed greater adsorption affinity, for instance, 35% (41%) of the terbutylazine originally included in the first wort [=3.2% (3.7%) of the total] was found in the spent hops (Figure 2). Table 1 shows that during beer production triazine reduction occurred mainly during the mashing process.

Fermentation. In our investigations with radiolabeled atrazine and terbutylazine, the formation of triazine metabolites was observed. The degradation

Table 1. Course of Triazine Concentrations during Beer Production in the Laboratory Scale (Bottom-Fermented Beer)[a]

	s-triazine concentration (μg/L)			
[^{14}C]-*s*-triazine	brewing liquor	first wort	finished wort	beer
TER	1.00[b]	0.12 ± 0.009	0.07 ± 0.010	0.07 ± 0.010
ATR	1.00[b]	0.45 ± 0.012[c]	0.42 ± 0.011	0.40 ± 0.010
OHA	1.00[b]	0.92 ± 0.021	0.93 ± 0.015	0.92 ± 0.014
DEA	1.00[b]	0.94 ± 0.018	1.01 ± 0.017	1.00 ± 0.016
DIA	1.00[b]	0.94 ± 0.019	1.02 ± 0.018	1.01 ± 0.017

[a] Concentrations were determined by measuring the radioactivity with liquid scintillation counting (LSC). [b] Spiked [^{14}C]-*s*-triazine concentration. [c] Standard deviation calculated from seven determinations.

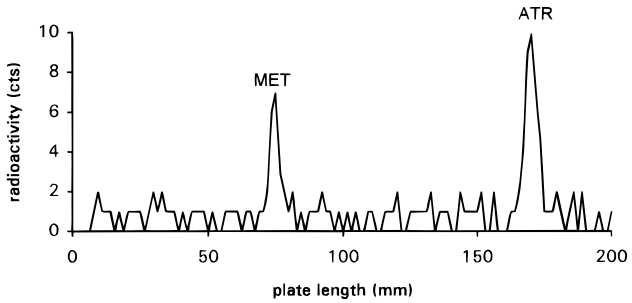

Figure 3. TLC chromatogram of a beer extract from top-fermented beer (after 10 weeks of storage).

products, which were identified as the hydroxy analogues (OHA and OHT), were mainly detected in top-fermented beers. When the fermented beer was stored without sheet filtration, nearly half of the total herbicide was converted after 10 weeks (Figure 3). In contrast to this, small amounts of metabolite were detected in bottom-fermented beers only after 4 months.

A detailed study on the course of the dechlorination reaction was carried out for atrazine. In the first 10 weeks after starting top fermentation, metabolite formation was determined weekly. Its course is illustrated in Figure 4. The metabolism of atrazine followed first-order kinetics. Half-life ($T_{1/2}$) was determined by ln(2/k) and was 98 days. By plotting the log (% atrazine) versus time, a straight line could be obtained, and the rate constant was derived from it ($R^2 = 0.9847$).

Fermentation studies showed that top-fermenting yeasts (*Saccharomyces cerevisiae*) had a much greater ability to convert triazines into their hydroxylated product than bottom-fermenting yeasts (*Saccharomyces carlsbergensis*). Ale yeasts are known to produce a wider spectrum of fermentation byproducts. Moreover, they can be differentiated from the lager yeasts by the higher metabolism activity and yeast propagation (Narziss, 1980). Considering these distinguishing characteristics, an explanation for the different behavior of the two brewery yeasts may be given.

Various microorganisms are capable of degrading atrazine and other triazines (Giardina et al., 1982; Behki and Khan, 1986, 1994; Kontchou and Gschwind, 1995; Van Zwieten and Kennedy, 1995). Metabolism of atrazine by yeasts was examined by Donhauser et al. (1992). In contrast to our findings they report the conversion to deethylatrazine in both top-fermented and bottom-fermented beer.

Identification of Degradation Compound. Due to its strong polarity, the isolation of the degradation product from beer caused some difficulties. Recovery for extraction with ethyl acetate was below 80%. The polarity of the extracting agent was increased by adding

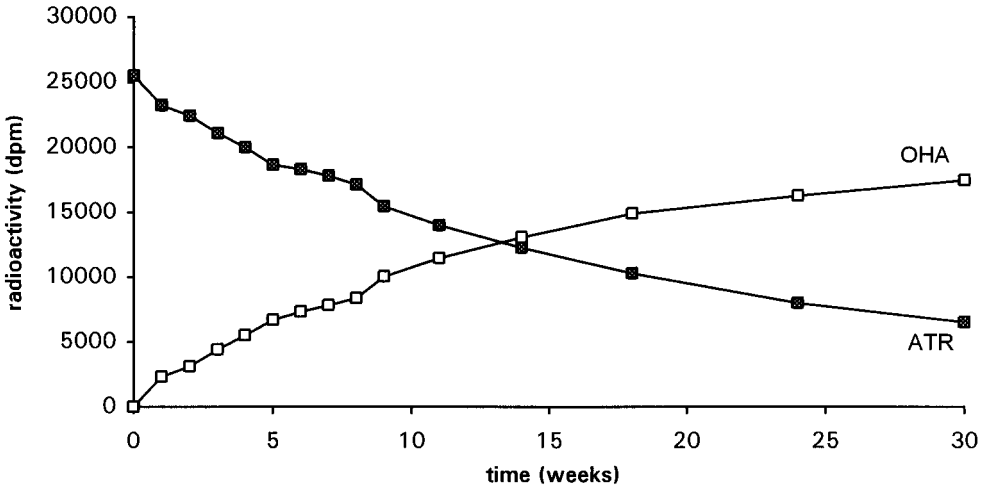

Figure 4. Course of atrazine degradation and hydroxyatrazine formation in top-fermented beer.

