Dipl.-Chem. David Siegel

Chemical Solutions to Current Issues in the Instrumental Quantification of Food Mycotoxins

BAM-Dissertationsreihe • Band 71 Berlin 2011 Die vorliegende Arbeit entstand an der BAM Bundesanstalt für Materialforschung und -prüfung.

Impressum

Chemical Solutions to Current Issues in the Instrumental Quantification of Food Mycotoxins

2011

Herausgeber: BAM Bundesanstalt für Materialforschung und -prüfung Unter den Eichen 87 12205 Berlin Telefon: +49 30 8104-0 Telefax: +49 30 8112029 E-Mail: info@bam.de Internet: www.bam.de

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Layout: BAM-Arbeitsgruppe Z.64

ISSN 1613-4249 ISBN 978-3-9813853-8-0

Chemical Solutions to Current Issues in the Instrumental Quantification of Food Mycotoxins

DISSERTATION

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) im Fach Chemie

eingereicht an der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin

von

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Tag der mündlichen Prüfung: 01.02.2011

"The simple truth is, that up to this period all analysis has failed; and until Von Kempelen chooses to let us have the key to his own published enigma, it is more than probable that the matter will remain, for years, *in status quo*.

All that yet can fairly be said to be known is, that 'pure gold can be made at will, and very readily from in lead in connexion with certain other substances, in kind and in proportions, unknown.'"

-Edgar Allen Poe, Von Kempelen and his discovery, 1850

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Preface

This dissertation was prepared from September 2007 to September 2010 at the Bundesanstalt für Materialforschung und –prüfung / Federal Institute for Materials Research and Testing (Division 1.2, Berlin, Germany) and the Humboldt-Universität zu Berlin (Department of Chemistry, Berlin, Germany) under the supervision of Prof. Dr. Ulrich Panne. The presented results were further published in international peer-reviewed journals [1-5] and included in a review article on mycotoxin analysis [6]. All relevant contributors appear in the respective author lists. Several relevant X-ray crystal structures were determined and published [7-10] as well. They will be shown below without further discussion. The reader is kindly referred to the respective publications¹ for experimental details.

To maintain the readability of the text, all instrumentation, methods, relevant compounds and mixtures thereof have been indexed. The instrumentation and method indexes will appear in curly brackets. {M2} indicates, for instance, that the concerned results were obtained using method M2. The corresponding index lists may be found in the materials and methods section. Relevant chemical compounds or mixtures are numbered consecutively according to the first occurrence in the dissertation. The index numbers are given in bold print, e.g. **2**, **3** or (**4**). Table 15 summarises all indexed compounds. Systematic names obtained by chemical nomenclature are given in Table 15 only and will not appear in the text.

Mycotoxins are commonly referred to using acronyms, e.g. ZON for zearalenone. If a commonly accepted acronym is available it is used in conjunction with the compound index, i.e. ZON **10**. Otherwise, only the index number is shown. Structures representing tautomers are labelled by the number of the principal compound followed by a small letter, e.g. **5a**, **5d**. Units of variables are given in square brackets, e.g. m_{sample} [g]. Molar concentrations of compounds are referred to by the compound identifier in square brackets, e.g. [ZON **10**].

The scientific literature is discussed as at August 2010.

¹ Available free of charge on http://journals.iucr.org/e/.

Abstract

Mycotoxins are toxic secondary metabolites of ubiquitously occurring moulds. Through the consumption of contaminated foods, they can cause acute or chronic intoxications in humans. Here, it is demonstrated how covalent hydrazine chemistry can be used to improve the performance of instrumental methods for the quantification of trace level food mycotoxins. In the case of the *Alternaria* mycotoxin tenuazonic acid, pre-column derivatisation with 2,4-dinitrophenylhydrazine resolved chromatographic issues due to the chemical properties of the analyte and allowed for its rapid, sensitive and selective quantification in cereals and beer by high performance liquid chromatography-ion-trap two stage mass spectrometry (HPLC-IT-MS²). Tenuazonic acid could be detected for the first time in beer and buckwheat flour. Although the encountered levels were too low to cause acute intoxications, the frequency of contamination indicated possible health risks due to chronic exposure.

In a second scenario, dynamic covalent hydrazine chemistry (DCHC) was exploited for a novel extraction and cleanup method applicable to the *Fusarium* mycotoxin zearalenone occurring in edible oils. Zearalenone was extracted by hydrazone formation on a hydrazine-functionalised polymer resin and subsequently released hydrolytically for quantification by HPLC-fluorescence detection (HPLC-FLD). The high selectivity of the approach allowed for the omission of MS detection and immunoaffinity cleanup. The DCHC method was superior to previously published methods in terms of handling efforts, cost, precision and selectivity and is well suited for the monitoring of the current European maximum level for zearalenone in refined maize oil.

In the second part of the dissertation, possible degradation routes of *Alternaria* mycotoxins upon storage and bread baking are discussed. In the frame of a kinetic study, it was shown that tenuazonic acid is degraded by two parallel processes, deacetylation and epimerisation, when stored in aqueous solution (half-life at 25 °C ~ 74 days). The primary degradation product deacetyl tenuazonic acid was less stable than its parent compound and degraded rapidly in beverage matrices. In model baking experiments it was furthermore revealed that alternariol, alternariol monomethyl ether and altenuene are stable under typical baking conditions. A newly identified degradation route, which is based on a sequence of hydrolysis and decarboxylation, caused only minor substance losses (< 1 %). Still, the degradation products could be detected in commercial rusk and crispbread by HPLC-tandem mass spectrometry (HPLC-MS/MS).

Deutschsprachige Zusammenfassung

Mykotoxine sind sekundäre Stoffwechselprodukte ubiquitärer Schimmelpilze. Über den Konsum belasteter Nahrungsmittel können sie beim Menschen, je nach Art und Ausmaß der Exposition, akute bzw. chronische Vergiftungen hervorrufen. Ziel der Arbeiten war es, durch chemische Methoden zwei Probleme aus dem Bereich der quantitativen Mykotoxinanalytik zu lösen. Hierbei ging es erstens um die Inkompatibilität des Alternaria Mykotoxins Tenuazonsäure mit üblichen Hochleistungsflüssigkeitschromatographie (HPLC) Säulen und zweitens um die selektive analytische Extraktion des in der Europäischen Union regulierten Fusarium Mykotoxins Zearalenon aus Speiseölen. Für beide Problemstellungen wurden Lösungen erarbeitet, die auf dem Einsatz kovalenter Hydrazinchemie im Rahmen der Probenvorbereitung beruhen. Die Verfahren wurden validiert und auf Verbraucherprodukte angewendet. Die Bestimmung der Tenuazonsäure erfolgte dabei nach Derivatisierung mit 2,4-Dinitrophenylhydrazin mittels HPLC-Ion-trap Massenspektrometrie (HPLC-IT-MS²). Für Zearalenon wurde eine neuartige Festphasenextraktionsmethode basierend auf dynamischer kovalenter Hydrazinchemie (DCHC) entwickelt und in Verbindung mit HPLC-Fluoreszenzdetektion (HPLC-FLD) eingesetzt. Beide Ansätze zeichnen sich durch hohe Selektivität, einfaches Handling und einen geringen Lösungsmittelverbrauch aus; alle Reaktionen erfolgen bei Raumtemperatur. So konnte Tenuazonsäure erstmals in Getränken (Bier) und Buchweizenmehl nachgewiesen werden.

Der zweite Teil der Arbeit beschäftigt sich mit möglichen Abbaureaktionen der *Alternaria* Mykotoxine bei der Lagerung und Zubereitung von Lebensmitteln. Im Rahmen einer kinetischen Studie wurde gezeigt, dass Tenuazonsäure in wässriger Lösung über zwei parallele Prozesse, hydrolytische Deacetylierung und Epimerisierung, abgebaut wird (Halbwertszeit bei 25 °C ~ 74 Tage). Während das vornehmlich gebildete Produkt, Deacetyl-Tenuazonsäure, in wässriger Lösung stabil war, wurde es in Getränkematrices innerhalb weniger Tage abgebaut.

In Modell-Backversuchen wurde weiterhin die Stabilität der Dibenzo-α-pyron Derivate Alternariol, Alternariol-monomethylether und Altenuen untersucht. Unter typischen Backbedingungen erwiesen sich die Verbindungen als stabil. Ein erstmals belegter Abbaumechanismus für Alternariol und Alternariol-monomethylether, der auf einer Sequenz aus Hydrolyse und Decarboxylierung beruht, führte nur zu geringen Substanzverlusten (< 1 %). Dennoch konnten die Abbauprodukte mittels HPLC-Tandem-Massenspektrometrie (HPLC-MS/MS) in extrudierten Produkten (Knäckebrot und Zwieback) nachgewiesen werden.

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Abbreviations

Refer to Table 15 (page 87) for abbreviations of indexed compounds.

| r wavelength | |
|---|--------|
| a.u. Arbitrary units | |
| AcOH Acetic acid | |
| ADI Acceptable daily intake | |
| ALA Alimentary toxic aleukia | |
| aq. Aqueous | |
| BP Boiling point | |
| bw Body weight | |
| CAD Collision gas (instrument setting) | |
| CE Collision energy (instrument setting) | |
| cf. Confer | |
| CID Collision induced dissociation | |
| CoA Coenzyme A | |
| cps Counts per second | |
| CUR Curtain gas (instrument setting) | |
| CXP Cell exit potential (instrument setting) | |
| DA Diode array | |
| DAD Diode array detector/detection | |
| DCHC Dynamic covalent hydrazine chemistry | |
| dG [‡] Gibbs energy of activation | |
| dH [‡] Enthalpy of activation | |
| DNA Deoxyribonucleic acid | |
| DP Declustering potential | |
| dS [‡] Entropy of activation | |
| DTA Differential thermo analysis | |
| DTG Differential thermo gravimetry | |
| E _A Activation energy | |
| EDTA Ethylenediaminetetraacetic acid | |
| EFSA European Food Safety Authority | |
| EP Entrance potential (instrument setting) | |
| ESI Electrospray ionisation | |
| EtOAc Ethyl acetate | |
| EU European Union | |
| Et ₂ O Diethyl ether | |
| FAO Food and Agriculture Organisation (of the United Na | tions) |

| FL | Fluorescence |
|----------------------------|---|
| FLD | Fluorescence detector/detection |
| GAP | Good agricultural practice |
| GC | Gas chromatography |
| GPC | Gel permeation chromatography |
| GS1/2 | GAS 1/2 (instrument setting) |
| h | Planck constant |
| HMBC | Heteronuclear multiple bond coherence |
| HMQC | Heteronuclear multiple quantum coherence |
| HPLC | High performance liquid chromatography |
| HSQC | Heteronuclear single quantum coherence |
| IA | Immunoaffinity |
| IAC | Immunoaffinity cleanup |
| i.e. | id est |
| IS | Ion-spray voltage (instrument setting) |
| IT | Ion-trap |
| IUPAC | International Union of Pure and Applied Chemistry |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| k | Rate constant |
| $k_{\scriptscriptstyle B}$ | Boltzmann constant |
| LD ₅₀ | Median lethal dose |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| ME | Matrix effect |
| MeCN | Acetonitrile |
| MeOH | Methanol |
| ML | Maximum level |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| MS^n | n-stage mass spectrometry |
| MSI | Minimum sample intake |
| NMR | Nuclear magnetic resonance spectroscopy |
| p.a. | pro analysi |
| PA | Peak area |
| рК _А | Acid dissociation constant |
| QT | Quantifier |
| QL | Qualifier |
| R | Gas constant |

| R^2 | Square of correlation coefficient |
|----------------|---|
| RASFF | Rapid Alert System for Food and Feed |
| RDS | Rate determining step |
| R _H | Hydrodynamic radius |
| rpm | Revolutions per minute |
| RSD | Relative standard deviation |
| RT | Room temperature |
| SAM | S-Adenosyl methionine |
| SCF | Scientific Committee for Food |
| SD | Absolute standard deviation |
| spp. | Several species |
| TA | Thermo analysis |
| TEM | Nebulisation temperature (instrument setting) |
| TFA | Trifluoroacetic acid |
| TG | Thermo gravimetry |
| THF | Tetrahydrofuran |
| T_{on}^{ex} | Extrapolated onset temperature |
| t _R | Retention time |
| USA | United States of America |
| UV | Ultraviolet |
| WHO | World Health Organisation |
| | |

1 Introduction

1.1 Fungi and mycotoxins

Fungi are eukaryotic organisms ubiquitously occurring as yeasts, mushrooms or moulds. By supporting the decomposition of organic matter like dead plant material or deceased animals, they take a vital part in the natural recycling processes which sustain evolution. However, the desirable fungal *biodegradation* may turn into a problematic *biodeterioration* if raw materials or food are infected. The apparent reasons for this are changes in looks, taste, smell and consistency of the infected produce. While these changes are usually easily recognised, fungal infection may also be accompanied by a release of toxic compounds. Such a contamination is not readily perceived and thus represents a significant non-obvious aspect of fungal *biodeterioration*.

Besides the products of their life-sustaining primary metabolism, fungi biosynthesise and release a wide range of biologically active secondary metabolites. Some of these compounds qualify as drugs, a famous example being the first antibiotic known to man, Penicillin G (1), a secondary metabolite of *Penicillium chrysogenum* discovered by Alexander Fleming in 1928. Other metabolites, however, are toxic and hence unsuited for medicinal uses (Figure 1).

Mycotoxins are, *per definitionem*, toxic secondary metabolites of Fungi imperfecti and ascomycota, including those fungal genera which are colloquially referred to as moulds. Compounds produced by poisonous mushrooms are not covered by this definition. Today, 300–400 mycotoxins and their respective biological conversion products [11] are known, belonging to approximately 300 fungal species [12-14]. Mycotoxins are mostly small (M < 1,000 g/mol), heat stable molecules [11], displaying a large structural and toxicological diversity. Pyrones, anthraquinones, coumarins, macrocyclic lactones, steroids and cyclic polypeptides are possible structural elements while the exerted pathogenic effects range from slight skin irritations to severe organ impairment to the genesis of malignant tumours [15].

The reasons for the production of mycotoxins by moulds are understood only to a limited extent [15]. Some mycotoxins trigger cellular differentiation processes in the fungal thallus [16], thus fulfilling a hormone like function. Pathogenic fungi are known to use phytotoxic mycotoxins to weaken plant hosts (cf. 1.8.1). Eventually, the antimicrobial or even antifungal activity [17] of certain mycotoxins suggests a defence mechanism directed against competitors like bacteria or other fungal species. In any case, the adverse impact on humans and mammals appears to be a merely collateral effect.



Figure 1

Exemplary structures of fungal secondary metabolites: a differentiation between mycotoxins and antibiotics is not always possible.

- 1: Penicillin G, a classical, non-toxic antibiotic drug of fungal origin
- 2: Streptomycin, an antibiotic drug of fungal origin showing toxic side effects
- 3: Patulin, a highly cytotoxic mycotoxin which also acts as an antibiotic
- 4: Aflatoxin B₁, a highly carcinogenic, non-antibiotic mycotoxin

1.2 Mycotoxicoses

Mycotoxicoses are intoxications caused by mycotoxins. They are usually triggered by the consumption of contaminated feed or food [18]. Almost all recent outbreaks of human mycotoxicoses can be related to a lack of GAP (good agricultural practice) favouring the growth of moulds, e.g. the wet storage of harvested grains (Table 1). For this reason, acute mycotoxicoses in humans are not of great concern to the industrialised Western world [18], where GAP is largely established. Here, the vast majority of fatalities related to foodborne disease are caused by bacterial pathogens [19].

Still, though seemingly contradictory, mycotoxins are recognised as a major concern in scientific, economic and political arenas [6, 20-22]. This is due to the high risk of subacute or chronic intoxications which is sustained by the ubiquitous occurrence of low mycotoxin quantities in foods as well as by the often severe carcinogenicity, mutagenicity or teratogenicity of the compounds [11]. A key event in this respect is the discovery of aflatoxin B₁ (4) in 1964 [13, 15]. This *Aspergillus* spp.¹ mycotoxin is considered the most potent natural carcinogen known [23] and has consequently motivated researchers as well as regulatory authorities to focus on the chronic effects caused by the repeated ingestion of low quantities of mycotoxins.

¹ spp. = several species

| Disease | Symptoms, progression | Incidences | Causative mycotoxins |
|--|--|--|--|
| Ergotism, also known as "Saint Anthony's Fire" or "Holy Fire" | Gangrenous (necrotic) form: Lassitude, prickling or icy cold sensation in the limbs, muscular pains, dulled intellect, inflammation and painful swelling of the limbs, burning pains, skin covered with red/violet vesicles. With the inset of gangrene toes and fingers become necrotic (black). In severe cases loss of fingers and toes or even of all four limbs. <i>Convulsive (neurological) form:</i> Sustained spasms, muscle cramps, a tingling sensation under the skin, constriction of the blood vessels followed by mortification of the limbs, hallucination. Mortality: 11–60 % [23]. | Commonly observed in the 9 th and 10 th century, with the first clear report being given in 1582. Hence, frequently thematised in medieval art [24]. Last occurrence in Europe as late as 1951/52 [15]. Outside Europe, 47 Ethiopians died in 1978 after consuming wild oat weeds infected by <i>Claviceps purpurea</i> [23]. Today extremely rare. | Ergot alkaloids produced by <i>Claviceps</i> spp., mainly <i>Claviceps</i> <i>purpurea</i> (gangreneous form) and <i>Claviceps paspali</i> (convulsive form) infecting rye and other grains [23] |
| Alimentary toxic aleukia (ALA) | First stage (duration: 3–9 days): burning sensations, emesis, diarrhoea, decrease of white blood cells. Second stage (3–4 weeks): disorder of bone marrow functions, progressive reduction of white blood cells. Final stage: haemorrhages, necrotic changes in the mouth, throat and oesophagus, bacterial infections, enlargement of the lymphatic glands. Death through strangulation due to swellings in one third of the cases. Mortality: 2–80 % [23]. | Endemic in Russia from 1932 to 1947. Reasons include mild winters with heavy snow in combination with alternate freezing and thawing. Also, grains were often left on field for too long due to the war-related lack of harvesters. ALA is held responsible for hundreds of thousands of fatalities in Russia [23]. Today extremely rare. | T-2 toxin and diacetoxy- scirpenol produced by <i>Fusarium</i> spp. infecting grains [23] |
| Acute aflatoxicosis | Vomiting, anorexia (lack of appetite), icterus, oedema of the lower extremities, pathological changes of the liver, in severe cases fulminant liver failure [23, 25]. Mortality: ~ 25 % [23]. | Most common modern mycotoxicosis, occurring mainly in developing countries. Recent, severe incidences in Kenya with 125 fatalities in 2004, 32 in 2005 and 21 in 2006 [25-27]. Previous incidences in north-west India in 1974 (104 fatalities) [23]. Usually related to bad agricultural practices, e.g. storage of maize under humid conditions. | Aflatoxins [23] produced by <i>Aspergillus</i> spp., mainly <i>Aspergillus</i> <i>flavus</i> infecting maize [25] |

Table 1 Overview of important mycotoxicoses.



Figure 2 Medieval ergotism patients suffering from the loss of limbs. Drawing of Pieter Bruegel the Elder, 1558, taken from [24].

1.3 Food mycotoxin risk assessment and regulation

On the world scale JECFA, the Joint Expert Committee on Food Additives, a scientific advisory board of the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO), evaluates mycotoxin related risks. In the European Union (EU), the mycotoxin issue is scientifically attended to by the European Food Safety Authority (EFSA), which advises the European Commission. In 2001 the Commission's Scientific Committee for Food (SCF) initially established maximum levels (MLs) for aflatoxins, ochratoxin A and patulin in food (EU regulation 466/2001) [28]. This regulation replaced former national legislation. It was updated several times and substituted in 2006 by EU regulation 1881/2006 [29] which was further updated in 2007 and 2010 [30, 31]. *Summa summarum*, the EU has implemented the most extensive and detailed regulations for food mycotoxins worldwide [11]. The MLs established to date (Table 2) are binding in all member states.

1

| Mycotoxins | ML range: lowest-highest [µg/kg] |
|---|---|
| Aflatoxins B_{1-2} , G_{1-2} (sum of 4) | 0.1 (infant food)–15 (unprocessed peanuts) |
| Aflatoxin M ₁ | 0.025 (infant food)–0.05 (milk) |
| Deoxynivalenol | 200 (infant food)–1,750 (unprocessed maize) |
| Fumonisins B ₁₋₂ (sum of 2) | 200 (infant food)-4,000 (unprocessed maize) |
| Ochratoxin A | 0.5 (infant food)–80 (liquorice extract) |
| Patulin | 10 (infant food)–50 (fruit juices) |
| Zearalenone (ZON 10) | 20 (infant food)-400 (refined maize oil) |

Table 2Current ranges of MLs for food mycotoxins in the EU (August 2010) [29-31].

1.4 Mycotoxin monitoring in the EU and Germany

Since 1979, ML violations in EU member states are entered into the database of the RASFF (Rapid Alert System for Food and Feed), which is publicly accessible [32]. RASFF notifications may be based on official market controls, border controls, own controls of companies, the consumer, the media or notification by non EU-countries [33]. From 2003 to 2008 mycotoxins were the hazard category with the highest number of RASFF notifications. This highlights the great importance of mycotoxins in the current European food safety discussion.

The actual number of official controls may vary between the EU member states. In Germany, it is the federal states' responsibility to conduct food control analyses. In 2007, 402,463 food samples were analysed; 5,919 (1.5%) of these samples violated existing regulations in the category of "mycotoxins, pesticides, acrylamide" [34].

In addition to the regulatory controls, federation and states have established a common foodstuffs monitoring programme supervised by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (Federal Office of Consumer Protection and Food Safety) [35]. In the frame of this programme, a product basket representative of a typical German diet is compiled. Each year, approximately 5,000 samples are sourced accordingly and analysed for various contaminants, including mycotoxins. In 2008, the programme identified aflatoxins in rice as well as ochratoxin A in liquorice and cocoa as possible, but not alarming health risks for the German population. Further monitoring was recommended [36].