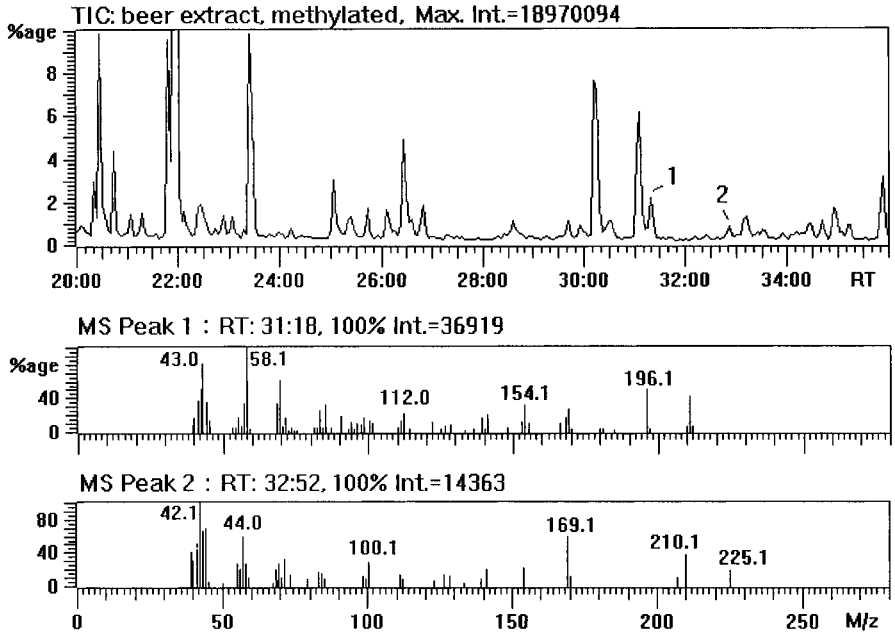

Figure 5. Determination of OHA and OHT after methylation to atratone and terbumetone by GC−MS: TIC chromatogram of the prepared beer extract; mass spectra (EI) of peaks 1 and 2 corresponded to atratone and terbumetone standard material, respectively.

20% 2-propanol, but this resulted in a co-extraction of small amounts of water. For this reason, sodium chloride was added to the beer sample. In the first cleanup step, the Dowex 50 W-X2 cation exchange resin proved to be suitable for removing the majority of the polar beer ingredients. Further cleanup by preparative thin-layer chromatography was sufficient to determine the retention time of the metabolite by HPLC with radiometric detection. An SCX cation exchange procedure, successfully employed for the dealkylated atrazine metabolites before, was the last cleanup step required for the determination of OHA and OHT by HPLC with UV (DAD) detection. After methylation (to atratone and terbumetone) the compounds were identified by GC−MS (Figure 5). Their mass spectra corresponded very well with those of atratone and terbumetone standard material.

Filtration. Assays were performed with kieselguhr containing folded filters, but no remarkable amounts of triazines were adsorbed. Thus, no further loss of triazines was noticed after fitration.

Behavior of Triazines in the Pilot Plant. The behavior of triazines during the brewing process in the pilot plant confirmed the results obtained from the laboratory plant. Figure 6 (Table 2) shows the course of triazines from water to beer during beer production (top-fermented beer). A sample was taken out after each individual step. The unlabeled herbicides were quantified by radioisotopic dilution analysis (Figure 7). The values corresponded well with those from the laboratory plant (Table 3). Due to hydrolysis, a constant loss of herbicides was noted during fermentation and storage of top-fermented beer. When the concentrations of the starting compounds were decreasing constantly, the concentrations of their corresponding hydroxylated products were increasing. From all metabolites that were expected in the beer after fermentation only OHA and OHT could be determined. For this reason, no confirmation could be given whether the dealkylated triazines underwent hydrolysis as well.