Figure 3 Graphical representation of RASFF notifications since the introduction of the harmonised EUwide mycotoxin regulations in 2001, data taken from [33, 37-42].

1.5 The importance of food mycotoxin analysis

Moulds are well known by consumers, as they visibly infect fruits and vegetables as well as water-containing food products like juices, yoghurt and cheese. Normally, obviously mouldy products are not consumed, so that ingestion of mycotoxins is avoided by default¹. However, mould growth may occur throughout the production chain of a food product (i.e. "from farm to fork"). Wheat, for instance, may be infected and contaminated with mycotoxins while still in the field. If milled and processed into bread, it is impossible for a bakery client to detect a possible contamination of his purchase. For these reasons, laboratory based chemical analysis is an essential requirement in the areas of risk assessment and consumer protection.

Furthermore, it can be pointed out that, according to applicable EU law [43], all food business operators have the "primary legal responsibility" for the safety of their products. With regard to the current EU regulations, this means that mycotoxin analysis is a mandatory quality control step for farmers, traders and the primary (e.g. millers, malsters) and secondary (e.g. bakers, brewers) processing industry.

Apart from that, mycotoxin analysis also offers a range of scientific challenges. Current research in the field primarily deals with the high number of possible analytes (over 400 known mycotoxins, *vide supra*) and the analytical issues encountered due to the chemical complexity,

¹ Certain toxicologically "safe" fungal species are used for food fermentation and are hence deliberately consumed. Examples of such fermented products include Roquefort, Gorgonzola or Stilton cheeses, as well as raw sausages and hams [15].

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inhomogeneity and variability of most food products [6, 44-46]. Driven by the regulatory and economic impact of food mycotoxins, the number of studies on efficient quantification methods, new sample preparation and detection principles as well as on chemical changes during storage and preparation of analytical samples has increased significantly. In the past decade, approximately two mycotoxin related articles were published per day, one of which was concerned with analytical aspects (Figure 4). The conclusion which can be drawn from all these aspects is that mycotoxin analysis and the topic of mycotoxins in general are vivid and highly relevant spheres of interest to a wide range of stakeholders, i.e. regulatory authorities, food business operators, scientists, manufacturers of analytical equipment etc.



Figure 4 ISI publication numbers¹.

1.6 The importance of food mycotoxin chemistry

Regulatory mycotoxin risk assessment generally requires data on toxicity, occurrence in foods and intake of the respective foods by the population [21]. In addition to these factors, the chemical behaviour of mycotoxins during food processing needs to be understood. As organic compounds mycotoxins may be subject to a variety of chemical processes. In principle, these may be irreversible or reversible and can lead to products being either more or less toxic than the parent compound. Chemical changes can be expected particularly upon thermal treatment (i.e. cooking, baking, extrusion processing etc.) [47].

A current example of possible issues evolving from mycotoxin chemistry are the so-called

¹ Data were obtained from ISI Web of Knowledge in August 2010. The utilised queries were "TOPIC= mycotoxin* AND analysis" and "TOPIC = mycotoxin* AND NOT analysis".

hidden or masked fumonisins, which are found mainly in processed maize products like cornflakes [48-51]. Fumonisins are primarily produced by *Fusarium* spp. [23] and are currently regulated in the EU (Table 2). The existence of masked fumonisins was inferred from the observation that, for a given food product, the detectable fumonisin content was higher, if an alkaline hydrolysis step was performed before quantification [49]. It was thus postulated that processed maize products may contain a significant fraction of fumonisin molecules which are not free, but covalently bound to starch or proteins [49, 52]. These masked fumonisins are not detected by routine analysis methods [49]. Even so, it can be speculated that they are at least partially released under the acidic conditions of the digestive system. The result is an underestimation of the true toxic potential of the concerned foods.

For the sum of these reasons, investigations on the chemical behaviour of mycotoxins during food storage and processing are of high importance. Generally, little is known in this respect, as most studies focus only on the quantitative aspects of mycotoxin degradation [47, 53, 54].

1.7 Important analytical concepts and techniques

1.7.1 Selectivity in the organic trace analysis of foods

Selectivity (cf. 5.4) is probably the most important property of a quantitative food analysis method [55]. A non-selective method is susceptible to variable errors caused by the sample matrix and thus inaccurate. Consequently, the more complex the sample matrix, the more important the method's selectivity. In food analysis, selectivity is usually achieved by (i) cleanup steps during sample preparation, (ii) chromatography and (iii) the detection principle. In the vast majority of methods, it is necessary to exploit all three elements in order to achieve a sufficient overall selectivity [55]. To give an example of the consequences of lacking selectivity, a look at Figure 29 (page 63) is justified. Upon inspecting the chromatographic traces for Sample #4, it can be seen that the analyte peak in the top trace (which was obtained by a non-selective method) has a slight shoulder and is larger than the one of the bottom trace (which was obtained by a selective method). This is attributed to a co-eluting matrix component. If the peak of the top trace would be evaluated, a higher, inaccurate analyte concentration would result. As the quantity of the co-eluting components may vary from sample to sample, this effect can not be compensated for by matrix-matched calibration.

In the field of food mycotoxins the pronounced need for selectivity has led to the success of

two techniques in particular: HPLC-MS/MS (high performance liquid chromatography-tandem mass spectrometry) and IAC (immunoaffinity cleanup)¹. These methodologies will be briefly introduced below. In any case, selectivity comes at a price. This can be the actual price of the analytical equipment needed. But also other method parameters (e.g. the precision, speed or practicality of the method) can be affected negatively, if the focus is set on selectivity only. Hence, a multitude of factors needs to be balanced upon establishing a quantitative method. All analytical methods presented in this dissertation fall back upon the above reflections.

1.7.2 HPLC-MS/MS

An important prerequisite for the success of HPLC-MS/MS in mycotoxin analysis was the method of ESI (electrospray ionisation), which was initially conceived in 1984 [61]. ESI is a "soft" ionisation technique applicable to a wide range of analytes, e.g. proteins, polymers or small organic molecules. It is easily coupled to HPLC and provides excellent sensitivity for many target molecules. The achievable LODs vary widely, depending on the chemical properties of the analyte. In food multi-mycotoxin analysis HPLC-MS/MS LODs usually range from 0.5 to 200 μ g/kg [62-64]. Lower LODs are possible when using preconcentration techniques or methods optimised for single analytes.

The predominant feature of HPLC-MS/MS, selectivity, is achieved by two stages of mass analysis. In the first stage, a precursor ion is selected from the sum of ions supplied by the ion source. This precursor ion is then fragmented and the fragments are eventually detected by a second mass analyser. Fragmentation can be done by a range of methods involving irradiation, collision or chemical reactions with neutral atoms or molecules. In any case the number, identity and relative intensities of the resulting fragments are compound specific properties which can be exploited for the sake of selectivity.

A further feature of HPLC-MS/MS with particular importance for mycotoxin analysis is its suitability for multi-analyte methods, which is based on the MS/MS detector's capability for the individual detection of co-eluting compounds. This has been exemplified by Sulyok *et al.*, who developed a semiquantitative HPLC-MS/MS method capable of determining more than 100 fungal metabolites in two chromatographic runs [14, 62]. For the sum of these reasons HPLC-MS/MS is currently seen as the most promising methodology in mycotoxin analysis [6].

¹ Several recent reviews on mycotoxin analysis are available for further reading [6, 44, 45, 56, 57], some of which are focused on HPLC-MS/MS [58, 59] or IAC [60] in particular.

Still, HPLC-MS/MS systems have disadvantages, including acquisition and maintenance costs as well as a lower precision compared to spectroscopic detection due to the delicate ionisation process. Also, while the MS/MS detector is highly selective, the ESI source is not. In fact, matrix effects (MEs) can enhance or suppress ionisation and need to be compensated for by a suitable method, e.g. stable isotope dilution [65]. However, stable isotope dilution requires costly isotope standards which are available only for a limited spectrum of analytes. If sample preparation and chromatography are sufficiently selective, it might thus be advantageous to rely on cheaper and more precise spectroscopic detection techniques like FLD (fluorescence detection). LODs of food mycotoxin HPLC-FLD methods are comparable to those achieved by HPLC-MS/MS [66-68], however, it should be mentioned that HPLC-FLD methods are usually optimised for a single analyte.

1.7.3 IAC

IA columns [60] consist of antibodies, which are immobilised on a gel. The antibodies are designed to bind to a specific analyte by antibody-antigen interactions. If a liquid food extract is put on an IA column, the analyte binds selectively to the antibodies, while all other matrix components remain in solution, eventually being washed away. The analyte can then be eluted by denaturing the antibodies with organic solvents. IAC offers a maximum in selectivity and preconcentration, thus allowing for the omission of MS/MS in favour of DAD or FLD. However, IA columns are costly (5–20 \in per single-use item) and implicitly incompatible with organic solvents. Also, their performance can vary significantly between different food matrices [46] and from lot to lot. Lastly, the spectrum of covered analytes is limited due to the time-consuming and costly development of suitable antibodies. Still, IAC is frequently utilised, with the issue of food mycotoxins being a major driving force [6, 60, 69].

1.8 Introduction to the relevant analytes

1.8.1 TA 5

Biosynthesis

Tenuazonic acid (TA **5**) is a tetramic acid which is biosynthesised by moulds of the genera *Alternaria, Aspergillus, Pyricularia* as well as by soil fungi of the *Phoma* genus. It was first described in 1959 [70]. The species *Alternaria alternata* is probably the most important TA **5** producer, as

its pathogenic capacities enable it to attack over 100 potential plant hosts [71]. TA **5** itself contributes to the pathogenicity of *A. alternata* by acting as a phytotoxin [72] inhibiting the plant's photosystem II [73]. The biosynthesis of TA **5** is not fully elucidated, however, it is assumed to proceed through N-acetoacetylation of the essential amino acid L-isoleucine by acetoacetyl-CoA (coenzyme A) and a subsequent ring formation (Figure 5) [74, 75].



Figure 5 Assumed biosynthetic production pathway of TA 5 in Alternaria tenuis [74, 75].

Occurrence in food

To date, TA **5** was detected in and on apples and derived products, beer, buckwheat flour, maize, mandarins, melons, oilseed rape, olives, peppers, rice, sorghum, sunflower seeds, tomato products and wheat. The usually encountered concentrations range up to $1,000 \mu g/kg$ [4, 5, 76, 77].

Toxicity

The data on the different aspects of TA **5** toxicity can be considered insufficient [78]. However, it is known that TA **5** is acutely toxic towards several mammalian species, i.e. mice, chickens and dogs with LD₅₀ (median lethal doses) of 81 and 168 mg/kg bw (body weight) in female and male mice, respectively [23, 76]. The mechanism of toxicity probably involves the inhibition of peptide bond formation during ribosomal protein biosynthesis [79]. Certain subacute effects of TA **5** have also been shown. In dogs, feeding of 10 mg/kg bw resulted in haemorrhages in several organs [76]. In mice, feeding of 25 mg/kg bw TA **5** per day resulted in precancerous changes in oesophageal mucosa, indicating a possible promotion of oesophageal cancer by TA **5** [80]. Other biological effects (antiviral, antibacterial, antifungal and antitumor) have been described as well [23].

1.8.2 The dibenzo-α-pyrone based *Alternaria* mycotoxins

Biosynthesis

The dibenzo-α-pyrone based *Alternaria* mycotoxins are alternariol (AOH **7**), alternariol monomethyl ether (AME **8**) and altenuene (ALT **9**). These compounds are produced by several *Alternaria* strains, e.g. *A. alternata*, *A. citri*, *A. cucumerina*, *A. dauci*, *A. kikuchiana* and *A. solani* [23]. AOH **7** and AME **8** were first described in 1953 [81] while ALT **9** was discovered in 1971 [82].

The first step of the biosynthetic pathway leading to AOH **7** is the chain extension of a starter acid like acetate, which is bound to a polyketide synthase (Figure 6). By successive additions of C_2 units from malonate, an enzyme-bound heptaketide chain (6) is created [83, 84] and subsequently cyclised. Eventually, AME **8** is biosynthesised from AOH **7** by an alternariol-O-methyltransferase under involvement of the cofactor SAM (S-adenosyl methionine) [85]. The biosynthesis of altenuene has not been studied yet.

Occurrence in food

To date, AOH **7** was detected in and on apples and derived products, barley, blackberries, gooseberries, lentils, mandarins, oats, oilseed rape meal, pecans, peppers, prune nectar, raspberries, red currant, red wine, rye, sorghum, strawberries, sunflower seeds, tomatoes and derived products, triticale and wheat [76, 77]. The matrices relevant for AME **8** are similar to those for AOH **7**. ALT **9** is screened for and found less frequently, however, it is known to occur in apples, maize, olives, ragi, red peppers, rice, sorghum, tomatoes and wheat [23, 77]. Similar to TA **5**, the usual concentrations lie in the range up to 1,000 μ g/kg.

Toxicity

AOH 7, AME 8 and ALT 9 are very weak acute toxins with an LD_{50} × 400 mg/kg bw (AOH 7, AME 8) and × 50 mg/kg bw (ALT 9) in female mice [88]. However, it was recently shown [89-91] that AOH 7 acts as a topoisomerase II poison thus inducing DNA (deoxyribonucleic acid) strand breaks. In the same studies, AME 8 showed an activity similar to AOH 7 while ALT 9 was not affecting DNA integrity. In competitive assays AOH 7 but not ALT 9 was shown to bind to the minor groove of DNA [90] (AME 8 not tested). AOH 7 also replaced 17β-estradiol from human oestrogen receptors α and β [92] (AME 8 and ALT 9 not tested).

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Figure 6 Assumed biosynthetic production pathway of AOH 7, AME 8 and ALT 9 in *Alternaria alternata* [83, 84, 86, 87] starting from an enzyme bound heptaketide chain 6.

In vitro studies with porcine granulosa cells furthermore revealed that AOH **7** and AME **8** inhibit the synthesis of progesterone [93], hence possibly affecting reproductive performance (ALT **9** not tested).

In summary, it should be stated that, similar to TA **5**, the toxicity of the dibenzo- α -pyrone based *Alternaria* mycotoxins is not adequately characterised. Although indications of mutagenic and estrogenic potential have been obtained, there is a particular lack of bioavailability studies, which are an essential requirement when assessing chronic or subacute toxicity. Hence, the risk for the consumer of contaminated foods remains unclear. None of the *Alternaria* mycotoxins are currently regulated.

1.8.3 ZON 10 and analogues

Biosynthesis

Zearalenone (ZON 10) was discovered in 1962 [94]. It is produced by a range of *Fusarium* species, e.g. *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense* and *F. semitectum* [95]. Similar to AOH 7, AME 8 and ALT 9, the biosynthesis of ZON 10 is based on polyketides and involves an aldol like condensation step yielding a resorcinol building block. However, due to the influence of reductases, the remaining part of the polyketide chain is largely saturated (Figure 7) [96].



Figure 7 Assumed biosynthetic production pathway of ZON 10 in *Gibberella zeae* [96].

Analogues

In mammals, ZON 10 is converted to α/β -zearalenol (α/β -ZOL 11/12) by ZON 10 reductases [23]. α/β -ZOL 11/12 are also produced by fungi and have been detected in small quantities in foods. By reduction of the non-aromatic carbon-carbon double bond of ZON 10 or α/β -ZOL 11/12 the analogues zearalanone (ZAN 13) and α/β -zearalanol (α/β -ZAL 14/15) are obtained. However, while α/β -ZAL 14/15 have been detected in rice cultures [97], none of the three compounds has been found in food so far.



Figure 8 Structures of ZON 10 analogues [95].

Occurrence in food

ZON **10** is a common food contaminant in temperate regions. Its production may take place in the field, during harvest or food processing. Particularly high ZON **10** levels are encountered when *Fusarium* spp. infected grains are stored in a moist environment [23]. Maize and derived

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products (cornflakes, breakfast cereals, maize based beer, maize oil, pop corn etc.) are the main commodities at risk. However, ZON **10** was also detected in bananas, barley and derived products, beans, beer, chilli powder, coriander, curry, fennel, millet, oats, edible oils, peppers, rice, rye and derived products, sorghum, soy, walnuts and wheat [23, 95]. Concentrations up to 10,000 μ g/kg are usually encountered, however, there are also reports on significantly higher concentrations, particularly from developing countries [95].

 α/β -ZOL 11/12 are commonly found in animal tissues [98] and were also detected in maize byproducts, soy and derived products [95, 99-101]. α/β -ZAL 14/15 and ZAN 13 were not yet detected in food [95]. In the United States of America (USA), α -ZOL 11 is used as an animal growth promoter due to its anabolic activity and may thus enter the food chain. In the EU this use was forbidden in 1985 and meat products, obtained from animals treated with α -ZOL 11 for non-vetinary purposes, may not be imported [98].

Toxicity

With an $LD_{50} > 2,000 \text{ mg/kg}$ bw in mice, rats and guinea pigs [23, 95], ZON 10 is not acutely toxic (LD_{50} of sodium chloride in rats: 3,750 mg/kg bw [23]). However, due to its structural resemblance to oestrogens, ZON 10 acts as an anabolic and causes a wide range of fertility related disorders like uterine enlargement, prolonged or interrupted oestrus, pseudo pregnancy and infertility [23]. Furthermore, there are several reports [102] on premature thelearche (puberty) in children, particularly girls, which implicate ZON 10 as a possible cause. The oestrogenic potential of ZON 10 and its analogues can be graduated in the order α -ZOL 11 > 17 β -estradiol > α -ZAL 14 $\approx \beta$ -ZAL 15 \approx ZON 10 > β -ZOL 12 [103]. It is notable that the mammalian ZON 10 metabolite α -ZOL 11 is a more potential oestrogen than 17β-estradiol, a factor which corroborates the high risk for the human health originating from ZON 10 contaminated foods. Besides its oestrogenic and anabolic activity, ZON 10 is genotoxic and was shown to form DNA adducts in various mammalian cells [95]. Reports on a possible carcinogenicity and immunotoxicity of ZON 10 exist as well, but can not be considered conclusive yet [95]. Lastly, ZON 10 was shown to inhibit the growth of other fungi (fungitoxicity) [17]. The ADI (acceptable daily intake) for ZON 10 is 0.5 μ g/kg bw [104]. Due to its pronounced impact on the human health a range of EU MLs for ZON 10 in different foods and raw materials has been established (Table 2).

2 Objectives and solution approaches

2.1 Superior objectives

Based on the considerations outlined in the introduction this dissertation had a twofold aim:

- the development of creative chemical solutions to current issues in instrumental mycotoxin analysis
- (ii) investigations on possible mycotoxin degradation mechanisms which may be of relevance during food processing and storage

For objective (i), two particular issues were selected. These were the quantification of the *Alternaria* spp. mycotoxin TA **5** in cereals and beer as well as the quantification of the *Fusarium* spp. mycotoxin ZON **10** in edible oils.

Objective (ii) was pursued in the frame of two studies on *Alternaria* spp. mycotoxins, i.e. AOH **7**, AME **8**, ALT **9** and TA **5**. The choice of this group of compounds was motivated by the increasing importance of the *Alternaria* mycotoxins in the current scientific discussion (*vide infra*) as well as by a general lack of data on stability and possible degradation mechanisms during food processing.

2.2 Quantification of TA 5 in cereals and beer

TA **5** is a small, polar, non-volatile compound with an acid dissociation constant (pK_A 3.5) [105] comparable to that of formic acid (pK_A 3.8). Its 1,3,5-triketone substructure enables TA **5** to chelate metal cations and allows for an extensive tautomerism involving keto/enol chemistry as well as bond rotation. In solution, TA **5** will hence be represented by a complex system of equilibrated structural species including different keto/enol tautomers, rotamers, protonated and deprotonated forms as well as various chelates, depending on the metal cation content of the solution (Figure 9). In chromatographic methods, each of these species will interact differently with the stationary phase, leading to severe peak-broadening. When using unmodified HPLC¹ eluents, this can even cause a complete loss of the TA **5** peak (Figure 10).

¹ Apart from HPLC, there have been several attempts to quantify TA **5** by GC (gas chromatography) after derivatisation [77, 106, 107], however, these approaches were not pursued to the validation stage. Thin layer chromatography based methods were developed as well [77, 108, 109] but used mainly on high content samples like fungal cultures.



Figure 9 Selected tautomeric structures and equilibria for TA 5. The percentages in parentheses correspond to the molar fractions in deuterated chloroform, which were estimated using NMR (nuclear magnetic resonance) data [110]. The structure of the metal chelate was derived from the crystal structure of copper(II) bis(tenuazonate) monohydrate [111].



Figure 10 HPLC chromatograms of identical TA 5 injections obtained using a 70:30 (v:v) water:MeCN (acetonitrile) eluent with varying modifications (own data) {11, 113}, the pH of the eluent water was determined using {110}.

If sharp peaks are to be obtained, the number of possible structural species needs to be limited by controlling the involved equilibria. This may be achieved by several means. The majority of methods involve eluent modification by excess $ZnSO_4$ [77, 112-115]. This ion pairing approach leads to the *in situ* formation of a stable zinc(II) bis(tenuazonate) complex with favourable chromatographic properties (*cf.* Figure 9: the equilibria are shifted towards the metal chelate).

Alternatively, the on-column ionisation of TA 5 may be suppressed by acidifying the eluent (ion suppression chromatography). This has been realised with NaH_2PO_4 [105, 116] and TFA

(trifluoroacetic acid) [117] based eluent modification. The addition of ethylenediaminetetraacetic acid (EDTA) further improves peak shapes, as metal impurities of the column are "masked" as EDTA chelates, thus avoiding complexation by TA 5 [118] (cf. Figure 9: the equilibria are shifted towards the structures **5a**–**d**, see also Figure 10). Detection is almost exclusively done with UV (ultraviolet) or DA (diode array) detectors at a wavelength (λ) of 280 nm [77]. At the outset of the works on this dissertation, there were no scientific articles on MS based methods for TA 5 in food. In view of the prevalence of MS in modern mycotoxin analysis [6], this is an intriguing fact. However, it may be rationalised by reconsidering the eluent modifiers necessary for TA 5 chromatography. Apart from TFA, they are all non-volatile and hence unsuited for common MS ion sources. TFA, on the other hand, causes ion suppression in MS ion sources due to its tendency to form stable, uncharged gas phase ion pairs with positively charged analyte ions [119]. Still, the use of MS or multistage MS techniques is highly desirable, as it implicates a significant increase in detection selectivity (cf. 1.7.1).

Consequently, a first aim was to enable the HPLC-MS based quantification of TA 5 in foods. The envisaged method should be straightforward, robust and cheap, while at the same time providing a maximum in selectivity. By developing and applying such a method, it could be expected to obtain new, instructive data on the occurrence of TA 5 in foods.

In order to achieve this, eluent modification was considered unsuited because of an apparent contradiction: both ion suppression and ion pairing chromatography rely on the formation of uncharged analyte species while the MS detector conversely requires the formation of ions. In other words: improving chromatography should impair detection and vice versa. It was thus decided to circumvent this contradiction by derivatising TA **5** prior to analysis. The novel method was intended to be applied to cereal products and beer, as cereals are considered to be the main contributors to the mycotoxin intake of the EU population [120].

The ideal derivatisation reagent should allow for a rapid, robust and quantitative reaction resulting in a single, stable product. It should furthermore be cheap, readily available and non-

toxic. In the light of the triketone substructure of TA 5, 2,4dinitrophenyl-hydrazine (DNPH 16) was considered most suited to meet these requirements. DNPH 16 (hazard codes: F, Xn) is a wellknown reagent with a range of applications in environmental and clinical analysis [121, 122]. Applications involving mycotoxins or cereals were, however, not yet reported.



Figure 11 DNPH 16.
2.3 Quantification of ZON **10** in edible oils

During the dry and wet milling of ZON 10 contaminated maize, the apolar mycotoxin is enriched in the maize oil fraction, resulting in high concentrations (up to 4.6 mg/kg) [123, 124]. For this reason, a specific EU ML for ZON 10 in refined maize oil was introduced in 2006 [125]. However, due to increased levels of ZON 10 in the maize harvests of 2005 and 2006, the initial limit of $200 \,\mu\text{g/kg}$ was raised to $400 \,\mu\text{g/kg}$ in 2007 [30] to avoid a disproportionate impact of regulation on maize oil availability. Despite the regulatory situation, a reference method for the quantification of ZON 10 in maize oil is not yet available.

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In the case of solid matrices like cereals, the analytical extraction of ZON **10** is usually done with mixtures of organic solvents and water followed by IAC (*cf.* 1.7.3) [126]. Edible oils, on the other hand, are apolar, liquid matrices composed of 95–98 % fatty acid triglycerides [127, 128]. Hence, extraction with organic solvents (in this case termed liquid-partitioning) is problematic, as a significant part of the matrix (about 10 % [128]) is co-extracted. Liquid-partitioning without further cleanup has thus been termed "far from adequate" for use in conjunction with modern GC or LC instruments [129] and the analytical extraction of apolar contaminants from edible oils has gained predicates from "formidable" [127] to "challenging" [129] to "tedious" [128] in recent reviews. Despite this, a range of liquid-partitioning based methods without cleanup steps were published [100, 123, 130] for ZON **10**. Only in one case was IAC applied after liquid-partitioning [131]. However, in many of the cited papers no chromatograms are shown and in the case a chromatogram is given [100, 130], the need for further cleanup can be inferred.

To date, the only published alternative to liquid-partitioning is GPC (gel permeation chromatography) [66, 132, 133]. This instrumental technique separates substances by their hydrodynamic volumes and is well suited for the separation of ZON **10** from the triglyceride matrix [66]. However, an additional IAC step is needed, as all molecules with hydrodynamic radii similar to ZON **10** are co-extracted. The IAC step can be omitted, if the selective MS/MS detector is used instead of FLD, but in that case isotope standards are required to achieve a precision comparable to FLD [66].

In summary it may be stated that the involvement of GPC, IAC, MS/MS or isotope standards causes the methods for the quantification of ZON **10** in edible oils to be costly and/or demanding in terms of the needed apparatuses or sample preparation procedures, while

procedures based solely on liquid-partitioning lack specifity and hence produce chromatographically not well-resolved peaks.

Consequently, there is the need for a novel, alternative solid phase principle for the extraction of ZON **10** occurring in edible oils. The new technique should:

- show a sufficiently high selectivity for ZON 10, allowing the quantification by HPLC FLD without further cleanup
- (ii) be more cost-efficient than IAC
- (iii) eliminate the need for GPC or MS instruments
- (iv) allow the direct application of the edible oil to the solid phase and thus combine extraction and cleanup to one step minimising the handling efforts
- (v) meet the performance criteria for ZON **10** methods required by the EU

While most solid phase based cleanup methods rely on rather unselective physisorption or ionion interactions, it seemed plausible that a cleanup procedure based on the reversible formation of a covalent bond between solid phase and analyte would significantly increase selectivity. The non-conjugated carbonyl group of ZON **10** enables a range of chemical reactions, inter alia the formation of a hydrazone with a hydrazine. As hydrazone formation is known to be reversible [134-137], it was considered suitable for the extraction and cleanup of ZON **10**.



Figure 12 Solid support based dynamic covalent hydrazine chemistry (DCHC), X = toluenesulfonyl linker.

Reversible hydrazone formation and other chemical reactions which involve the formation and cleavage of covalent bonds under equilibrium control were reviewed by Rowan *et al.* [138], introducing the concept of dynamic covalent chemistry. Recent applications of dynamic covalent hydrazine chemistry (DCHC) in particular can be found in the fields of profragrances [139, 140], dynamic covalent polymers [141] and drug discovery [142]. In a particularly interesting application [143] a hydrazine moiety was covalently attached to glass beads. The functionalised

beads were then used to bind peptides modified by the lipid peroxidation product 4-hydroxynoneal. After the beads were washed, the enriched peptides were liberated by hydrolysis and analysed by different MS techniques. However, DCHC has not yet been used for quantitative instrumental analysis. Hence, it was a second aim to develop a novel approach for the combined extraction and cleanup of the *Fusarium* mycotoxin zearalenone (ZON **10**) occurring in edible oils, which is based on reversible hydrazone formation on a hydrazinefunctionalised polymer resin.

2.4 Degradation of Alternaria mycotoxins upon food processing

Generally, there is little literature on the chemistry of the *Alternaria* mycotoxins. In the case of TA **5**, it is known that standing for several months or boiling in 0.1 M NaOH [70, 105] leads to epimerisation yielding a mixture of TA **5** and its diastereomer *u*-TA **19**. A plausible epimerisation mechanism is shown in Figure 13. However, there is no information on the occurrence of *u*-TA **19** in food. This can be attributed to the fact that most HPLC-UV methods for the quantification of TA **5** do not achieve separation of TA **5** and *u*-TA **19** but rather detect the sum of both epimers.



Figure 13 Suggested mechanism for the epimerisation of TA 5 and *u*-TA 19 in boiling NaOH (not all conceivable enol intermediates are shown).

Apart from epimerisation, TA **5** may be degraded by boiling the compound in hydrochloric acid [70]. Depending on the conditions, two degradation products are formed (Figure 14). Similar to epimerisation, actual degradation rates and information on the identity of expected products under mild hydrolysis conditions are not available. In the case of AOH **7**, AME **8** and ALT **9**, no degradation products have been identified at all. Still, several solely quantitative stability studies for TA **5**, AOH **7** and AME **8** were published:

Combina *et al.* [144] investigated the stability of TA **5** in common organic solvents by UV spectrometry. The study period was 28 weeks. At -20 °C, TA **5** was stable in the tested solvent systems {MeOH (methanol), MeOH:water, benzene and benzene:MeCN}. At 4 °C and 25 °C

however, degradation occurred in all cases. TA **5** was least stable in MeOH and MeOH:water. A kinetic evaluation of the data or an identification of possible degradation products was not performed. Combina *et al.* also investigated the stability of AOH **7** and AME **8** in sunflower flour upon thermal treatment [145]. While both substances were stable in flour heated to 100 °C, a significant degradation could be observed at 121 °C (i.e. 75 % of AOH **7** and 100 % of AME **8** were degraded after 60 min). Information on the chemical fate of the toxins was not given. Lastly, Scott and Kanhere studied the stability of AOH **7** and AME **8** in different fruit juices and white wine [146], concluding that the compounds are stable over a period of 27 days.



Figure 14 Previously reported degradation reactions for TA 5 [70].

In light of the available literature the aim was to identify the actual degradation pathways of TA **5** in aqueous solution and to quantify the relative contribution of each pathway. The approach taken was based on the development of a methodical canon for the quantification of TA **5**, *u*-TA **19** and other possible degradation products as well as the application of that canon to a four month kinetic study.

As for AOH **7** and AME **8** stability data for aqueous matrices were already available [146], it was decided to investigate a possible degradation during bread baking. This choice was motivated by the high relevance of baking as a food preparation procedure and its rather harsh conditions (heating to ~ 200 °C in an oxidative atmosphere and complex chemical matrix), which make a degradation more likely [47]. Also, the occurrence of AOH **7**, AME **8** and ALT **9** on wheat is known [147-149]. Therefore, a series of quantitative model experiments using spiked wheat flour was designed. In the case of AOH **7**, a large supply of standard substance obtained through total synthesis [150] allowed further experiments on possible degradation products as well as TA (thermo analysis)-MS measurements.

3 Results and discussion

3.1 Quantification of TA 5 in cereals and beer

3.1.1 Derivatisation chemistry

Even at room temperature (RT), TA 5 reacted readily with DNPH 16 in aq. (aqueous) HCl {M16}. Although several reaction products are conceivable, a single tenuazonic acid 2,4dinitrophenylhydrazone (TA-DNPH 22), formed (Figure 15). This was exclusive regioselectivity is in accordance with the results of Gelin et al. [151], who investigated the reaction of phenylhydrazine with analogous 3-acetyl-tetramic acids. The identity of TA-DNPH 22 was confirmed by NMR and X-ray crystallography [9]. In solution, TA-DNPH 22 apparently occurs as two species, the interconversion of which is slow on the NMR timescale, thus causing the splitting of certain signals {M16}. Yamaguchi et al. made similar observations for Schiff bases of TA 5 analogues [152, 153] and identified the two species to be rotamers occurring due to the rotation of the imine/enamine sidechain. This is consistent with the tautomerism of TA 5 (Figure 9). The corresponding TA-DNPH 22 rotamers are given by structures **22a–b** and **22c–d** (Figure 16); in the crystal tautomer **22b** occurs exclusively.



Figure 15 Derivatisation of TA 5 by DNPH 16. A single product, the hydrazone TA-DNPH 22, is obtained {M16}. For details on the crystal structure of TA-DNPH 22 see [9].



Figure 16 Suggested formation mechanism [134] and tautomeric equilibria for TA-DNPH 22. Due to precipitation of the product, the water elimination step is irreversible. The molar ratios of the rotameric species was determined by NMR {M16}.

With respect to derivatisation, the rapid formation of a single product, TA-DNPH 22, is favourable. It is furthermore advantageous that TA-DNPH 22 precipitates from the reaction mixture, thus ensuring a complete conversion even at RT. Concerning the issue of the rotamers, it will be shown below that the interconversion between species 22a–b and 22c–d does not negatively affect chromatography.

3.1.2 Sample preparation routines for cereals and beer

DNPH 16 shows a reduced reactivity towards sugars, other α -hydroxy-carbonyls, the carbonyl function of carbonic acids and their amides or esters [154, 155]. Hence, a direct application of a huge excess of the aqueous derivatisation reagent {M13} to both solid and liquid matrices was considered a suitable strategy. This approach is advantageous because it avoids time-consuming pre-extraction steps. The optimisation of the sample preparation routine was done by repeated analyses of a naturally TA 5 contaminated buckwheat flour, with single sample preparation parameters being varied. The effect of the variations was determined from the resulting TA-DNPH 22 peak areas {M2}. A combination of ultrasonication and shaking proved most efficient for treating the reaction mixture and making TA 5 available for reaction. Longer shaking with or without repeated ultrasonication steps did not improve the results. The overall reaction time could be limited to 30 min.

Following derivatisation, an extraction of the aqueous reaction mixture was necessary as the derivatisation product TA-DNPH 22 is not water soluble. Apolar solvents (hexane, heptane, toluene etc.) were found to be unsuited for re-extraction by liquid-partitioning due to poor solubilisation of TA-DNPH 22. As the use of chlorine containing solvents (dichloromethane, chloroform) should be avoided for ecological and health reasons and as Et_2O (diethyl ether) was deemed unsuited due to its low BP (boiling point) complicating analytical handling, EtOAc (ethyl acetate) was chosen for re-extraction. After a 10 min re-extraction period, the EtOAc could be taken off for injection into the HPLC-ESI-IT-MSⁿ (HPLC-ESI-ion-trap n-stage mass spectrometry) system {12}. The derivatisation yield (ratio of TA 5 converted to TA-DNPH 22) of the sample preparation routines was determined to be 100.6 ± 1.2 % {M2}.

Unfortunately, EtOAc co-extracts the excess DNPH **16** present in the aqueous reaction mixture. DNPH **16** is non-volatile under the conditions inside the ESI source (nebulisation at T = 350 °C) and will thus contaminate the instrument. To keep DNPH **16** from reaching the ESI source, an excess of undecanal (BP = 109–115 °C) was introduced to the reaction mixture along with the EtOAc. Consequently, free DNPH **16** reacts to form the undecanal 2,4-dinitrophenylhydrazone. The latter is highly retained on the employed HPLC column {I12}. Hence, the ESI source could be protected by bypassing it after elution of TA-DNPH **22** and before elution of the undecanal 2,4-dinitrophenylhydrazone through an automated switching valve.

After establishing the sample preparation routine, the ideal sample intake was determined. In order to save organic solvents and reagents, it is generally desirable to miniaturise the sample preparation routine by using the smallest possible sample portion. However, especially in the case of solid food matrices, there is a lower limit to the sample intake, i.e. the minimum sample intake (MSI), *cf.* 5.4. Using two naturally TA **5** contaminated flour samples, the MSI was determined to be 2 g (Figure 17). This sample intake was used for all analyses of ground solid samples. In the case of beer, the influence of the sample portion weight was much less pronounced due to the intrinsical homogeneity of liquid samples. Here, the sample intake could be reduced to 0.4 g, allowing for the whole sample preparation to be conducted in a discardable 2 mL plastic safelock tube.

The overall sample preparation routines for cereal based samples and beer are shown in Figure 18. In both cases, the sample preparation takes less than 50 min and does not require heating or any solvent evaporation steps. The total analysis time was less than 90 min.



Figure 17Effect of the sample intake on the precision of method {M2} evaluated by repeated analyses of
two naturally TA 5 contaminated flour samples. The TA 5 contents were $c_{TA} = 851 \pm 41 \mu g/kg$
(buckwheat flour) and $c_{TA} = 168 \pm 27 \mu g/kg$ (rye flour).



Figure 18 Flowcharts of the sample preparation routines for TA 5 in cereal based samples $\{M2\}$ and beer $\{M3\}.$

3.1.3 HPLC-ESI-IT-MS² conditions

TA-DNPH **22** did not exhibit the problematic chromatographic behaviour of TA **5** (*cf.* 2.2 and Figure 10). Using mildly acidified eluents $\{0.1 \% (v) \text{ formic acid}\}$ TA-DNPH **22** was well retained by the employed reversed phase-HPLC column $\{112\}$ and eluted as a sharp peak (Figure 20). Problems such as peak splitting, which might be expected due to the injection of an organic phase into an aqueous eluent were not observed.

The fragmentation behaviour of TA-DNPH **22** upon CID (collision induced dissociation) was studied on IT-MS² and FTICR-MS² instruments using ESI(+). The most abundant product ion of the pseudomolecular ion $[22+H]^+$ had an odd electron count and was presumably formed by the homolytic cleavage of the N-N bond and the heterolytic cleavage of the isobutyl sidechain (Figure 19).





While TA-DNPH 22 was also observable in ESI(-), the negative polarity resulted in a significantly narrower linear range. Hence, the use of ESI(-) was not considered further. As the mechanism shown in Figure 19 implies a derivatisation specific part (loss of the 2,4-dinitrophenyl group) and an analyte specific part (loss of the TA 5 isobutyl sidechain), the transition m/z 378 \rightarrow 140 was judged to be of high selectivity and used as the QT (quantifier) transition for all further analyses. The ESI-IT-MS² parameters for this transition were optimised by repeated injections of an EtOAc solution resulting from a sample preparation of a naturally

TA **5** contaminated buckwheat flour. Reducing the trap-drive level from 100% to 75% resulted in the highest increase in signal intensity. The trap-drive represents the field strength inside the ion trap during accumulation. Lower field strengths are preferred for measuring lighter ions. Chromatograms obtained using the optimised method settings are summarised in Figure 20. For beer, similar chromatograms were obtained [4].

| m/z (rel. intensity) IT-MS ² {I2} | m/z FTICR-MS ² {M10} | Suggested formula | Calculated m/z | Error [mDa] | Electron count |
|--|---------------------------------------|----------------------|----------------|----------------|-------------------|
| 140 (100 %) | 140.0581 | $C_6H_8O_2N_2$ | 140.0580 | 0.1 | Odd |
| 180 (33 %) | 180.1021 | $C_{10}H_{14}O_2N_1$ | 180.1019 | 0.3 | Even |
| 333 (14 %) | Not observed | $C_{16}H_{21}O_4N_4$ | 333.1557 | - | Even |
| 221 (11 %) | 221.0673 | $C_9H_9O_3N_4$ | 221.0669 | 0.4 | Even |

Table 3Product ions of $[22+H]^+$ (m/z 378.141) yielded by CID.

3.1.4 Validation

Basic in-house validation was performed for the presented methods. The obtained data are summarised in Table 4. For solids and liquids, linearity was given over a range of two decades.

Table 4Limits of detection and quantification (LOD, LOQ), linearity and apparent recoveries of
methods {M2} and {M3}.

| Sample matrix | Cereal based solids {M2} | Beer {M3} |
|--|--|--|
| LOD [µg/kg] | 10 | 2 |
| LOQ [µg/kg] | 50 | 8 |
| Linear range [µg/kg] | 50–10,000 | 8–1,000 |
| Typical calibration curve ¹ (no sample matrix) | y = 376.2 (± 4.5) x + 23.6 (± 17.7) × 10 ³ | $y = 1,376 (\pm 14) x + 5,000 (\pm 4,690)$ |
| ditto, R ² (square of correlation coefficient) | 0.9990 | 0.9992 |
| Typical calibration curve ¹ (spiked sample matrix) | $y = 281.8 (\pm 2.5) x + 13.6 (\pm 9.0) \times 10^{3}$ | $y = 1,630 (\pm 34) x$ + 6,154 (± 10,913) |
| Ditto, R ² | 0.9994 | 0.9964 |
| Average apparent recovery [%] (samples analysed) | 79 ± 11 (6) | 90 ± 22 (16) |

¹ General form: $y [\mu g/kg] = a [\mu g/kg/a.u.] x + b [\mu g/kg]; x = peak area [a.u.]; a.u. = arbitrary units$

Since an amount of TA **5** standard equivalent to the approximate natural TA **5** content in the sample was added, the determinable natural TA **5** contents were limited by the middle of the linear range (i.e. 5,000 μ g/kg for cereal based solids and 500 μ g/kg for beer). However, these limitations were not exceeded by any of the samples analysed.

Further validation data are summarised in Tables 5 and 6 (cf. 5.4). The slight loss in trueness and precision observed for the beer method {M3} is attributed to the miniaturisation of the sample preparation routine. To further interpret the obtained data, they were compared to the analyte-specific performance criteria for quantitative food mycotoxin methods issued by the European Commission in 2006 [156]. However, as there are no provisions for TA 5, the strictest criteria given for any other mycotoxin were considered. In this respect, the cited regulation requests that a method's typical relative standard deviation (RSD) should not exceed 20 % (or 15 % in the special case of the compound patulin at concentrations \geq 50 µg/kg). Furthermore, apparent recoveries should be in the range of 75–105 %. These criteria are well met by the methods suggested here.

| с _{тл} [µg/kg] | 50 (LOQ) | 500 (LOQ × 10) | 5,000 (LOQ × 100) |
|-------------------------------------|------------|----------------|-------------------|
| RSD [%] (Intraday, n = 5) | 9.4 | 11.4 | 12.7 |
| RSD [%] (Interday, n = 3, m = 5) | 2.2 | 2.0 | 3.8 |
| Bias [%] | 5.5 | -2.8 | 2.0 |
| Table 6 Validation data for | beer {M3}. | | |
| с _{тл} [µg/kg] | 8 (LOQ) | 50 (LOQ × 6.3) | 500 (LOQ × 63) |
| RSD [%] (Intraday, n = 5) | 15.9 | 12.9 | 10.8 |
| RSD [%] (Interday, n = 3, m = 5) | 4.0 | 7.1 | 8.0 |
| Bias [%] | 16.6 | 5.8 | 15.4 |

Table 5 Validation data for cereal based solids {M2}.