CONCLUSION

As the studies performed in the laboratory scale could be applied to the pilot scale, they should as well be transferable to the technological scale. With the results of this study, predictions can be made about the fate of the used triazines in the brewing process. Measures

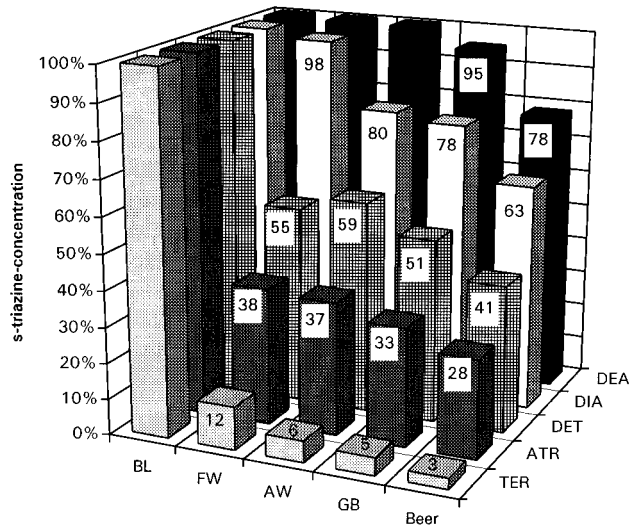

Figure 6. Decrease of the different *s*-triazine herbicides from brewing liquor to beer in the pilot plant (top-fermented beer): BL = brewing liquor, FW = first wort, AW = finished wort, GB = green beer.

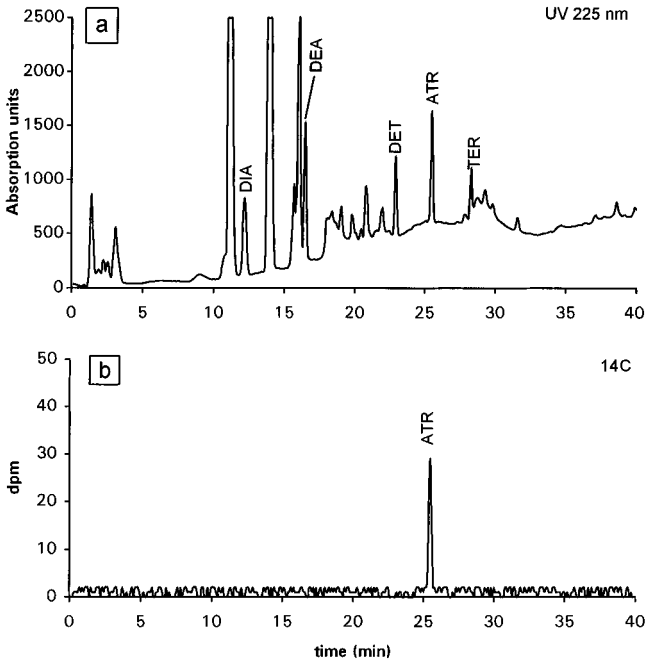

Figure 7. Liquid chromatogram of a beer sample determined with isotopic dilution analysis; simultaneous detection by (a) UV and (b) radioactivity.

Table 2. Course of Triazine Concentrations during Beer Production in the Pilot Plant (Top-Fermented Beer)[a]

	s-triazine concentration (μg/L)				
s-triazine	brewing liquor	first wort	finished wort	green beer	beer
TER	5.00[b]	0.60 ± 0.063	0.30 ± 0.034	0.25 ± 0.042	0.15 ± 0.060
ATR	5.00[b]	1.90 ± 0.033	1.85 ± 0.039	1.65 ± 0.051	1.40 ± 0.033
DET	5.00[b]	2.75 ± 0.058	2.95 ± 0.052	2.55 ± 0.063	2.05 ± 0.032
DEA	5.00[b]	4.90 ± 0.135	4.00 ± 0.189	3.90 ± 0.176	3.15 ± 0.085
DIA	5.00[b]	5.00 ± 0.146	5.15 ± 0.223	4.75 ± 0.275	3.90 ± 0.262

[a] Concentrations were determined by HPLC-UV after extraction and cleanup. [b] Spiked *s*-triazine concentration.

can be taken in order to prevent that triazine herbicides become beer pollutants. Breweries often have their own wells. In 1991 and 1992, almost half of the examined well samples in Germany were contaminated with atrazine (Glas, 1993). Particular attention has to be paid to the polar atrazine degradation products. In recent years, their determination in drinking water has gained more importance. Monitoring of these substances in the brewing water is essential, since their almost complete transition into the beer could be foreseen. In view of the possible damaging to one's health, triazines and their degradation products in contaminated water have to be eliminated or at least reduced. This could be accomplished by different methods, i.e., filtration with activated carbon, adsorption on ion exchange resins in deionization units, reverse osmosis (Glas, 1993), UV/ozone photolysis (Meesters et al., 1995; Zwiener et al., 1995), and photodecomposition with salts of ferric ion (Larson et al., 1991; Sun and Pignatello, 1993).

Table 3. Comparison of Triazine Concentrations during Production of Bottom-Fermented Beer in the Laboratory Scale (LS) and in the Pilot Plant (PP)

	s-triazine concentration (%)							
	brewing liquor		first wort		finished wort		beer	
s-triazine	LS	PP	LS	PP	LS	PP	LS	PP
ATR	100	100	45	39	42	37	40	39
TER	100	100	12	13	7	8	7	6
DEA	100	100	94	100	101	103	100	100
DIA	100	100	94	98	102	98	101	97

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