- C Same wheat flour, spiked to $c_{TA} = 10 \ \mu g/kg (LOD)$
- D Same wheat flour, spiked to $c_{TA} = 50 \ \mu g/kg (LOQ)$
- E Naturally TA 5 contaminated rye flour, $c_{TA} = 168 \pm 27 \,\mu g/kg$ (measured)

3.1.5 Sample survey

The optimised, validated methods were applied to a range of commercial consumer food products obtained from a local supermarket. A total of 27 flour and bakery products as well as 43 beers were analysed. TA 5 was detected in 13 flour and bakery product samples and in 37 beers. These data constitute the first report on the occurrence of TA 5 in buckwheat flour as well as in beer and beverages in general. The detailed results for cereals are summarised in Table 7. Beers were grouped as shown in Figure 21, whereas one sample could be member of several groups. With an average content of 11 μ g/kg over all 43 beer samples, the level of contamination is low. The mean TA 5 level of the alcohol free beer group was not reduced compared to the other groups, indicating that common alcohol removal procedures like reverse osmosis or vacuum distillation [157] do not reduce the TA 5 content of the final product. It is furthermore noticeable, that the bock beer group showed the highest average TA 5 content, even if the top sample containing 174.6 \pm 12.5 μ g/kg TA 5 was omitted as an outlier. A possible explanation might be elevated original gravity of bock beers (over 16 % original gravity) compared to pilsener beers (11.8-12.7 % original gravity). The original gravity is a measure for the amount of malt used in brewing. However, a direct correlation of original gravity and TA 5 content was not observed. It is thus concluded that the TA 5 contents found essentially depend on the contamination of the raw materials used for brewing.



Figure 21 Diamond box plot of the sample survey results for TA 5 in beer, total samples = 43, sample numbers in parentheses (one beer could be in several groups). The diamonds contain the middle 50 % of the data, the horizontal line indicates the median and the black spot (•) the mean of the respective dataset. The whisker bars indicate the lowest and highest concentrations observed.

Concerning the comparatively low mean TA **5** content of the wheat beer group, possible explanations are a different raw material composition (i.e. wheat in addition to barley) as well as a varying, top-fermented brewing style. Although beer is the predominant alcoholic beverage of central and northern Europe [158], with a consumption of 117 L per person in Germany as at 2006 [159], the levels of TA **5** found do not indicate acute health risks (*cf.* 1.8.1). The same can be said for the cereal based solid food products. Still, it is noteworthy that 48% of the solid samples and 86% of the beers were contaminated. Presuming an equally frequent contamination of other food products, the daily intake of TA **5** by the German population might be sufficient to trigger subacute or chronic effects. Hence, further food monitoring data as well as toxicological studies are needed. This is of particular concern since TA **5** is not commonly included in multi-mycotoxin methods applied to foods due to its unfavourable chromatographic properties.

| Sample | Organic product | c _{ta} [µg/kg] |
|--|------------------------|-------------------------|
| Above LOQ | | |
| Buckwheat flour | No | 851 ± 41 |
| Rye flour, type 997 | Yes | 168 ± 27 |
| Rye crispbread | Yes | 61 ± 9 |
| Below LOQ | | |
| Bread crisps (2 \times), buckwheat flour, crispbread, crispbread with amaranth, rye wholemeal flour, spelt crispbread, spelt wholemeal flour, wheat wholemeal flour | Yes (6 ×) No (4 ×) | $\approx 30^{1}$ |
| Not detected | | |
| Wholemeal wheat flour, wheat flour (6 \times), wholemeal spelt flour, oat flakes (2 \times), ground maize, maize flour, crispbread (2 \times) | Yes (1 ×) No (13 ×) | < 10 |

Table 7Sample survey results for TA 5 in cereal based solids.

3.1.6 Critical discussion

There are no previous articles on the HPLC-MS based quantification of TA **5**. However, in a poster presentation [160] Kocher *et al.* suggested an ESI(+)-HPLC-MS/MS method for TA **5** in cereals, which comprised extraction by MeCN:MeOH:water 40:10:50 (v:v:v) for 120 min, dilution, deep-freezing, thawing and centrifugation. Using 10 g of sample, a LOD of 30 μ g/kg (LOQ = 90 μ g/kg) was achieved. The eluents were modified by ammonium bicarbonate and

 $^{^{1}}LOD + 0.5 (LOQ - LOD) = (10 + 0.5 \cdot 40) \, \mu g/kg = 30 \, \mu g/kg$

the transition m/z 198 \rightarrow 153 was monitored for quantification. The average apparent recovery was 69 % (three cereal matrices tested).

Compared to the DNPH **16** method (analysis time: 90 min), the Kocher method is time consuming due to a long extraction period as well as freezing an thawing steps (analysis time > 4 h). Furthermore, the LOQ is approximately double that of the DNPH **16** method, while five times as much sample is used. It may also be noted that the results of Kocher *et al.* could not be reproduced on {I2} and {I12} with bicarbonate modified eluents (no TA **5** peak observed). Only when the eluents were modified with TFA, acceptable peak shapes could be obtained {M2}. In that case, however, the resulting LOD was as high as 2,000 µg/kg due to ion suppression by TFA. Therefore, the advantages of the DNPH **16** based method can be summarised as follows:

- (i) DNPH 16 can be mixed directly with solid and liquid food matrices. No heating, solvent evaporation or freezing is required, resulting in a short total analysis time of ~ 90 min
- (ii) Derivatisation resolves the chromatographic issues commonly obstructing effective quantification of TA 5 by HPLC-MS
- (iii) The LOQ in ESI(+)-IT-MS² is improved significantly as TFA can be omitted
- (iv) A sufficient selectivity is achieved by monitoring a m/z transition implying a derivatization specific part (cleavage of N-N bond) and a TA 5 specific part (loss of isobutyl side chain)
- (v) In the case of solid cereal based products, the average apparent recovery (79 %) is higher compared to the one of the previously published Kocher method (69 %) [160]

It seems acceptable to conclude from all these aspects that the proposed method is well suited for the quantification of TA **5** in food samples. The derivatisation with DNPH **16** is facile and fast, allowing for the straightforward monitoring for the analytically neglected mycotoxin TA **5** in consumer food products. As a consequence, TA **5** could be detected in buckwheat flour and beverages (beer) for the first time.

3.2 Quantification of ZON 10 in edible oils

3.2.1 DCHC

The approach presented here is based on the reversible reaction of ZON **10** with a hydrazine moiety anchored to a polystyrene resin (Figure 12). This DCHC method can be subdivided into three major steps:

- (i) Coupling (i.e. covalent binding) of ZON 10, present in an diluted edible oil, to the polymer resin 17 through hydrazone formation
- (ii) washing of the polymer resin to remove the edible oil matrix
- (iii) Hydrolysis of coupled ZON 18 yielding ZON 10 for quantification by HPLC-FLD

This sequence should allow an overall recovery between 70 % and 120 % to meet the typical performance criteria for ZON **10** requested by the European Commission [156, 161]. In this respect, the inherent equilibrium character of hydrazone formation is disadvantageous: during coupling, a fraction of ZON **10** will not react and is hence lost in the washing step. Similarly, during decoupling, a fraction of coupled ZON **18** will not hydrolyse and is thus not available for quantification. Also, it can be expected that hydrazone formation and hydrolysis are slower than physisorption or liquid-partitioning processes.

For the sum of these reasons, a first aim was to establish conditions which allow for the quantitative control of the ZON 10/coupled ZON 18 equilibrium (Figure 12). At the same time, the kinetics of the equilibrium had to be optimised in order to achieve practicality.

3.2.2 Optimisation of coupling and decoupling

Both hydrazone formation and hydrolysis are catalysed by acids or bases [134]. However, as ZON **10** is unstable under alkaline conditions [130], only acidic catalysis was considered (*cf.* Figure 16). In the case of coupling, the acid may be introduced with the solvent. Commonly used aqueous solvents (e.g. aq. HCl or aq. sulfuric acid) should be avoided because they shift the hydrazone formation equilibrium to the undesirable product side. Carbonic acids like AcOH (acetic acid) on the other hand, which may be used in water free mixtures, tend to cross-react with the hydrazone groups (acetylation) [162] and are also unsuited for coupling. Alternatively, the required catalytic protons may be introduced conveniently to the reaction mixture by converting the resin's hydrazine groups **17** to their hydrochlorides **23** before use. Hydrazine

hydrochlorides liberate HCl *in situ* $[162]^1$ and are compatible with non-aqueous solvents. A comparison of the coupling rates using **17** (non-activated, hydrazine groups) and **23** (activated, hydrazine hydrochloride groups) is shown in Figure 22. In both cases, the concentration of ZON **10** in the solvent was described by an exponential decay curve, indicating pseudo first order coupling kinetics. The pseudo first order rate constants were $134 (\pm 2) \times 10^{-4} \text{ min}^{-1}$ (**17**) and 772 (± 14) $\times 10^{-4} \text{ min}^{-1}$ (**23**). Hence, a sixfold increase in the reaction rate was achieved by the activation procedure.





C: ZON 10 and non-activated resin (17)

D: α -ZOL **11** and activated resin (**23**)

In the case of decoupling, the presence of water in the reaction mixture is desirable. Therefore, rapid phenylhydrazone hydrolysis is usually done with aqueous solvents at high acid strengths, e.g., in sulphuric acid [163] or levulinic acid [164]. However, to protect analytes and resin, it was intended to employ a milder pH and to use only completely volatile solvents. While mixtures of MeOH and dilute aq. HCl did not give acceptable results (Figure 23), ZON 10 decoupled readily at RT if acetone was used instead of MeOH. The rationale behind this effect is that acetone itself reacts with the resin yielding coupled acetone. As acetone is introduced in a high excess relative to the hydrazine sites 23, ZON 10 is eventually displaced into solution.

¹ The release of HCl from 23 prior to hydrazone formation with ZON 10 is an essential mechanistic requirement, as in 23 no nucleophilic electron lone pair is available at N_1 [134].



Figure 23 Hydrolysis of coupled ZON 18 in different solvents {M18}. The curves connecting the datapoints are spline based (no fit).
 A: solvent 24 {acetone:0.13 M aq. HCl 70:30 (v:v)}
 B: solvent 25 {MeOH:0.13 M aq. HCl 70:30 (v:v)}

The ZON 10 analogues (Figure 8) were tested under the optimised DCHC conditions as well. Only one analogue, ZAN 13, was reactive, the pseudo first order coupling rate constant being 784 (\pm 33) × 10⁻⁴ min⁻¹ (Figure 22). As ZAN 13 differs from ZON 10 only by the absence of a C=C bond, the rate constants for coupling are not significantly different and upon decoupling a concentration curve congruent with the one of ZON 10 was obtained. It should also be mentioned that ZAN 13 {t_R (retention time) = 13.1 min} partly co-eluted with ZON 10 (t_R = 13.2 min) under the employed HPLC conditions. This co-elution is commonly observed and may be exploited by using ZAN 13, which is not occurring in food, as an internal standard for ZON 10 quantification in combination with MS or MS/MS detectors [165-167].

The lacking reactivity of α -/ β -ZOL 11/12 and α -/ β -ZAL 14/15 is explained by the absence of a non-conjugated carbonyl group (the non-conjugated keto group of ZON 10 is reduced to the alcohol). As the benzene conjugated lactone carbonyl group is unreactive due to resonance stabilisation, there is no potential site for hydrazone formation and hence no reaction with the resin¹. Hence, DCHC separates ZON 10 and ZAN 13 from α -/ β -ZOL 11/12 and α -/ β -ZAL 14/15.

¹ The lacking reactivity of ZAN **13** can also be interpreted as indirect evidence for the structure of coupled ZON **18**. It furthermore confirms that extraction takes place by DCHC and not by some other mechanism (physisorption etc.).

3.2.3 Nanofiltration

To make use of all available hydrazine sites, ZON **10** needs to enter the macropores of the polymer resin (diameter: 3–6 nm). Its crystal structure indicates that the ZON **10** molecule is sufficiently small [168], however, the hydrodynamic volume of a small molecule is not adequately characterised by plain molecular dimensions. In fact, hydrogen bonds with surrounding solvent molecules can increase the hydrodynamic radius (R_H) and volume. This could cause ZON **10** to be excluded from the macropores through nanofiltration. In addition to the number of hydrogen bonds, the density and the size of the solvent molecules as well as their dipolar moment can also affect R_H : solvents with high dipolar moments are more likely to be attached to ZON **10**. Hence, the R_H of ZON **10** in different solvents was estimated on the basis of molecular simulation data (Table 8 and Figure 25). As expected, the averaged number of hydrogen bonds is maximal in water. Here, hydrogen bond formation increases R_H to an extent, which makes the rejection of ZON **10** by the macropores likely (Figure 24). In MeCN and EtOAc equally high R_H values are obtained due to the high dipole moments of these solvents. In the case of EtOAc, the size of the solvent molecule is thought to increase R_H further.



Figure 24 Comparison of calculated hydrodynamic radii for ZON 10 and the resin pore size.

However, in hexane, MeOH and THF (tetrahydrofuran) the described effects are significantly less pronounced, resulting in fourfold lower R_H values. In summary, the shown data support the hypothesis that ZON **10** is able to freely enter the macropores of the polymer resin, if hexane, MeOH or THF are chosen as coupling solvents. When using these solvents, all hydrazine sites, and not only those on the resin surface or in larger pores, should participate in the coupling reaction.



Figure 25 Hydrodynamic radii for ZON 10 in different solvents {M21}.

| Table 8 | Comparison of hydrodyna | mic parameters and I | DCHC results for ZON 10. |
|---------|-------------------------|----------------------|--------------------------|
|---------|-------------------------|----------------------|--------------------------|

| So | Solvent | | ation {M21} | DCHC | DCHC {M18} | |
|--------|---|---------------------------------|---|--|--|--|
| | Dipole moment [10 ⁻³⁰ C m] | Average number of H-bonds | Hydrodyn. radius (R _H) [nm] | Reaction rate [10 ⁻⁴ min ⁻¹] | Apparent recovery ¹ [%] | |
| EtOAc | 6.27 | 0.11 | 2.52 | 171 ± 9 | 73.5 ± 0.6 | |
| Water | 6.07 | 2.92 | 2.09 | - | - | |
| MeCN | 11.48 | 0.21 | 2.04 | 573 ± 34 | 75.8 ± 0.6 | |
| Hexane | 0 | 0.01 | 0.44 | - | - | |
| МеОН | 5.67 | 1.47 | 0.43 | 772 ± 14 | 79.6 ± 0.6 | |
| THF | 5.84 | 0.06 | 0.42 | 70 ± 1 | 63.9 ± 0.8 | |

¹ If used as coupling solvent with a spiked sample (c = 2 mg/kg) according to {M4}.

3.2.4 Sample to resin ration and choice of coupling solvent

To test the influence of the coupling solvent experimentally, the DCHC sample preparation was conducted with MeCN, EtOAc or THF instead of MeOH and all corresponding coupling rate constants were determined (Table 8). Hexane and water could not be investigated due to the limited solubility of ZON **10** in these solvents. MeOH afforded the highest coupling rate and the highest apparent recovery if applied with a sample matrix. The lower rate constants for MeCN and EtOAc may be attributed to the significantly higher R_H of ZON **10** in these solvents, causing a lower accessibility of the resin pores and reactive hydrazine sites. However, this cannot be considered the only factor of importance, as THF gave the lowest coupling rate, although the respective R_H was smallest.

The ideal sample to resin ratio was determined by evaluating the recoveries obtained by sample preparations with varying resin amounts (Figure 26). The ideal ratio was \geq 500 mg resin per mL oil. If this ratio is maintained, with the amount of sample being increased or reduced, the method may be up- or downscaled to achieve a better LOD or to save resin, respectively.



Figure 26Effect of the resin/oil ratio on the ZON 10 relative apparent recovery (750 mg/mL set to
100 %) as determined by analysing a blank matrix spiked to $c_{ZON} = 5 \text{ mg/kg} \{\$M4\}.$

3.2.5 Regeneration of the polymer resin

As DCHC is reversible by principle, it seemed plausible to reuse resin 17 in order to lower analysis costs. Hence, several recycling routines were evaluated. Best results were obtained when the resin was continuously washed overnight. Using this approach, a ZON 10 spiked blank matrix ($c_{ZON} = 5 \text{ mg/kg}$) was worked up on 12 days starting with new resin. After each day, the

used resin was recycled in bulk and used for the next day's determinations (Figure 27). It can be seen that resin performance decreased gradually after the third regeneration cycle. For the first three days, the two-sided t-test (f = 18, P = 95 %) showed no statistically significant differences in the obtained recoveries. Also, the F-test ($f_1 = f_2 = 9$, P = 95 %) detected no statistically significant inhomogeneity if applied to the highest and lowest RSD obtained throughout the trial. Hence, it may be concluded that the resin can be used up to three times without losses in recovery or precision. Based on the experimental results outlined above, a sample preparation routine was derived (Figure 28). Due to miniaturisation, the whole sample preparation can be conducted in a 2 mL safelock tube.



Figure 27 Relative performance of polymer resin 23 after regeneration, recovery at cycle 0 set to 100 %. Datapoints are connected by straight lines (no fit).

3.2.6 Validation

To assess linear range and apparent recovery, two 12 point calibration curves were constructed: curve A by dissolving ZON **10** in solvent **24** and curve B by spiking a blank sample and subsequently conducting the DCHC sample preparation for each spiking level. For both curves linearity was given up to $c_{ZON} = 20,000 \,\mu g/kg$. The curve equations in the range 10–20,000 $\mu g/kg$ (ZON **10** per oil) or 6–11,250 $\mu g/kg$ (ZON **10** per **24**) were:

Curve A (solvent):
$$y = (0.786 \pm 0.002) x - (3.911 \pm 5.268), R^2 = 0.9999$$

Residual standard deviation (s_y) = 14.9
Curve B (spiked sample): $y = (0.626 \pm 0.004) x - (4.922 \pm 14.250), R^2 = 0.9998, s_y = 41.3$



Figure 28 Flowchart of the sample preparation routine for ZON 10 in edible oils {M4}.

with $x = c_{ZON}$ [µg ZON 10 per kg elution solvent] and y = peak area [a.u.]. From the two slopes, the method's apparent recovery for the spiked blank matrix computes as 79.6 (± 0.6) %. The recovery (without sample matrix) was 92.6 (± 2.1) % (Figure 23). Further apparent recoveries and RSDs for contaminated edible oils are given below. The average apparent recovery of the spiked blank matrix and the four positive samples was 89 (± 10) % (in fact, the apparent recovery of the spiked blank matrix was lowest; however, it still fell into the acceptable range discussed below). Using curve B, LOD and LOQ were determined to be 10 and 30 µg/kg, respectively. Trueness and precision were characterised by the bias and RSD obtained for a blank matrix spiked to different ZON 10 concentrations using the methods of external calibration or standard addition (Table 9). It can be seen that the application of standard 60 BAM-Dissertationsreihe

additions improves trueness, particularly at low ZON **10** concentrations. To satisfy the specific official requirements (EU) for ZON **10** analysis, RSDs should not exceed 25 %. Also, the method's apparent recovery should be in the range of 70–120 % [156, 161]. These criteria are well met, regardless of whether external calibration or standard additions are considered. Characteristic chromatograms are given in Figure 29.

| c _{zon} spiked [µg/kg] | 30 (LOQ) | 301 (LOQ × 10) | 3,003 (LOQ × 100) |
|---|------------|----------------|-------------------|
| c _{zon} measured (external calibration, n = 5, recovery corrected) [µg/kg] | 32 ± 1 | 296 ± 8 | 3017 ± 8 |
| RSD [%] | 3.1 | 2.7 | 0.3 |
| Bias [%] | 6.7 | 1.7 | 0.5 |
| c _{zon} measured (using standard addition) [μg/kg] | 30 ± 1 | 299 ± 10 | 2950 ± 26 |
| RSD [%] | 3.3 | 3.3 | 0.9 |
| B ias [%] | 0 | 0.7 | 1.8 |
| Injection RSD, n = 5 [%] | 0.8 | 1.5 | 0.4 |

Table 9Validation data for ZON 10 in a maize oil matrix.

3.2.7 Sample survey

A total of 44 edible oils based on various agricultural commodities was sourced from local supermarkets as well as online shops and analysed by HPLC-FLD using standard additions. For further confirmation HPLC-MS/MS was employed. The MS/MS detector afforded an LOD and LOQ of 5 and 15 µg/kg, respectively, as well as a linear range of 5–1,000 µg/kg.

ZON 10 was detected and quantified in four samples. All contaminated samples were maize oils (Table 10). ZON 10 was not detected in the following samples: grape core oil (4×), linseed oil, maize oil (2×), olive oil (5×), peanut oil (2×), pumpkin seed oil, rapeseed oil (8×), rice oil, salad oil (mixture), sesame oil (3×), soy oil, sunflower oil (6×), thistle oil, walnut oil and wheat germ oil (3×).

Previous publications on ZON **10** in consumer maize oils reported average contents of 170 μ g/kg (total samples: 38, positive samples: 38) [66] and 505 μ g/kg (total samples: 17, positive samples: 9) [100]. Thus, the mean ZON **10** content found in the present study is comparatively low. Also, in contrast to the previous studies, a violation of the current EU ML

 $(400 \ \mu g/kg)$ for ZON **10** was not observed. This may be attributed to a higher awareness for the problem of ZON **10** in maize oil amongst producers due to the introduction of the EU ML in 2005. Still, it is noteworthy that 67 % of all maize oils were contaminated.

| | DCHC-HPLC-FLD | | DCHC-HP | DCHC-HPLC-MS/MS | | Liquid-partitioning- HPLC-FLD | |
|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------------|--|
| Sample | c _{zon} [µg/kg] | Apparent recovery [%] | c _{zon} [µg/kg] | Apparent recovery [%] | c _{zon} [µg/kg] | Apparent recovery [%] | |
| #1 | 57 ± 1 | 96 ± 1 | 58 ± 2 | 107 ± 10 | 93 ± 35 | 69 ± 44 | |
| #2 | 86 ± 1 | 102 ± 5 | 80 ± 5 | 89 ± 6 | 299 ± 63 | 36 ± 31 | |
| #3 | 135 ± 3 | 84 ± 3 | 135 ± 7 | 102 ± 5 | 128 ± 4 | 80 ± 9 | |
| #4 | 117 ± 3 | 82 ± 2 | 104 ± 5 | 75 ± 4 | 92 ± 5 | 131 ± 9 | |
| Mean | 99 | 91 ± 10 | 94 | 93 ± 14 | 153 | 79 ± 40 | |
| Ø RSD [%] | 1.4 ± 0.5 | - | 9.7 ± 2.9 | - | 16.7 ± 16.1 | - | |

Table 10ZON 10 contents in the positive maize oil samples as determined by various methods.

3.2.8 Selectivity

In contrast to the methods for TA **5** discussed above, ZON **10** was quantified by FLD rather than MS. This was possible due to the high selectivity of the DCHC sample preparation. While FLD offers sensitivity comparable to MS/MS, it is significantly more precise (*cf.* 1.7.2). Also, FLD systems are comparatively cheap and widely available. In the case of ZON **10**, the major weak spot of FLD, selectivity, could be successfully compensated for by establishing a selective sample preparation routine (DCHC). Although other compounds will be co-extracted by DCHC, a matrix component, critically interfering with the quantification of ZON **10**, would have to meet the following criteria:

- (i) Ability to pass the resin pores
- (ii) Presence of a reactive group (e.g. a non-conjugated carbonyl group)
- (iii) Sufficient reactivity during coupling
- (iv) Sufficient reactivity during decoupling
- (v) Same HPLC retention window as ZON 10
- (vi) Fluorescence properties similar to those of ZON 10



Figure 29 HPLC-FLD chromatograms for ZON 10 obtained for different extraction methods.

Carbonyl groups, which are conjugated to heteroatoms (as in lactones, carbonic acid esters and amides etc.) exhibit a reduced reactivity towards phenylhydrazines [154, 169-171] and are thus of no major concern under the mild reaction conditions employed here. Hence, a significant cleanup effect is achieved by DCHC (Figure 29). In combination with the high precision of FLD, this ensures accurate results, which are highly desirable for analyses in the frame of current ZON **10** regulations (*cf.* 2.3).

3.2.9 Critical discussion

To date, there is no reference method for the determination of ZON **10** in edible oils. However, the DCHC method was compared to the most recently published liquid-partitioning method [130]. For this purpose, the ZON **10** contents in the positive maize oil samples were determined by both methods, including the determination of RSDs and recoveries (Table 10). For qualitative comparison of the resulting chromatograms, GPC was performed as well Figure 29. Table 11 gives a general overview of previous methods, including the performance characteristics given in the respective publications.

| Ref. | Extraction Cleanup Detection | Sample intake [g] | LOD [µg/kg] | Recov. [%] | Solvent [§] [mL] | Ø RSD (samples evaluated) |
|-------|-------------------------------------|-------------------------|----------------|---------------|------------------------------|---|
| - | DCHC DCHC FLD | 0.2 | 10 | 89 | 17 | $1.4\%(4)^{\dagger}$ $2.0\%(3)^{\ddagger}$ |
| | DCHC DCHC MS/MS | 0.2 | 5 | 93 | 17 | $9.7~\%~(4)^{\dagger}$ |
| [130] | Liquid-partitioning - FLD | 2 | 10 | 87 | 20 | $3.1 \% (1)^{\dagger}$ $2.8 \% (1)^{\ddagger}$ |
| [100] | Liquid-partitioning - FLD | 5 | 3 | 86 | 140 | $5.7\%(2)^{\ddagger}$ |
| [66] | GPC IAC FLD | 4 | 3 | 85 | 202 | 3.1 % (3) [‡] |
| | GPC - MS/MS* | 4 | 0.3 | 91 | 196 | 2.3 % (3) [‡] |

 Table 11
 Comparison of the DCHC method to previously published methods for ZON 10 in edible oil.

 $^{\$}$ organic solvent consumed per sample preparation, * internal standard: α -d4-ZOL, † naturally contaminated samples, ‡ spiked blank samples

Upon applying liquid-partitioning to the four ZON **10** positive maize oil samples, a series of problems was experienced. First of all, the separation of layers was not always easily achieved and required varying centrifugation speeds and times (no details on the centrifugation process

are given in [130]). Secondly, the RSDs were unacceptable for two samples and thirdly, recoveries were outside the 70–120 % range in three cases (Table 10). For our blank matrix spiked to 30 μ g/kg, no ZON **10** was recovered at all (Figure 29). These results indicate that consumer maize oils differ significantly in their chemical composition and that a liquid-partitioning based sample preparation is not robust enough to account for these variations. Hence, liquid-partitioning cannot be considered methodologically sound and poses no serious alternative to GPC or DCHC.

Comparing GPC and DCHC, the latter technique features a twelvefold lower organic solvent consumption. Also, as GPC does not provide cleanup (Figure 29), it has to be combined with IAC (5–20 \notin per single use column) or MS/MS and isotope standards. By choosing the selective DCHC method these expensive techniques can be avoided.

While the DCHC sample preparation requires coupling and decoupling for 2 h each, it involves little manual labour. All involved reactions were optimised to proceed quantitatively at RT. Also, the use of small, disposable reaction vessels minimises the need for glassware and allows a laboratory assistant to carry out approx. 30 simultaneous sample preparations per day.

It can furthermore be noted, that DCHC features the lowest RSDs compared to pre-existing methods (Table 11). This precision is attributed to the summary of extraction and cleanup to one straightforward procedure. As the complete DCHC sample preparation is carried out in a single safelock tube, uncertainties due to handling, solvent transfer etc. are minimised.

The conclusion, which can be drawn from all these aspects is, that DCHC is well suited as a sample preparation technique for purposes of quantitative instrumental analysis. For ZON **10** in edible oils DCHC can be a valuable alternative to the extraction by liquid-partitioning or GPC because it minimises the need for laboratory equipment, being highly selective and accurate at the same time. For these reasons, DCHC is considered to be well suited for the monitoring of the current EU ML for ZON **10** in refined maize oil as well as for the cost-efficient analysis of other oils or fatty matrices.

3.3 Kinetic study on the degradation of TA 5 in aq. solution

3.3.1 Method development

Chromatographic separation of the TA **5** epimers was previously achieved by ion- and ligandexchange chromatography [118] as well by a high carbon load octadecylsilane column and phosphate buffered eluents [105]. However, all mentioned methods did not achieve base line separation of the epimers and operated at high flow rates (1–2 mL/min). Thus, in order to optimise separation and eluent expenditure, a novel HPLC-DAD method for the simultaneous quantification of TA **5** and *u*-TA **19** was developed¹. In this method, a silica-free, graphite based Thermo Hypercarb column was employed. To suppress TA **5** ionisation on column, eluents were modified with 0.1 % (v) TFA. Lower TFA concentrations led to peak broadening. The addition of a small amount of EDTA to the aqueous eluent further improved peak shapes (*cf.* Figure 10). The optimised method allowed separation of TA **5** ($t_R = 12.8$ min) and *u*-TA **19** ($t_R = 13.5$ min) with a chromatographic resolution of 2.2 (full baseline separation, Figure 30) and a total run time of 23 min. The LOD was 0.3 µmol/kg (60 µg/kg).

The epimeric mixture of DTA **20** and *u*-DTA **26**, iso-DTA (*cf.* 5.3), was quantified by HPLC-IT-MS² as it lacks the major UV absorption maximum of TA **5** ($\varepsilon_{280} = 12,980 \text{ L cm}^{-1} \text{ mol}^{-1}$ [105]), featuring rather weak absorption maxima (Figure 31). Unfortunately, a separation of DTA **20** and *u*-DTA **26** could not be achieved.



Figure 30 Sample HPLC-DAD (λ = 280 nm) chromatogram (week 3 at pH 3.5, T=40°C).

¹ The DNPH **16** based method discussed above did not allow for a separation of TA **5** and *u*-TA **19**.



Figure 31Analytical data for DTA 20: UV spectrum (in MeOH) and ORTEP representation of the crystal
structure [8]. The pKA of DTA 20 was 6.8 ± 0.1 {M12}. UV extinction coefficients:
 $\epsilon_{265} = 1,416 \pm 134$ and $\epsilon_{208} = 5,279 \pm 208 \, L \, cm^{-1} \, mol^{-1}.$

3.3.2 Design of the kinetic study

The kinetic study was conducted at two pH levels and three temperatures, respectively, resulting in a total of six datasets. A pH of 3.5 was chosen as it is representative for beverages potentially contaminated with TA **5** (e.g. apple juice, pH 2.8–3.9 [172], tangerine juice: pH 3.2–3.6 [173, 174]). A pH 7 control was done as well. Typical storage temperatures representative for refrigeration (T = 4 °C) and RT storage (T = 25 °C) were selected. As limited degradation was expected at T = 4 °C, a T = 40 °C series was done to obtain appropriate datasets for the calculation of kinetic parameters. The average weight loss of the stored solutions, which is attributed to solvent evaporation, was found to be 2.08 ± 0.09 % for 40 °C, 0.45 ± 0.05 % for 25 °C and 0.08 ± 0.06 % for 4 °C at the end of the study period.

3.3.3 Results of the kinetic study

TA **5** was stable at pH 7.0, $T = 4 \,^{\circ}C$ and pH 7.0, $T = 25 \,^{\circ}C$. At pH 7.0, $T = 40 \,^{\circ}C$ and pH 3.5, $T = 4 \,^{\circ}C$ minor degradation occurred. Significant decay was observed at pH 3.5, $T = 25 \,^{\circ}C$ and pH 3.5, $T = 40 \,^{\circ}C$. After establishing the TA **5** decay curves (Figure 33), the kinetic study vials were screened for possible degradation products {I2}. Whereas DDTA **21** (deacetyl decarboxyl tenuazonic acid) was not detected, iso-DTA was found for all datasets

shown in Figure 33. As the molar balance (sum of [TA 5], [*u*-TA 19] and [iso-DTA]) was retained, iso-DTA is assumed to be the only hydrolytically formed TA 5 degradation product. Furthermore, it can be concluded that iso-DTA is not degraded further under the conditions of the kinetic study.

3.3.4 Kinetics of hydrolysis

During the kinetic study the concentrations [TA 5] and [iso-TA] decreased exponentially indicating pseudo first order kinetics. Consequently, the ratio of TA 5 in the epimeric mixture ([TA 5]/[iso-TA]) decreased exponentially as well, leading to a simple kinetic scheme (Figure 32) in which [TA 5] is reduced by two reactions: epimerisation and hydrolysis to DTA 20.



Figure 32 Kinetic scheme for the degradation of TA 5 in aq. buffer (pH 3.5).

Hydrolysis proceeds significantly faster under acidic conditions (Figure 33). Thus an acid catalysed reaction following either an A1 or A2 mechanism is indicated. With $[H^+]$ and $[H_2O]$ being constant due to the use of an aqueous, buffered solvent, both mechanisms equally explain the observed rate law. However, as most acid-catalysed hydrolyses of carbonic acid analogues¹ proceed via the A2 mechanism [175] and very dilute solutions are considered, an A1 mechanism is unlikely.

¹ TA **5** can be seen as an AcOH derivative.

Figure 33

Kinetic study results, starting from pure TA **5**. Datapoints are connected by straight lines (no fit).

A (squares): molar balance B (circles): [iso-DTA] {M6} C (diamonds): [iso-TA] {M5} D (triangles): [TA **5**] {M5} E (inverted triangles): [u-TA **19**] {M5}



This is supported by the fact that dS^{\ddagger} (entropy of activation) has a negative sign for the hydrolysis process (Table 12), indicating a loss of entropy in the RDS (rate determining step)¹; in the A2 mechanism, the RDS corresponds to the nucleophilic attack of the protonated TA **5** intermediate by water (Figure 34).

As epimers can show different chemical behaviour, the hydrolysis rates of TA 5 and u-TA 19 might differ as well. However, based on the obtained datasets the rate constants k_{H} and k_{H} ' were not found to be significantly different (two sample t-test, unequal variances, $\alpha = 0.001$). This is reasonable, as the stereogenic part of the molecule is not directly involved in the hydrolysis reaction.

Lastly, the E_A (energy of activation) of the hydrolysis reaction was compared to literature values for similar processes: Kennon reported an average E_A for the decomposition of 38 solubilised pharmaceuticals to be 82.9 kJ mol⁻¹ [176] and Connors *et al.* reported an average value of 87.9 kJ mol⁻¹ for 147 drugs [177]. The activation energies determined for TA **5** hydrolysis are in reasonable agreement with these values.



Figure 34 Suggested mechanism for the formation of DTA 20 from TA 5 in aq. buffer (pH 3.5).

 $^{^{1}\}Delta S^{\ddagger}$ values of known A2 reactions lie in the range of -60 to -120 J mol⁻¹ K⁻¹ [175].

Table 12

Kinetic and thermodynamic data for the degradation of TA **5** in aq. buffer (pH 3.5), $dG^{\ddagger} =$ Gibbs energy of activation, $dH^{\ddagger} =$ Enthalpy of activation.

| | Hydrolysis | | |] | Epimerisation | | |
|--|----------------------------|----------------|---------|--|----------------|---------|--|
| T [°C] | 4 | 25 | 40 | 4 | 25 | 40 | |
| Datapoints, n | 16, 2 | 16, 2 | 8,2 | 16, 2 | 16, 2 | 8,2 | |
| R ² (1 st order linear fit) | 0.9912 | 0.9994 | 0.9999 | 0.9879 | 0.9998 | 0.9992 | |
| k | 9.9 | 72.1 | 343.1 | 2.2 | 22.6 | 154.6 | |
| $[10^{-4} d^{-1}]$ | (± 0.2) | (± 0.8) | (± 4.0) | (± 0.1) | (± 0.1) | (± 1.6) | |
| Half life | 703.7 | 96.2 | 20.2 | 3,155.6 | 306.3 | 44.8 | |
| [d] | (± 16.6) | (± 1.1) | (± 0.3) | (± 87.3) | (± 1.1) | (± 0.5) | |
| dG [‡] | 83.7 | 85.3 | 85.6 | 87.1 | 88.1 | 87.7 | |
| [kJ/mol] | (± 1.9) | (± 0.6) | (± 0.4) | (± 2.4) | (± 0.4) | (± 0.9) | |
| Interpretation (cf. Figure 32) | $k = k_{H} \approx k_{H}'$ | | | $\mathbf{k} = \mathbf{k}_1 - \mathbf{k}_{-1} \approx \mathbf{k}_1$ $(\mathbf{k}_1 >> \mathbf{k}_{-1})$ | | | |
| R ² of Arrhenius plot | | 0.9963 | | | 0.9946 | | |
| E _A [kJ/mol] | 70.3 (± 4.3) 84.4 (± 6.2) | | | | | | |
| dH [‡] [kJ/mol] | 67.9 (± 4.3) 81.9 (± 6.2) | | | | | | |
| dS^{\ddagger} $[J mo\Gamma^{1} K^{-1}]$ | | -58.4 (± 14.6) | | | -20.9 (± 20.9) | | |

3.3.5 Kinetics of epimerisation

By inversion of the stereogenic centre at C_5 , TA **5** is converted to *u*-TA **19** (Figure 13). At pH 3.5, T = 25 °C, epimerisation is approximately three times slower than hydrolysis. Due to the less negative dS[‡], the epimerisation rate increases with respect to hydrolysis at higher temperatures. Whereas epimerisation of TA **5** was previously reported for alkaline pH [70, 105], the presented results show that there also is an acid catalysed epimerisation mechanism, which probably involves enol formation in the neutral TA **5** molecule, analogous to Figure 13.

In kinetic terms, the ratio [TA 5]/[iso-TA] should decrease following pseudo-first order kinetics until the equilibrium ratio is reached. The rate constant of this process is $k = k_1 - k_{-1}$. However, none of the obtained datasets allowed the direct observation of the equilibrium composition as either epimerisation was too slow or hydrolysis was too fast, driving the TA 5 and *u*-TA 19 levels below the LOQ of the employed HPLC-UV method. The indirectly obtained

values for k_1 and $k_{.1}$ {M19} were 0.54 (± 0.82) x 10⁻⁴ d⁻¹ and 3.30 (± 4.83) x 10⁻⁴ d⁻¹ for pH = 3.5, T = 25 °C and pH = 3.5, T = 40 °C, respectively. $k_{.1}$ is thus not significantly different from zero (one sample t-test, $\alpha = 0.001$), given the available precision, and the observed rate constant for epimerisation may be approximated as $k \approx k_1$. Under alkaline conditions (0.1 M aq. KOH, RT) epimerisation is fast and the equilibrium composition may be observed directly. Here, the fraction of *u*-TA **19** was 62.4 ± 0.8 %, corresponding to an equilibrium constant K = 1.66 ± 0.04.

3.3.6 Stability and occurrence in food matrices

Due to the extended time frame of the kinetic study, it was not possible to investigate actual beverage matrices like apple juice or beer, as these would have been subject to microbial or fungal decay¹. However, stability data for the mentioned matrices were obtained on a short timescale (Figure 35) by analysing spiked beverages. It can be seen that DTA 20 was degraded rapidly while TA 5 was stable. DTA 20 and *u*-TA 19 could not be detected upon screening the available food samples (*cf.* 3.1.5) using methods {M5} and {M6}. However, it should be mentioned that method {M5} was not sensitive and selective enough to be applied to the naturally TA 5 contaminated beer samples (*cf.* 3.1.5). Hence, further method development directed towards higher sensitivity is necessary in order to assess the natural occurrence of *u*-TA 19. The low stability of DTA 20 (Figure 35) renders an occurrence in beverages unlikely.



Figure 35 Stability of TA 5 and DTA 20 in spiked beverage matrices $\{M20\}$, n = 3. Datapoints are connected by straight lines (no fit).

¹ Pasteurisation equipment was not available at the time of the study.
3.3.7 Discussion

Beverages are typically stored between 4–25 °C. Under the conditions of the kinetic study the half lives of TA 5 were 575.4 \pm 13.4 d (4 °C) and 73.8 \pm 0.4 d (25 °C) (comprising degradation by epimerisation as well as by hydrolysis). It is thus fair to assume that TA 5 is sufficiently stable to be encountered in beverages made from contaminated raw materials. However, degradation may occur to a certain degree, yielding the TA 5 epimer *u*-TA 19 and, in higher quantities, the hydrolysis product DTA 20. While DTA 20 was stable in aqueous buffer, it was highly unstable in beverage matrices. This is explained by the enol moiety of DTA 20, enabling a wide range of reactions with other beverage components (Figure 36) [178]. Also, contrary to TA 5, DTA 20 can not be stabilised by complexation of metal cations (*cf.* Figure 9). The compound is thus not expected to be readily detectable in beverages.

With respect to the toxicity of *u*-TA **19** and DTA **20** there is very limited information available. The only relevant study [72] dealt with phytotoxicity which was tested by observing rice root growth inhibition and rice leaf browning induction. *u*-TA **19** and DTA **20** exhibited a significantly lower phytotoxicity when compared to TA **5**. Although this indicates that the stereochemistry at C_5 as well as the presence of the acetyl sidechain have an influence on TA **5** toxicity, it is relevant for rice plants only and cannot be extrapolated to humans or animals. At present, it thus remains unclear, whether epimerisation or hydrolysis of TA **5** can be considered as "detoxifying" processes.



Figure 36 Reactivity of DTA 20.

3.4 Degradation of AOH 7, AME 8 and ALT 9 upon bread baking

3.4.1 TA-MS of bulk AOH 7

Initially, the behaviour of bulk AOH 7 upon dry heating was investigated. To do so, a TA-MS coupling device [179, 180] was employed. In the present case, this device allows for the detection of endo- or exothermic processes (e.g. isomerisation) by DTA (differential thermo analysis) while the TG (thermo gravimetry) curves show mass loss due to evaporation or chemical split off reactions. Furthermore, IC curves recorded by the coupled quadrupole mass spectrometer allow for a qualification of liberated gaseous species. TA-MS experiments were performed in argon and air (Figure 38) {M11}. In argon, the constant sample mass indicates absence of any dynamics up to 270 °C. The subsequent mass loss is attributed mainly to the evaporation of AOH 7. The endothermic effect at T_{on}^{ex} (extrapolated onset temperature) 349 °C is the melting peak (literature value: 350 °C [81]) followed by stronger evaporation. Even when sublimed at 380 °C (ambient pressure, argon atmosphere) AOH 7 deposits in the form of crystalline needles (Figure 37), thus further confirming the high thermal stability of the compound. In air, the mass loss above 300 °C is due to a burning process (as indicated by m/z 18 (H₂O⁺) and m/z 44 (CO₂⁺)). Both in argon and air the temperatures required for a substantial mass loss exceed typical food processing conditions. Hence, the conclusion which can be drawn at this stage is that bulk AOH 7 is thermally stable at common baking temperatures both in oxidising and non-oxidising atmospheres.



Figure 37 Line structure of AOH 7 and ORTEP representation of its crystal structure [10].



Figure 38 TA-MS curves for bulk AOH 7 with the ionic current curves for m/z 18 (H₂O⁺) and 44 (CO₂⁺).

3.4.2 Degradation products of AOH 7/AME 8

In pilot studies AOH **7**, AME **8** and ALT **9** were refluxed separately in (i) aqueous phosphate/citrate buffer (0.15 M, pH 5), (ii) 0.1 M KOH and (iii) aqueous phosphate/citrate buffer (0.18 M, pH 7) for 5 h with the solutions being analysed by HPLC-DAD. While all three compounds were stable in (i), complete degradation accompanied by browning of the solution occurred in (ii), however, no well-defined degradation products could be identified by HPLC-DAD. In (iii), ALT **9** was stable, but in the case of AOH **7** and AME **8** degradation took place. For both compounds a novel DAD peak, corresponding to the degradation products AOD **27** (degraded AOH **7**) and AMD **28** (degraded AME **8**), was observed. Subsequently, AOD **27** and AMD **28** were synthesised and purified {M17}. The assignment of structures was done on the basis of ¹H, ¹³C, HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multiple bond coherence) spectra as well as exact mass measurements (Figure 39).



Figure 39 Selected HMBC correlations for AMD 28.

The mechanism suggested for the formation of AOD **27** and AMD **28** is shown in Figure 40. It is initiated by hydrolysis of the lactone group and thus favoured by an elevated pH. The resulting hydrolysed intermediate is finally decarboxylated. Decarboxylation is favoured by the presence of a hydroxyl group in an *ortho* position of the carboxylic acid moiety, allowing for a cyclic decarboxylation intermediate [181]. This may explain why decarboxylation takes place at the relatively low temperature dictated by the BP of the aqueous solvent. That ALT **9** is not susceptible to the shown mechanism is possibly due to the lack of aromaticity in the C1–C6 ring (Figure 39). If this ring is not aromatic, the charged, hydrolysed intermediate (Figure 40) lacks resonance stabilisation, thus disfavouring hydrolysis. Furthermore, the steric shielding of the ALT **9** lactone group by the diastereotopic methyl moiety, which is not present in AOH **7** and AME **8**, might reduce reactivity¹.

It can furthermore be noted that the reaction rates of AOH **7** and AME **8** are limited by their low solubility in water. In the employed phosphate/citrate buffer (pH 7) the approximate solubilities were 1.1 mg/kg (AOH **7**) and 0.1 mg/kg (AME **8**). This explains the significantly longer reaction time needed for quantitative conversion of AME **8** {M17}.

Since the degradation of AOH 7 and AME 8 by the suggested mechanism requires water, heat and a pH \geq 7, no AOD 27 was formed during the TA-MS experiments. Also, AOD 27 and AMD 28 could not be detected in preliminary boiling experiments with tomato soup (pH 4.3) and apple pure (pH 3.6) spiked with AOH 7 and AME 8.

¹ The same arguments apply to the structurally comparable ZON **10**, which was also unreactive under the conditions that led to the formation of **5** and **6**.



Figure 40 Suggested formation mechanism of AOD 27/AMD 28 in aqueous citrate/phosphate buffer (pH 7).

3.4.3 Design of the baking experiments

Temperature is an essential parameter in the degradation of chemical substances. However, the temperature inside an oven differs from the temperature inside the dough being baked. A steep temperature gradient of $200 \rightarrow 120$ °C is observed only in the outermost layer of the dough, whereas the inside (i.e. the bulk of the material) does not exceed 106 °C even at the end of the baking process [157]. This should be considered when evaluating baking studies.

During the study presented here, two experimental designs were employed: one using spiked flour only (dry baking) and one using spiked flour and water (wet baking). Oven temperatures (170–230 °C), baking times (0–60 min) as well as the amounts of flour and water were chosen in a way that all stages of the baking process could be reproduced (i.e. whereas baking for 15 min at 170 °C afforded a moist sponge, baking for 60 min at 230 °C caused the complete evaporation of all added water, yielding a rusk like product). The average water content of most bread types lies between 37–42 % [157]. To obtain such a water content with the recipe used in our baking study, baking for 45–60 min at 200 °C or baking for 30–45 min at 230 °C are most suited and may thus be considered most realistic.

In this context, the experiments done with flour only are less realistic, as the absence of water causes the bulk flour to attain the actual oven temperature, which is substantially higher than the $106 \,^{\circ}C$ usually reached. However, these experiments have some relevance for the processes occurring inside the outer layer of the dough and can show some general tendencies in compound stability.

3.4.4 Method development

During baking, a range of chemical processes occur, e.g. the denaturation of proteins, starch gelatinisation, the release of dextrins, mono- and disaccharides as well as caramelisation and non-enzymatic browning reactions [157]. This implies that a baked product will have chemical and physical properties significantly different from the dough. This is also the case for the different products to be analysed during a quantitative time-resolved baking study. If the same quantitative analytical method is used on all baking products, whether they were obtained after 30 min at 170 °C or 60 min at 230 °C, it is of important to ensure that the chemical changes during baking do not falsify the quantitative data through varying MEs. Ultimately this means that either a reliable internal standard (e.g. an isotope standard when using MS) has to be found, or that the apparent recovery of the employed method needs to be established for each baking time and temperature. As isotope standards are not yet commercially available for AOH 7, AME 8 and ALT 9, it was decided to employ the method of standard additions. Standard additions were done for each baking time and temperature.

The MRM (multiple reaction monitoring) transitions for the quantification of AOH 7, AME 8, ALT 9, AOD 27 and AMD 28 were chosen on the basis of both intensity and selectivity. E.g. for AME 8 the most intense ESI(-) transitions were: $m/z 270.9 \rightarrow 255.9$ (100%) and $270.9 \rightarrow 228.0$ (24%) as opposed to $m/z 273.1 \rightarrow 128.1$ (70%) and $273.1 \rightarrow 115.0$ (47%) for ESI(+). However, as the most intense transition corresponds to a mere demethylation, ESI(+) was chosen for the sake of selectivity.

For the sample preparation, the routine previously established for TA 5 (*cf.* 3.1) was adapted. The recoveries obtained for the spiked wholemeal wheat flour used for all further experiments are summarised in Table 13. In the case of AME 8 the apparent recovery was elevated to 141 %. The reason for this could be identified as signal enhancement in the MS/MS ion source due to a ME (Table 13). Such MEs are commonly observed. Lattanzio *et al.* [182] communicated, for example, that upon analysing mycotoxins in cereals by HPLC-MS/MS a statistically significant increase in the signal due to MEs occurred for 20 out of 32 analyte/cereal sample combinations. Although recoveries > 120 % are generally not desirable for quantitative analysis, the elevated

apparent recovery for AME 8 was judged to be acceptable for the frame of this study, as it was consistently compensated for by standard additions.

| Table 13 | Recovery | data | of | the | employed | analytical | method | (determined | for | the | untreated, | spiked |
|----------|----------|--------|-------|-------|------------|-------------|------------|----------------|------|-------|------------|--------|
| | wholemea | l flou | r use | ed in | the baking | studies), s | ee [2] for | further valida | tion | data. | | |

| Analyte | Apparent recovery [%] | ME [%] | Recovery [%] |
|--------------|-----------------------|-------------|--------------|
| AOH 7 | 99 ± 9 | 113 ± 3 | 88 ± 8 |
| AME 8 | 141 ± 3 | 160 ± 5 | 88 ± 3 |
| ALT 9 | 102 ± 3 | 120 ± 2 | 85 ± 3 |
| AOD 27 | 95 ± 4 | 98 ± 4 | 97 ± 6 |
| AMD 28 | 92 ± 3 | 106 ± 4 | 87 ± 4 |

3.4.5 Stability of AOH 7, AME 8 and ALT 9 upon wet and dry baking

The results are summarised in Figure 41. Under the most realistic conditions (wet baking for 45–60 min at 200 °C or 30–45 min at 230 °C), no degradation was observed. Still, AOH **7** and ALT **9** were degraded slightly after 1 h at 230 °C.

Upon dry baking, degradation was much more pronounced. Here, a clear graduation in compound stabilities could be observed with AME 8 being the most stable, followed by AOH 7 and ALT 9. ALT 9 was almost fully degraded after dry baking at 230 °C for 1 h. The dry baking results show, that degradation mechanisms different from the one given in Figure 40 exist, because no free water is available for solubilisation and hydrolysis during dry baking (also, no AOD 27 and AMD 28 were detected). These additional mechanisms can be expected to involve compounds originating from the flour matrix, as the TA-MS results for bulk AOH 7 (no matrix present) indicated stability up to 270 °C. While dry baking at 200 °C and 230 °C caused a significant degradation of all considered toxins, it was quite naturally accompanied by changes in colour, consistency and smell of the heated flour. Hence, dry heating is not suitable as a detoxifying pre-treatment for flour.



Figure 41 Graphical representation of the baking study results, n = 3. The curves connecting the datapoints are spline based (no fit).

3.4.6 Formation and stability of AOD 27 and AMD 28 upon wet baking

AOD 27 was present at concentrations $< 20 \,\mu g/kg$ in those baking products obtained from AOH 7 spiked flour after wet baking at 170 °C and 200 °C. It was not found for 230 °C. AMD 28 was not detected at all. The detected quantities of AOD 27 are low and correspond merely to approx. 1% conversion of AOH 7. Therefore, no significant decrease of the AOH 7 concentration was detectable (Figure 41).



Figure 42 Excerpts of HPLC-MS/MS chromatograms illustrating the formation of AOD 27 upon the wet baking of AOH 7 spiked flour.

To understand why AOD **27** was not found in the baking products prepared at 230 °C, the wet baking study was repeated with a wholemeal flour batch spiked exclusively with AOD **27** and AMD **28** (Figure 43). While both AOD **27** and AMD **28** were stable at 170 °C, significant degradation occurred at 200 °C and 230 °C with AMD **28** being more stable than AOD **27**.

interpreting Upon the results further, it can be seen that the degradation rates of AOD 27 and AMD 28 at 200 °C and 230 °C are higher than the degradation rates of AOH 7 and AME 8 under the same conditions. This means that AOD 27 and AMD $\mathbf{28}$ are degraded faster than they are formed and explains their absence at 230 °C. That no AMD 28 was formed at all is attributed to the low water solubility of AME 8, entailing a lower hydrolysis rate. In summary, AOD 27 is the stable end product of AOH7 degradation upon wet baking at 170 °C, while it can be considered a mere degradation intermediate at higher temperatures. The further chemical fate of AOD 27 is yet unclear.



Graphical representation of the baking study results, n = 3. The curves connecting the datapoints are spline based (no fit).

3.4.7 Sample survey

A total of 24 baking product samples was obtained from a local supermarket. The samples were: wholemeal bread rolls (6×), wholemeal bread (3×), rusk (3×), wholemeal rusk (3×), crispbread (2×), wholemeal crispbread (2×), rye crispbread, spelt rusk, wholemeal rye bread roll, pumpernickel bread and buckwheat cookies.

The encountered levels (Table 14) of AOD 27 and AMD 28 are low, which is probably due to low levels of the parent compounds AOH 7 and AME 8. That the degradation products could be detected at all might be attributed to more favourable formation conditions compared to the ones of the baking study. In this context it is noteworthy, that the majority of AOD 27/AMD 28 positive samples are rusks or crispbreads. The latter can be produced by extrusion, a process which relies on high temperatures and the application of pressure [157]. Extrusion has been shown to promote the degradation of a range of *Fusarium* mycotoxins [183-185] and might thus also favour AOD 27/AMD 28 formation.

3.4.8 Discussion

The toxicological properties of AOD **27** and AMD **28** are yet unknown, as these compounds are described for the first time. In this respect, further studies are necessary. However, in view of the low acute toxicity of AOH **7**, AME **8** and ALT **9** and the low levels encountered in the sample survey, a risk of acute intoxications is not indicated. To quantify the risk of chronic and subacute effects caused by the repeated ingestion of low toxin quantities, food monitoring data and further toxicological studies (particularly concerning bioavailability) are required. Not least because the presented data indicate that consumers of bakery products will be exposed to the full quantity of AOH **7**, AME **8** and ALT **9** originally present in the flour.

| Analyte | Samples above LOD | Samples above LOQ | Detected in (concentration [µg/kg]) |
|--------------|----------------------|----------------------|--|
| AOH 7 | 0 | 0 | - |
| AME 8 | 1 | 0 | Buckwheat cookies (< 15) |
| ALT 9 | 2 | 0 | Wholemeal bread roll (< 30), rusk (< 30) |
| AOD 27 | 2 | 0 | Rye crispbread (< 45), wholemeal rye bread roll (< 45) |
| AMD 28 | 2 | 0 | Spelt rusk (< 45), wholemeal crispbread (< 45) |

Table 14 Sample survey of bakery products for AOD 27, AMD 28 etc.

4 Conclusion and outlook

The physicochemical properties of many analyte/matrix combinations relevant in the organic trace analysis of foods require creative analytical solutions which go beyond the mere application of the instrumentation available today. Simple reversible and irreversible chemistry, applied in the frame of analytical sample preparation, can be a powerful asset in this respect. A major reason for this is the high selectivity inherent to most covalent reactions. If an analyte is chemically "pre-selected" before quantification, significant enhancements of a methods performance can be expected, however powerful the utilised instrumentation might be in itself.

Hence, by taking advantage of the multitude of organic reactions available today, "tailormade" analytical solutions to almost any problem are feasible. In the present dissertation, this was demonstrated using the example of covalent hydrazine chemistry. From a simple derivatisation reaction to a more sophisticated dynamic covalent chemistry based solution for analyte extraction: in all cases the application of covalent chemistry during sample preparation greatly improved almost every relevant method performance parameter.

However, designing tailor-made solutions requires time and effort. To further illustrate the high potential available through the combination of well-known chemistry with the possibilities of modern analytical instruments, additional studies would be desirable. A major task in this respect is the evaluation of robust, straightforward reactions compatible with the special demands of the analytical chemist (i.e. ease of use, inexpensiveness and (non-)toxicity of the involved reagents as well as their applicability in the presence of food matrices).

While the development of the analytical methods presented in this dissertation can be considered complete, inter-laboratory validation and ring trials would be a consequent next step in order to better assess their performance and robustness¹. Also, an automation of the sample preparation routines using common robotic sample handling equipment or scripted autosamplers could be considered in order to lower handling efforts thus enabling a higher sample throughput.

Lastly, following up on the sample survey results, further studies on the occurrence of tenuazonic acid in an extended food product basket, representing a typical consumer diet, are needed as a first step towards a comprehensive risk assessment. Such studies should also feature

¹ In the case of ZON **10** in edible oils, a ring trial will be organised by the Federal Institute of Materials Research and Testing (BAM 1.2) in the 4th quarter of 2010.

an increased number of samples and look at regional variations.

Besides the *desired* analyte reactions exploited in the method development part of this dissertation, a great deal of work was directed towards understanding the *undesired* analyte reactions possibly occurring even before analysis, during food processing and storage. Even so, the high number of potentially reactive food constituents as well as the multitude of relevant food processing techniques (e.g. cooking, baking, extruding, fermentation etc.) make this a very challenging field with many variables and unknowns. As a consequence, efforts were focused on basic, "universal" degradation reactions, based on first order and pseudo-first order chemistry (e.g. hydrolyses or decarboxylations). It was, however, clear from the results obtained, that additional degradation routes must exist. As indicated by the current literature, these routes could involve bimolecular reactions with food constituents like monosaccharides, starch or proteins. This is a good starting point for further studies, which should investigate the effect of such food constituents on the mycotoxin degradation by means of suitable model experiments.

A last aspect, which could not be covered in the frame of this dissertation, concerns the biological activity and toxicity of the newly identified degradation products. To understand the impact of mycotoxin degradation on the health risks associated with the consumption of contaminated foods, toxicological studies, ideally comparing parent and product compounds, are needed. These studies should cover both acute and chronic effects.

In conclusion, it should be acknowledged that mycotoxins present an abundance of challenges to a wide variety of scientific disciplines, e.g. the agricultural sciences, biology, chemistry, toxicology or risk analysis. It is thus clearly impossible to treat the "mycotoxin issue" comprehensively in the frame of a single dissertation. However, the present work illustrates that mycotoxin analysis is by no means trivial and that "chemical creativity" is an excellent tool to overcome the current issues in the field. It would be greatly rewarding if the presented solutions inspired more scientists to harness the potential of chemistry when approaching their analytical challenges.

5 Materials and methods

5.1 Materials and instruments

Unless stated otherwise, all standard solvents were of HPLC grade and all standard chemicals of analytical reagent grade. Deionised water was sourced from a Milli-Q Synthesis A10 system equipped with a Quantum EX Ultrapure Organex cartridge (Millipore, Billerica, Massachusetts, USA) or from a Seralpur PRO 90 CN system equipped with a Supur DCF 0.2 μ m filter (Seral, Ransbach-Baumbach, Germany). Undecylic aldehyde (97%) and Dowex 50WX8-200 cation-exchange resin (H⁺ form) were purchased from Sigma-Aldrich (Schnelldorf, Germany). The sources of all indexed compounds (1–28) are shown in Table 15.

AOH 7, TA 5 (Na⁺ salt), DTA 20 and DDTA 21 were synthesised by the workgroup of Prof. Dr. R. Faust (Universität Kassel, Germany) according to literature procedures [72, 150]. The stereochemistry of TA 5 and DTA 20 was confirmed by x-ray crystallography [8, 9]. Purities of TA 5 (84.3 \pm 1.3 %) and DTA 20 (98.4 \pm 1.0 %) were obtained by Dr. Dietmar Pfeifer (BAM Federal Institute for Materials Research and Testing, Berlin, Germany) through quantitative nuclear magnetic resonance spectroscopy. Synthetic TA 5 and DTA 20 were used without further purification for selected applications only. TA 5 stock solutions for quantification purposes were prepared from commercial TA 5 by UV spectrometry {M14}. The AOH 7 crude product was used for synthesis only and purified by preparative HPLC prior to analytical measurements.
 Table 15
 Compounds (solvent: solvent used for the preparation of stock solutions) SAL: Sigma-Aldrich, Schnelldorf, Germany, Merck: Merck, Darmstadt, Germany and Faust: workgroup of Prof. Dr. Rüdiger Faust, Kassel, Germany (vide supra).

| Short | Trivial name | Systematic name | Solvent | Sources (purity) |
|-----------------|---|---|---------|---|
| 1 | Penicillin G | (25,5R,6R)-3,3-Dimethyl-7- oxo-6-[(phenylacetyl) amino]- 4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid | | |
| 2 | Streptomycin | - | | - |
| 3 | Patulin | 4-Hydroxy-4 <i>H</i> -furo[3,2-c] pyran-2(6 <i>H</i>)-one | | |
| 4 | Aflatoxin B_1 | - | | |
| TA 5 | Tenuazonic acid | (55)-5-[(25)-Butan-2-yl]-3-(1- hydroxyethylidene) pyrrolidine-2,4-dione | MeOH | Na ⁺ salt: Faust (84 %), Cu ²⁺ salt: SAL (-) |
| 6 | Enzyme bound heptaketide (illustration) | - | | - |
| AOH 7 | Alternariol | 3,7,9-Trihydroxy-1-methyl- 6H-benzo[c]chromen-6-one | EtOAc | SAL (96 %), Faust (-) |
| AME 8 | Alternariol Monomethyl Ether | 3,7-Dihydroxy-9-methoxy-1- methyl-6H-benzo[c]chromen- 6-one | EtOAc | SAL (-) |
| ALT 9 | Altenuene | (25,35,4a5)-2,3,7-Trihydroxy- 9-methoxy-4a-methyl- 2,3,4,4a-tetrahydro-6 <i>H</i> - benzo[<i>c</i>]chromen-6-one | EtOAc | SAL (-) |
| ZON 10 | Zearalenone | (3 <i>S</i> ,11 <i>E</i>)-14,16-Dihydroxy-3- methyl-3,4,5,6,9,10- hexahydro-1 <i>H</i> -2- benzoxacyclotetradecine- 1,7(8 <i>H</i>)-dione | MeCN | SAL (99.3 %) |
| α-ZOL 11 | α-Zearalenol | (35,75,11E)-7,14,16- Trihydroxy-3-methyl- 3,4,5,6,7,8,9,10-octahydro- 1 <i>H</i> -2-benzoxacyclo-tetradecin- 1-one | MeCN | SAL (-) |
| β-ZOL 12 | β-Zearalenol | (35,7R,11E)-7,14,16- Trihydroxy-3-methyl- 3,4,5,6,7,8,9,10-octahydro- 1H-2-benzoxa-cyclotetradecin- 1-one | MeCN | SAL (-) |
| ZAN 13 | Zearalanone | (35)-14,16-Dihydroxy-3- methyl-3,4,5,6,9,10,11,12- octahydro-1 <i>H</i> -2- benzoxacyclotetradecine- 1,7(8 <i>H</i>)-dione | MeCN | SAL (-) |

| α-ZAL 14 | α-Zearalanol | (35,75)-7,14,16-Trihydroxy-3- methyl- 3,4,5,6,7,8,9,10,11,12- decahydro-1 <i>H</i> -2- benzoxacyclotetradecin-1-one | MeCN | SAL (97 %) |
|-------------------------|---|---|-------|---|
| β-ZAL 15 | β-Zearalanol | (35,7R)-7,14,16-Trihydroxy- 3-methyl- 3,4,5,6,7,8,9,10,11,12- decahydro-1 <i>H</i> -2- benzoxacyclotetradecin-1-one | MeCN | SAL (98 %) |
| DNPH 16 | 2,4- Dinitrophenyl- hydrazine | 2,4-Dinitrophenylhydrazine | - | SAL (p.a., 50 % water), Merck (p.a., 33 % water) |
| 17 | MP-TsNHNH ₂ | Toluenesulfonylhydrazine, polymer bound, macroporous, pore size: 3–6 nm, typical loading: 1.5–3.0 mmol/g | - | SAL (-) |
| 18 | ZON resin bound | - | - | - |
| и-ТА 19 | u-Tenuazonic acid | (3Z,5R)-5-[(2S)-Butan-2-yl]-3- (1-hydroxyethylidene) pyrrolidine-2,4-dione | - | - |
| DTA 20 | Deacetyl tenuazonic acid | (55)-5-[(25)-Butan-2-yl] pyrrolidine-2,4-dione | - | Faust (98 %) |
| DDTA 21 | Deacetyl decarboxyl tenuazonic acid | (35,45)-3-Amino-4- methylhexan-2-one | - | Faust (-) |
| TA-DNPH 22 | Tenuazonic acid 2,4-dinitro- phenyl- hydrazone | (55)-3-[N-(2,4-Dinitrophenyl) ethanehydrazonoyl]-5-[(15)-1- methylpropyl]pyrrolidine-2,4- dione | EtOAc | Synthesis {M16} |
| 23 | MP- TsNHNH ₃ ⁺ Cl ⁻ | Toluenesulfonylhydrazine hydrochloride, polymer bound | - | Synthesis {M15} |
| 24 | DCHC solvent mixture | Acetone:0.13 M aq. HCl 70:30 (v:v) | - | - |
| 25 | DCHC solvent mixture | MeOH:0.13 M aq. HCl 70:30 (v:v) | - | - |
| <i>u</i> -DTA 26 | <i>u</i> -Deacetyl tenuazonic acid | (5 <i>R</i>)-5-[(2 <i>S</i>)-Butan-2-yl] pyrrolidine-2,4-dione | - | - |
| AOD 27 | Degraded AOH | 6-Methylbiphenyl-2,3',4,5'- tetrol | EtOAc | Synthesis {M17} |
| AMD 28 | Degraded AME | 5'-Methoxy-6-methylbiphenyl- 2,3',4-triol | EtOAc | Synthesis {M17} |

| # | Short name | Description |
|-----|---|---|
| I1 | HPLC-DAD/FLD | 1200 series HPLC tower equipped with 1200 series DA- and FL- detectors (Agilent, Böblingen, Germany) |
| 12 | HPLC-DAD/FLD/ESI-IT- MS ⁿ | 1100 series HPLC tower equipped with 1100 series DA-, FL- and MS- extended capacity trap-detectors (Agilent, Böblingen, Germany); the ion-trap was operated with an ESI source |
| 13 | HPLC-ESI-MS/MS | 1200 series Ultra Performance Liquid Chromatography (UPLC) tower (Agilent, Böblingen, Germany) directly linked to a QTRAP 4000 MS/MS system equipped with a TurboSpray ion source (Applied Biosystems, Foster City, California, USA) |
| I4 | ESI-FTICR-MS ² | LTQ mass spectrometer (Thermo-Finnigan, Dreieich, Germany) coupled to a NanoMate 100 Robot ESI source (Advion, Ithaca, New York, USA). Direct infusion of analyte solutions by syringe pump |
| 15 | ESI-TOF-MS | Micromass Q-TOF Ultima mass spectrometer (Waters, Milford, Massachusetts, USA) equipped with an ESI ion source |
| I6 | UV/VIS | Unicam 5625 spectrometer (Unicam Instruments, Cambridge, United Kingdom) |
| I7 | UV/VIS | Cary 5000 spectrometer (Varian Inc., Paolo Alto, California, USA) |
| 18 | GPC | LC-Tech GPC Vario system, equipped with a FW-20 fixed wavelength detector, a GPC 1122 solvent delivery system and a GPC10011 column, dimension 500 × 40 mm, Bio-Beads S-X3 filling (all LC-Tech, Dorfen, Germany) |
| 19 | Automated Karl-Fischer titration | KF 756 coulometer, a KF 728 stirrer and a KF oven (METROHM, Filderstadt, Germany) |
| I10 | pH electrode | Sentix 81 pH electrode attached to an inoLab 740 terminal (WTW, Weilheim, Germany) |
| I11 | TA-MS | STA 409 C <i>Skimmer</i> system (Netzsch, Selb, Germany) equipped with a QMG 421 (Balzers, Balzers, Liechtenstein) quadrupole MS |
| I12 | HPLC column I | Acclaim Polar Advantage II, dimensions 2.1 × 150 mm, particle size: 3 μm (Dionex, Idstein, Germany) |
| I13 | HPLC column II | Gemini NX C18 column, dimensions 2 × 150 mm, particle size: 3 μm (Phenomenex, Torrance, California, USA) with precolumn |
| I14 | HPLC column III | Hypercarb column, dimensions: 2.1 × 150 mm, particle size: 5 μm (Thermo, Waltham, Massachusetts, USA) with precolumn |
| I15 | HPLC column IV | Nucleodur 100-5 C18 ec column, dimensions: 250 × 7.8 mm, particle size: 5 μm (Macherey-Nagel, Düren, Germany) |

5.2 Software

Structures were drawn with ChemSketch 10.02 (Advanced Chemistry Development, Toronto, Canada). Diagrams and graphs were prepared with Origin 8G (OriginLab Corporation, Northampton, Massachusetts, USA). Flowcharts were constructed with Diagram Designer 1.21 (MeeSoft, Denmark). Crystal structure ORTEP plots were generated with ORTEP-III (Oak Ridge National Laboratory, Tennessee, USA) by Dr. Franziska Emmerling (BAM Federal Institute for Materials Research and Testing, Berlin, Germany). Citation management was done with Endnote X3 (Thomson Reuters, New York, New York, USA).

5.3 Chemical nomenclature

According to IUPAC (International Union of Pure and Applied Chemistry) recommendations [186], the *l*,*u*-nomenclature is used to differentiate the two epimers of tenuazonic acid. However, to maintain consistency with the literature, the *l*-prefix is omitted and only the uprefix is shown (i.e. *l*-TA is referred to as TA 5 whereas *u*-TA is designated as *u*-TA 19). A mixture of TA 5 and *u*-TA 19, with its exact composition unknown, is termed iso-TA in accordance with the literature [70, 105]. The same system of prefixes is used for deacetyl tenuazonic acid (DTA 20).

5.4 Analytical terminology

(In)accuracy

According to the IUPAC definition [186], the term inaccuracy is used "to describe the (lack of) accuracy of a chemical measurement process." Inaccuracy is a two-component quantity, comprising the RSD (measure of precision) and the bias (measure of trueness).

LOD (limit of detection) and LOQ (limit of quantification)

The LOD is defined as the concentration at which the signal to noise ratio, observable e.g. in a typical chromatogram, is approximately 3. The LOQ is initially defined as the LOD \times 3, however, if precision or trueness of a method are not sufficient at that level, the LOQ can be higher. LODs and LOQs always refer to the concentration of the analyte in the sample.

MSI (minimum sample intake)

Food matrices are intrinsically heterogeneous and sufficiently large sample intakes are needed to eliminate the effect of this heterogeneity. The MSI is the minimum amount of material that is still representative for the average of the sample. If the sample intake of an analytical procedure is less, differences between the analytical portions will significantly influence the variability of the results.

Precision and RSDs

According to the IUPAC definition [186], the term precision is used to refer to "the closeness of agreement between independent test results obtained by applying the experimental procedure under stipulated conditions." Hence, precision characterises the random part of the experimental errors affecting an analytical result. Precision is a qualitative concept. A measure of a method's precision is its RSD.

Recovery, apparent recovery and ME (matrix effect)

In the context of analytical methods the term recovery is used to refer to the yield of a sample preparation procedure—as recommended by IUPAC [187]. The term ME is used to refer to the combined effect of all components of the sample other than the analyte on the quantification of the analyte (from sample preparation to detection) [188]. Slightly deviating from the general IUPAC definition, the term apparent recovery is used to refer to the product of recovery (e.g. 80%) and ME (e.g. 120%): apparent recovery = $0.8 \cdot 1.5 = 1.2 = 120\%$. The apparent recovery is always determined by analysing an actual sample matrix.

Selectivity and specifity

According to IUPAC recommendations [189], the term selectivity is used to refer to the extent to which an analytical method can determine particular analyte(s) in a complex mixture without interference from other components. The term specifity is used to designate the ultimate value of selectivity.

Trueness and bias

According to the IUPAC definition [186], the term trueness is used to refer to the "closeness of the agreement between the average value obtained from a large series of test results and an

accepted reference value." This implies that trueness is a qualitative concept (i.e. trueness can never be quantified). A measure of trueness is the quantity bias Δ . Δ corresponds to the difference between a true value τ and the limiting mean μ (a statistical estimate of τ obtained by a series of measurements):

(1)
$$\mu = \tau + \Delta$$

Consequently, Δ can only be determined if an accepted value for τ is available. Certified reference materials provide such accepted values [190]. If no reference material is available, τ may be set by spiking a blank matrix with pure analyte. The latter method was applied throughout this dissertation.

5.5 General procedures

The solvents used for the preparation of analytical stock solutions may be taken from Table 15. All stock solutions were stored at -20 °C. All shaking was done on a Promax 2020 horizontal shaker (Heidolph, Kelheim, Germany) at 400 rpm (revolutions per minute). The term safelock tube is used with respect to a 2 mL semi-transparent Eppendorf SafeLock tube. Centrifugation of safelock tubes was done by means of an Eppendorf Minispin plus centrifuge (Eppendorf, Hamburg, Germany) at 14.5 × 10³ rpm (14.1 × 10³ g). All uncertainties correspond to absolute standard deviations (SDs) if not stated otherwise. Generally, all liquids (solvent, oil) directly involved in the sample preparation processes were weighed and all calculations were performed using only gravimetric data.

5.6 Methods

5.6.1 Analytical methods

M1: General standard addition procedure

m_{sample} weight of the subsample used for a single sample preparation [g]

- $m_{solvent}$ weight of the solvent containing the equivalent of m_{sample} at the end of the sample preparation procedure (injection solvent) [g]
- c* concentration of analyte A in the sample, not recovery corrected [mg/kg]
- c concentration of analyte A in the sample, recovery corrected [mg/kg]
- dc change of c in a given subsample due to addition of standard substance [mg/kg]
- a slope of the linear standard addition curve [a.u. kg/mg]
- b y-axis intercept of the linear standard addition curve [a.u.]
- s_{a-c} RSDs of a, b and c, respectively
- PA peak area obtained by analysis of a given subsample [a.u.]

Initially, c* is determined by a single sample preparation being evaluated by external calibration. Subsequently, graduated amounts of a stock solution containing the analyte are weighed into four out of five sample preparation vessels, with the solvent being removed in a gentle nitrogen stream. The amounts of analyte are chosen, so that the highest value of dc equals c*. Now, equally weighted (m_{sample}) subsamples are added to all five vessels and the typical sample preparation routine is conducted. After analysis, a linear standard addition curve is obtained by plotting dc (x-axis) against *PA* $m_{solvent}$ m_{sample}^{-1} (y-axis) for each of the five sample preparations. The implicitly recovery corrected value for c is finally calculated according to $c = -b a^{-1}$. The value of s_c is obtained from s_a and s_b by error propagation according to $s_c = (s_a^{-2} + s_b^{-2})^{0.5}$.

Although not required for further calculations, the methods apparent recovery for a given sample may be obtained according to: *apparent recovery* $[\%] = 100 a a_{CAL}^{-1}$, where a_{CAL} is the slope of a five-point calibration curve covering the concentration range of the standard addition curve. Calibration curves are constructed by addition of pure analyte (weight: $m_{analyte}$) to pure solvent (weight: $m_{CALsolv}$). The solvent used equals the injection solvent of the sample preparation procedure. The calibration curve is obtained by plotting $m_{analyte}$ (x-axis) against *PA* $m_{CALsolv}$ (y-axis).

M2: Derivatisation based quantification of TA 5 in cereals by HPLC-IT-MS²

Sample preparation

Before analysis, bakery product samples were ground manually in a mortar. 2 g (exact weight recorded) of the ground sample were mixed with 15 mL derivatisation reagent {M13} in an Erlenmeyer flask. The reaction vessels were ultrasonicated for 10 min followed by 20 min of horizontal shaking. Subsequently, 10 mL of EtOAc:undecylic aldehyde 95:5 (v:v) were added (exact weight recorded) and the mixture was shaken for another 10 min. A 1.5 mL portion of the EtOAc layer was taken off and transferred to a safelock tube which was then centrifuged for 1 min. The centrifuged solution was filtered through a 0.2 µm SPARTAN 13/02 syringe filter (filter material: regenerated cellulose, Whatman, Maidstone, United Kingdom), transferred to an HPLC vial and injected directly. Given the 5 µL injection volume, the equivalent of 1 mg matrix was injected. Two injections were performed for each vial and the resulting peak areas were averaged.

HPLC-IT-MS² conditions

HPLC system {12} was equipped with column {I12}, the column oven being thermostated at 40 °C. The chromatographic parameters were as follows: solvent A water, solvent B MeCN, both modified with 0.1 % (v) formic acid. The following linear gradient was used: 0 to 100 % B in 10 min at 0.4 mL/min, 100 % B for 1.5 min at 0.4 mL/min, 100 % B for 4 min at 0.8 mL/min (MS ion source bypassed), 100 % B for 4 min at 0.4 mL/min (MS ion source on-line), 100 % A for 4 min. at 0.4 mL/min (re-equilibration). Total runtime was 23.5 min. The injection volume was 5 μ L.

The CID transition utilised for quantification was $m/z 378 \rightarrow 140$. By means of a switching valve, the ESI source was automatically bypassed after 11.5 min of the chromatographic run to avoid contamination with quenched derivatization reagent (eluting at approx. 12 min.). The instrument parameters were as follows: nebuliser: 40 psi, dry gas (N₂): 9 L/min, drying temp.: 350 °C, mode: standard extended, ICC: 200,000, max. accumulation time: 200 ms, scan range: m/z 138 to 143, averaging off, SPS-target: m/z 140, compound stability: 100 %, trap drive: 75 %, optimise: wide, isolation bandwidth: m/z 2, fragmentation amplitude: 100 %, cut-off: default, smart-fragmentation on (30 to 200 %), delay: 0 ms, time 40 ms, width > m/z 10.

For the quantification of TA **5** without derivatisation, the chromatographic and MS parameters were identical apart from the following differences: both eluents where modified by 0.05 % (v) TFA and 0.1 % (v) AcOH. The most abundant transition (m/z 198 \rightarrow 153) was

observed upon using ESI(+) and hence fixed as the QT reaction. The scan range was m/z 150 to 156 and the SPS-target was m/z 153.

Calibration, derivatisation yield and quantification

Variable portions of a TA **5** stock solution (e.g. 100, 200, 400, 800, 1,600 mg of a 1 mg/kg stock solution as an equivalent to 50, 100, 200, 400, 800 μ g/kg sample concentration) were weighed into Erlenmeyer flasks and the solvent was removed in a gentle nitrogen stream. Subsequently, the sample preparation given above was conducted, omitting the filtration step. For the calculation of the derivatisation yield a second calibration curve in the same range was constructed by dissolving pure TA-DNPH **22** in EtOAc. The derivatisation yield corresponds to the slope of the latter curve divided by the slope of the former curve \times 100.

Quantification was done by standard addition according to {M1}. E.g. 0, 250, 500, 750, 1,000 mg of a 1 mg/kg TA **5** stock solution were added to a rye flour sample of $c*_{TA} = 500 \,\mu\text{g/kg}$, corresponding to approximate final TA **5** concentrations in the sample of 500, 625, 750, 875, 1,000 $\mu\text{g/kg}$). Hence, five sample preparations are done per sample, resulting in a total sample amount of 10 g.

Validation and MSI

The LOQ was defined as the LOD \times 5 as at the LOD \times 3 level, RSDs were found to exceed 20 %. Linearity in the range of 50–10,000 µg/kg was demonstrated by constructing 9 point calibration curves with and without an uncontaminated wheat flour matrix, respectively. Bias and RSDs were evaluated by analysing an uncontaminated wheat flour matrix spiked to different TA **5** contents (50, 500 and 5,000 µg/kg) on three consecutive days. For each concentration and day the standard addition scheme {M1} was conducted. To determine the MSI, the sample preparation was performed with differing sample weights (0.05, 0.1, 1, 2 and 5 g) and six replicates, respectively. The amounts of solvents, reagents etc. were scaled according to the sample weight. The MSI was defined as the point after which an increase in sample weight did not afford an improved RSD.

M3: Derivatisation based quantification of TA 5 in beer by HPLC-IT-MS²

Sample preparation

Beer samples were ultrasonicated to remove the carboxylic acid and frozen for storage. For sample preparation, 0.4 g of thawed beer were weighed into a safelock tube and 0.80 mL derivatisation reagent {M13} were added. The reaction vessels were ultrasonicated (10 min)

and shaken (20 min). Subsequently, 0.60 mL of EtOAc:undecylic aldehyde 95:5 (v:v) were added (exact weight recorded). The safelock tubes were then shaken for further 10 min and centrifuged for 1 min. The EtOAc layer was collected, filtered through a 0.2 μ m SPARTAN 13/02 syringe filter (filter material: regenerated cellulose, Whatman, Maidstone, United Kingdom), transferred to an HPLC vial and injected directly. Given the 10 μ L injection volume, an equivalent of 6.7 mg matrix was put on the column. Two injections were performed for each vial and the resulting peak areas were averaged.

HPLC-IT-MS² conditions

The HPLC-IT-MS² conditions were identical to $\{M2\}$. The injection volume was 10 μ L.

Calibration and quantification

Five point calibration curves were constructed using a 95:5 (v:v) water:ethanol mixture as a synthetic matrix. Variable portions of TA **5** stock solutions (e.g. 100, 200, 400, 800, 1,600 mg of a 40 μ g/kg stock solution as an equivalent to 10, 20, 40, 80, 160 μ g/kg sample concentration) were weighed into safelock tubes and the solvent was removed by a gentle nitrogen stream. Subsequently, 0.4 g of the synthetic beer matrix were added gravimetrically and the sample preparation was performed as described above.

Quantification was done by standard addition according to {M1}. E.g. 0, 200, 400, 600, 800 mg of a 40 μ g/kg TA **5** stock solution were added to a beer sample of c*_{TA} = 80 μ g/kg, corresponding to approximate final TA **5** sample concentrations of 80, 100, 120, 140, 160 μ g/kg. This results in a total of five workups per sample using a total sample amount of 2 g.

Validation

The LOQ was defined as the LOD \times 4, due to a bias > 20 % at the usual LOD \times 3 level. Linearity was demonstrated in the range of 8 to 1,000 µg/kg by constructing 8 point calibration curves in synthetic and natural beer matrices, respectively. Bias and RSDs were evaluated by analysing an uncontaminated pilsener beer matrix spiked to different TA 5 concentrations (50, 500 and 5,000 µg/kg). For each concentration and day the standard addition scheme {M1} was conducted.

M4: Quantification of ZON 10 in edible oils by DCHC-HPLC-FLD / DCHC-HPLC-MS/MS

Sample preparation (DCHC)

100 \pm 2 mg of resin **23** obtained according to {M15} were weighed into a safelock tube. Then, 0.8 mL MeOH and 0.2 mL of the oil sample (approx. 0.18 g, exact weight recorded) were added (the phase separation can be ignored). The safelock tube was shaken for 1.5 h. Subsequently the supernatant was taken off and discarded. 1.8 mL MeOH were added and the safelock tube was vortexed for 10 s on a IKA Lab Dancer vortex (IKA, Staufen, Germany). After removal of the MeOH, 1.8 mL heptane were added and the resin was vortexed again. After removal of the heptane, the resin was dried in a gentle nitrogen stream for 20 min. Then, 400 μ L (approx. 0.32 g, exact weight recorded) of solvent **24** were added and the safelock tube was shaken for 2 h at 400 rpm. The supernatant was taken off and transferred to an HPLC vial through a Phenex 4 mm syringe filter (pore size: 0.2 μ m, filter material: regenerated cellulose, Phenomenex, Aschaffenburg, Germany). Given the injection volumes stated below, equivalents of 6.9 mg / 7.5 μ L (FL detection) or 2.3 mg / 2.5 μ L (MS/MS detection) matrix were put on the HPLC column. The solvent remaining in the safelock tube was removed and the resin was washed with 1.8 mL of MeOH after which it was dried in the nitrogen stream and stored until the next activation {M15}.

HPLC-FLD conditions

Analyses were done using instrument {I1} and column {I13}. The chromatographic parameters were as follows: oven temperature: 50 °C, injection volume: 15 μ L, flow rate: 0.4 mL/min, solvent A: water + 0.1 % (v) formic acid, solvent B: MeCN + 0.1 % (v) formic acid. The following linear gradient was used: 0 to 70 % B in 14 min, followed by 100 % B for 5 min and 100 % A for 7 min (re-equilibration). FLD detection of ZON **10** (t_R = 13.2 min) was done at $\lambda = 464$ nm after excitation at $\lambda = 232$ nm, the photomultiplier-gain was 15. The same parameters were used for the ZON **10** analogues (Figure 8).

HPLC-MS/MS conditions

Analyses were done using instrument {I3} and column {I12}. The chromatographic parameters were as follows: oven temperature: 40 °C, injection volume: 5 μ L, flow rate: 0.4 mL, solvents were identical to the FLD method, the following linear gradient was used: 0 to 100 % B in 10 min followed by 100 % B for 5 min and 100 % A for 5 min (re-equilibration). ZON **10** eluted at t_R = 10.4 min.

The ESI(+) m/z transitions were $m/z 319 \rightarrow 301$ (quantification) and $m/z 319 \rightarrow 283$ (qualification). The MS parameters for these transitions were optimised by using the instruments compound optimisation and flow injection analysis functions. The ion source parameters were as follows: CUR (curtain gas): 55 a.u., TEM (temperature): 500 °C, GS1 (gas 1): 50 a.u., GS (gas 2): 30 a.u., IS (ion spray voltage): 5500 kV, CAD (collision gas): medium, interface heater: on. The optimised compound specific parameters were (QT/QL): DP (declustering potential): 61/61 V, entrance potential (EP): 10/10 V, CE (collision energy): 15/19 V, CXP (cell exit potential): 8/8 V, dwell time: 50/50 ms.

Calibration and quantification

Calibration curves were constructed on the day of the analysis by weighing variable portions of the ZON 10 stock solution into HPLC vials. After removal of the solvent by a gentle nitrogen stream, solvent 24 was added gravimetrically. E.g. 30, 60, 120, 240 and 480 mg of a ZON 10 stock solution (c = 1.0 mg/kg) were taken up in 1 g of 24 after removal of the stock solution solvent, to obtain a calibration curve with datapoints corresponding to 30, 60, 120, 240 and 480 µg ZON 10 per kg 24.

Quantification was done by standard addition according to {M3} with a slight variation. E.g., for an oil of the approximate natural ZON **10** content of 500 μ g/kg, 0, 22.5, 45.0, 67.5 and 90 mg of a ZON **10** stock solution (c = 1.0 mg/kg) were added to safelock tubes with the solvent being removed. Differently to {M3}, the tubes were ultrasonicated for 5 min and then shaken overnight after addition of the oil subsamples to ensure complete dissolution of ZON **10**. After shaking, the sample preparation was conducted, to obtain a standard addition curve with datapoints corresponding to 500, 600, 700, 800, 900 and 1000 μ g ZON **10** per kg oil. The same procedure was performed for the standard addition curves to be measured by HPLC-MS/MS.

Validation

ZON 10 spiked blank matrices (c = 30, 300 and 3,000 µg/kg) were prepared as described under "Calibration and quantification". The precision was characterised by the RSD obtained from five independent sample preparations for each of the three concentrations as well as one standard addition scheme per concentration. The trueness was determined in terms of bias by evaluating the deviation of the obtained ZON 10 concentrations from the known, spiked concentration. RSD values were also obtained for all positive samples (n = 5, no standard addition). Alternative extraction methods (GPC and liquid-partitioning)

GPC was done using {18}. The GPC eluent was cyclohexane:EtOAc 1:1 (v:v). The flow rate was 5 mL/min and the total runtime 40 min. 5 mL sample were injected. The ZON 10 fraction was collected from $t_R = 17$ min to $t_R = 26$ min. Before analysis, 5 mL of the oil sample (approx. 4.5 g, exact weight recorded) were diluted by 5 mL of the GPC eluent. After the GPC run, the solvent of the collected ZON 10 fraction was removed and the fraction was reconstituted in 5 mL (approx. 4 g, exact weight recorded) of solvent 24. This solution was injected into the HPLC system (injected matrix equivalent identical to DCHC sample preparation).

Liquid-partitioning based sample preparations were done exactly as described in [130]. After the final evaporation step, the residue was taken up in 1.1 mL solvent **24** and injected directly (injected matrix equivalent identical to DCHC sample preparation). For each ZON **10** positive sample, six sample preparations were done with the one increasing the resulting RSD most being omitted. The apparent recovery was calculated by standard addition and results were corrected accordingly.

M5: HPLC-DAD method for the separate quantification of TA 5 and u-TA 19 (kinetic study)

Instrument {I1} and column {I14} were used. The column oven was thermostated at 20 °C. The chromatographic parameters were as follows: solvent A water modified with 50 mg/L disodium EDTA and 0.1 % (v) TFA, solvent B MeCN modified with 0.1 % (v) TFA. The flow rate was 0.4 mL/min. The following linear gradient was used: 0 to 100 % B in 16 min, 100 % B for 4 min, 100 % A for 3 min (re-equilibration). Total run time was 23 min. The injection volume was 10 μ L. TA **5** (t_R = 12.8 min) and *u*-TA **19** (t_R = 13.5 min) were detected at $\lambda = 280$ nm. The DAD spectra of the two separated species were identical. The LOD and LOQ were 0.3 μ mol/kg and 0.9 μ mol/kg, respectively.

As there was no pure *u*-TA **19** standard available at the time of the study, the response of TA **5** and *u*-TA **19** was assumed to be equal and the TA **5** calibration data were used also for *u*-TA **19**. This approximation was found to be acceptable, as the sum of TA **5** and *u*-TA **19** peak areas remained constant upon following the epimerisation of pure TA **5** in 0.1 M NaOH.

M6: HPLC-IT-MS² method for the quantification of iso-DTA (kinetic study)

Instrument {I2} and column {I14} were used. The column oven was thermostated at 25 °C. The eluents, gradient, flow rate and injection volume were identical to {M5} (exception: solvent A not modified with disodium EDTA). The transition m/z 156 \rightarrow 128 was used for the

quantification of iso-DTA **20** ($t_R = 8.7 \text{ min}$). LOD and LOQ were 1.0 μ mol/kg and 3.0 μ mol/kg, respectively. The MS instrument parameters were as follows:

nebuliser: 40 psi, dry gas (N₂): 9 L/min, drying temp.: 350 °C, mode: standard extended, ICC: off, accumulation time: 1900 ms, scan range: m/z 126 to 131, averaging off, SPS-target: m/z 156, compound stability: 100 %, trap drive: 100 %, optimise: normal, isolation bandwidth: m/z 2, fragmentation amplitude: 100 %, cut-off: default, smart-fragmentation on (30 to 200 %), delay: 0 ms, time 40 ms, width > m/z 10. DTA **20** was used as a calibration standard (a separation of DTA **20** and *u*-DTA **26** was not achieved).

M7: Baking studies

Wet baking

Three glass bowls were charged with 7 g of spiked flour (exact weight = m_{flour}) and 14 mL of water each. After manual mixing, the open bowls were put into a preheated oven. At t = 30, 45 and 60 min one bowl was removed from the oven and allowed to cool to RT. This experiment was done in triplicate for three oven temperatures (170, 200 and 230 °C), respectively. After cooling, the weight of the baked product ($m_{product}$) was recorded and the contents of the glass bowls were homogenised individually in a kitchen mill. The quantification of AOH **7**, AME **8** and ALT **9** was done by a one-point standard addition scheme, both points were determined three times. For each glass bowl six 20 mL brown glass vessels were charged with $m_{sample} = m_{product} / 7$ (the equivalent of 1 g flour) of the homogenised product, respectively. The remaining product was discarded.

Dry baking

For the dry baking study, 18 brown glass vessels (20 mL) were charged with 1 g of spiked flour, respectively. The open vessels were put into a preheated oven. At t = 30, 45 and 60 min six vessels were removed from the oven and allowed to cool to RT. This experiment was done for three oven temperatures (170, 200 and 230 °C).

Common sample preparation

Both dry and wet baking resulted in six equally prepared 20 mL brown glass vessels for each baking time and temperature. These six vessels were used for one standard addition procedure: Depending on baking time and temperature, a varying amount of water was added to the vessels $(m_{water} = 8 \text{ g} - m_{sample})$ in order to compensate for the varying amount of residual water in the baked product. In the case of dry baking m_{water} was fixed to 7 g. Subsequently, 2 mL of 2 M aq.

HCl were added. Eventually, three of the six vessels were supplemented with 5 mL EtOAc, while for the other three 5 mL of an EtOAc stock solution containing AOH 7, AME 8 and ALT 9 ($c_{aack} = 0.4 \text{ mg/L}$, respectively) were used. The weight of the EtOAc (m_{EtOAc}) was recorded. The resulting ternary phase systems were shaken for 45 min, ultrasonicated for 10 min and shaken again for 45 min. Then, 2 mL of the upper EtOAc layer were transferred into a safelock tube and centrifuged for 10 min to achieve complete phase separation. The EtOAc was eventually transferred to an HPLC vial through a Minisart RC 4 regenerated cellulose syringe filter (Sartorius, Göttingen, Germany) and stored at -20 °C, resulting in a total of six vials per baking time, temperature and replicate. After quantification by {M8}, the following variables were used for further data processing:

- PA peak area of the analyte
- SF scaling factor = $7 m_{EtOAc} m_{flour}^{-1} \approx 4.51$

Standard addition curves were constructed by plotting the terms PA (y-axis) against $c_{sock}SF$ (xaxis) for all six sample preparations and conducting linear least squares regression (the latter term equals zero for the sample preparations without addition of standard substance). Further calculations were performed as described in {M3}. The semi-quantitative determination of the AOD **27** formed during the baking study was done by external calibration.

Calibration, determination of MEs and recoveries

For the calculation of MEs, two five-point curves in the concentration range 0.2-3.2 mg/kg were constructed. For curve 1, the solvent was pure EtOAc. For curve 2, the solvent was EtOAc originating from a sample preparation of the non-spiked, non-contaminated wholemeal wheat flour used throughout this study (as described above). The ME equals $100 \times slope \ curve 2$ / $slope \ curve 1$. For the determination of the apparent recoveries, curve 3 was constructed from the standard addition data obtained during the baking study. The x-values of curve 3 corresponded to the concentration of the analyte in the EtOAc added during the sample preparation (either 0 or 0.4 mg/L) and the y-values to the obtained peak area. The apparent recovery equals $100 \times slope \ curve 3$ / $slope \ curve 1$. The recovery was calculated by dividing the overall recovery by the ME.

<u>M8:</u> HPLC-MS/MS multi-method for AOH 7, AME 8, ALT 9, AOD 27 and AMD 28 in cereals and bakery products

HPLC-MS/MS conditions

Instrument {13} and column {112} were used. The chromatographic parameters were as follows: oven temperature: 40 °C, flow rate: 0.4 mL, eluents: water (A) and MeOH (B), both modified with 400 mg/L NH₄OAc. The following linear gradient was used: 0 to 100 % B in 10 min followed by 100 % B for 6 min and 100 % A for 5 min (re-equilibration). The injection volumes were 2.5 μ L (baking study) and 10 μ L (sample survey). Two injections were performed for each vial, with the resulting peak areas being averaged. The MS parameters were optimised by using the instruments compound optimization and flow injection analysis functions. To apply the optimised parameters, the chromatogram was subdivided into four periods. The complete parameters are given in the table below. Parameters, which were identical for all compounds: EP: +/- 10 V, interface heater: on, CUR: 55 a.u., TEM: 500 °C and dwell time: 100 ms.

| Period | 7.0–9.4 min | | 9.4–11 | .0 min | 11.0–21.0 min | | |
|----------------------|---------------|--------|----------|----------|---------------|--------|--|
| Analyte | AOI | AOD 27 | | AMD 28 | AOH 7 | AME 8 | |
| t _R [min] | 8.7 | | 9.9 | 9.9 | 12.4 | 12.9 | |
| ESI polarity | - + | | - | | + | | |
| m/z Q1 [Da] | 231.0 | 233.1 | 290.9 | 244.9 | 259.1 | 273.1 | |
| m/z Q3 A [Da] | 187.0 | 122.9 | 202.7 | 229.6 | 185.0 | 128.1 | |
| m/z Q3 B [Da] | 188.8 | 215.0 | 247.0 | 200.9 | 128.1 | 115.0 | |
| DP [V] | -75 | 66 | -75 | -75 | 101 | 96 | |
| CE (A, B) [V] | -22, -24 | 27, 25 | -46, -16 | -25, -26 | 49, 57 | 67, 87 | |
| CXP (A, B) [V] | -29, -13 | 8,16 | -37, -15 | -17, -13 | 14, 10 | 10, 8 | |
| GS 1, GS 2 [a.u.] | 50, 50 50, 90 | | 50, 50 | | 50, 70 | | |
| CAD [a.u.] | 12 4 | | 12 | | 4 | | |
| IS [kV] | -3,500 | 5,000 | -3,500 | | 3,500 | | |

M9: Quantification of water in flour and baking study products

Instrument {I9} was used. Water was extracted from the flour for 10 min at 165 °C and a nitrogen gas flow of 60 mL/min. 50 mg of flour were used for each determination. The water content in the baking study products of {M7} was approximated by assuming the weight loss (m_{loss}) due to baking to be caused by water evaporation only. The total amount of water in the baking product (m_{water}) was then calculated according to $m_{water} = water added to the flour + water$

naturally present in the flour - m_{Los} , e.g. for wet baking $m_{water} = 14.0 \text{ g} + 1.2 \text{ g} - m_{los}$. All measurements were done in triplicate.

M10: ESI-FTICR-MS² measurements

Instrument {I4} (capillary voltage: 1.5 kV, backpressure 0.5 psi, Advion, Ithaca, New York, USA) was used in conjunction with a syringe pump (flow rate: $5 \,\mu$ L/min) infusing a solution of the analyte (approx. concentration = 10^{-5} mol/L) in 75:25 (v:v) MeOH:water + 0.5 % (v) formic acid. Fragmentation was done by infrared multiphoton dissociation with the parameters being intensity: 80 a.u. and irradiation time: 80 ms.

M11: TA-MS

All TA-MS measurements were done by Dr. Michael Feist (Humboldt-Universität zu Berlin, Germany). Instrument {I11} was used to record T, DTA, TG and DTG (differential TG) curves together with the ionic current curves in the multiple ion detection mode [179, 180]. Further experimental details were as follows: DTA-TG sample carrier system; Pt/PtRh10 thermocouples; platinum crucibles (0.8 mL); sample mass 9–15 mg (measured versus empty reference crucible); constant purge gas flow of 70 mL/min argon 4.8 or synthetic air; constant heating rate 10 K/min; the manufacturer's software packages PROTEUS (v. 4.3) and QUADSTAR 422 (v. 6.02) were used for raw data evaluation. The determination of the T_{on}^{ex} was done following international recommendations [191].

M12: pH titration

The pK_A of DTA **20** was determined as follows: 8 mg of DTA **20** were dissolved in 5 mL water and titrated with a total of 30 mL 3 mM aq. KOH in 1 mL increments, the pH being measured after each addition using {I10}. Then, the volume of added KOH (x-axis) was plotted against the pH (y-axis). The pK_A was defined as the mean y-value of the 5 datapoints closest to the inflection point of the resulting curve.

5.6.2 Synthetic methods

M13: Preparation of a DNPH 16 hydrochloride based derivatisation reagent

Similar to the procedure suggested by Brady and Elsmie [162], stabilised DNPH **16** (0.3 g, 1.0 mmol) was suspended in 2 M aq. HCl (3 mL). Consequently, 2 mL concentrated aq. HCl (2.4 g) were added and the suspension was ultrasonicated for 2 min. The crystalline DNPH **16** hydrochloride was re-dissolved in another 60 mL (61.2 g) of 2 M aq. HCl. The resulting solution was ultrasonicated until it cleared up and used directly as the derivatisation reagent (DNPH **16** concentration: 15.4 mmol/L).

M14: Preparation of TA 5 stock solutions from copper(II) bis(tenuazonate)

TA **5** is commercially available only as its stable copper (II) salt. Stock solutions of the free acid were obtained by two equally suitable methods:

Method A [192]: Approx. 5 mg copper(II) bis(tenuazonate) were dissolved in 10 mL chloroform. 10 mL 2 M aq. HCl were added and the organic layer was removed. The aq. layer was re-extracted twice with 10 mL chloroform. The united chloroform fractions were washed with 20 mL 2 M aq. HCl. Subsequently, the solvent was removed *in vacuo* at 30 °C. The solid residue was taken up in MeOH to obtain the stock solution.

Method B [105]: Dowex 50WX8-200 resin (H⁺ form) was left to stand in deionised water for 2 hours. A minicolumn (4 \times 1.5 cm) was packed with the conditioned resin and washed with MeOH three times. Approx. 5 mg of copper(II) bis(tenuazonate) were dissolved in 2 mL MeOH, put on the column, and eluted with 15 mL MeOH. The resulting colourless solution was diluted further to obtain the stock solution.

The actual concentrations of the stock solutions were determined by UV-spectroscopy {I6} using the extinction coefficient $\epsilon_{277} = 12,980$ cm mol L⁻¹ for TA 5 in MeOH [105].

M15: Activation of polymer resin MP-TsNHNH₂ 17 by conversion to MP-TsNHNH₃⁺Cl⁻ 23

5 g of MP-TsNHNH₂ **17** (either new or used, sufficient for 50 sample preparations) were put into a glass column and washed with 50 mL heptane. Subsequently, 500 mL of MeOH:0.4 M aq. HCl 90:10 (v:v) were passed through the column overnight. The column was then washed twice with 50 mL of Et_2O and dried in a gentle nitrogen stream to be stored at 4 °C. The activated resin **23** was stable for at least two months.

<u>M16: TA-DNPH 22</u>

TA **5** (10 mg, 51 μ mol, 1 eq.) was prepared from its copper salt {M14} and dried in a gentle nitrogen stream. Subsequently, the derivatisation reagent {M13} was added (30 mL, 468 μ mol, 9.2 eq.). After ultrasonication, a yellowish white solid precipitated. The mixture was shaken for further 30 min. The precipitate was collected, washed with water and recrystallised from ethanol to obtain TA-DNPH **22** (10 mg, 27 μ mol, yield: 53 %). The latter occurs in two rotameric forms which are differentiable in the ¹H-NMR spectrum. Their ratio at T = 25 °C is rotamer A (rot. A):rotamer B (rot. B) 62:38. Affected ¹H-NMR signals are split accordingly. NMR measurements at elevated temperature (heating to 90 °C in 10 °C steps) resulted in a successive loss of the rotameric signals.

TA-DNPH 22 Exact mass ({I4}, ESI(+)): 378.1410 Da, calculated mass: 378.1408 Da, Δ mDa: 0.2.

¹H-NMR (600 MHz, DMSO- d_6): δ 0.84 (m, 3H, CH₃), 0.93 (m, 3H, CH₃), 1.19 (m, 1H, CH₂), 1.30 (m, 1H, CH₂), 1.91 (m, 1H, CH), 2.42 (s, 3H, CH₃, rot. A), 2.47 (s, 3H, CH₃, rot. B), 3.64 (d, 1H, CH, rot. A), 3.68 (d, 1H, CH, rot. B), 7.18 (m, 1H, CH_{arom.}), 7.75 (s, 1H, lactam-NH, rot. B), 8.02 (s, 1H, lactam-NH, rot. A), 8.40 (m, 1H, CH_{arom.}), 8.88 (m, 1H, CH_{arom.}), 10.42 (s, 1H, amine-NH), 11.57 (s, 1H, H-bond, rot. A), 11.66 (s, 1H, H-bond, rot. B) ppm.

¹³C-NMR (151 MHz, DMSO-*d*₆): δ 11.9 (CH₃), 12.6 (CH₃), 15.8 (CH₃), 23.1 (CH₂), 36.7 (CH), 65.8 (CH), 96.6 (C_{quart}), 115.4 (CH_{arom}), 122.9 (CH_{arom}), 130.4-130.7 (CH_{arom}, C_{arom}), 137.5 (C_{arom}), 147.8 (C_{arom}), 168.1 (C_{quart}), 173.6 (C_{quart}), 196.5 (C_{quart}) ppm.

M17: AOD 27 and AMD 28

104 mg (0.40 mmol) of crude synthetic AOH **7** or 30 mg (0.11 mmol) of commercial AME **8** were suspended in 15 mL aq. phosphate buffer (pH 7, prepared by mixing 10 mL of 0.1 M KH₂PO₄ with 5.8 mL 0.1 M NaOH). The suspension was refluxed until it cleared up (AOH **7**: ~ 30 h, AME **8**: ~ 100 h), cooled to RT and extracted three times with 10 mL of Et₂O. The ether was removed by a gentle nitrogen stream and the residue was taken up in 15 mL of MeCN:water 60:40 (v:v). Cleanup was done by semi-preparative HPLC (Instrument {I1}, column {I15}). Eluents were water (A) and MeCN (B). The method was isocratic with 60:40 (v:v) A:B from 0 to 8 min., 0:100 A:B from 8 to 12 min and 3 min 60:40 A:B from 12 to 15 min (re-equilibration). Total runtime: 15 min. The flow rate was 5 mL/min and the injection volume 100 µL. Fractions were collected from $t_R = 5$ to 6 min (AOD **27**) and $t_R = 7$

to 8 min (AMD **28**). The collected fractions were kept in the dark under a gentle nitrogen stream and were left to dry under nitrogen after completion. AOD **27** (yield: 67 %, 63 mg, 0.27 mmol) and AMD **28** (yield: 82 %, 22 mg, 0.09 mmol) were obtained as white solids. Analytical data (see Figure 39 for atom-numbering):

AOD 27 Exact mass ({I5}, ESI(-)): 231.063 Da, calculated mass: 231.066 Da, ΔmDa: 3.

¹H-NMR (300 MHz, DMSO-d₆): δ 1.91 (s, 3H, H13), 5.97 (d, 2H, H8 + H12), 6.11 (m, 2H, H2 + H4), 6.19 (d, 1H, H10) ppm.

¹³C NMR (75 MHz, DMSO-d₆): δ 20.8 (C13), 100.5 (C10), 100.9 (C2), 108.1 (C4), 109.2 (C8 + C12), 120.9 (C6), 137.4 (C5), 140.2 (C7), 155.6 (C1), 156.8 (C3), 158.2 (C9 + C11) ppm.

HMQC (Heteronuclear multiple quantum coherence, 300 MHz, DMSO-d₆): C2-H2, C4-H4, C8-H8, C10-H10, C12-H12, C13-H13.

HMBC (300 MHz, DMSO-d₆): C2-(H2 + H4), C4-(H2 + H4), C10-H10, (C8 + C12)-(H8 + H12), C13-H13.

AMD 28 Exact mass ({I5}, ESI(-)): 245.078 Da, calculated mass: 245.082 Da, ΔmDa: 4.

¹H-NMR (600 MHz, DMSO-d₆): δ 1.91 (s, 3H, H13), 3.67 (s, 3H, H14), 6.08 (m, 1H, H8), 6.10 (d, 1H, H4), 6.11 (t, 1H, H12), 6.18 (d, 1H, H2), 6.21 (t, 1H, H10), 8.69 (s, 1H, H15), 8.96 (s, 1H, H16), 9.17 (s, 1H, H17) ppm.

¹³C NMR (150 MHz, DMSO-d₆): δ 20.3 (C13), 54.6 (C14), 99.0 (C10), 100.0 (C2), 107.0 (C8), 107.6 (C4), 110.2 (C12), 120.1 (C6), 136.8 (C5), 139.8 (C7), 155.0 (C1), 156.5 (C3), 157.7 (C11), 159.8 (C9) ppm.

HSQC (600 MHz, DMSO-d₆): C2-H2, C4-H4, C8-H8, C10-H10, C12-H12, C13-H13, C14-H14.

HMBC (600 MHz, DMSO-d₆): C1-H2, C2-(H4, H15, H16), C3-(H2, H4, H13), C4-(H2, H13, H16), C5-H13, C6-(H2, H4, H13, H15), C8-(H10, H12), C9-(H8, H10, H14), C11-(H10, H12, H17), C12-(H8, H10, H17), C13-H4, C10-(H8, H12, H17).

5.6.3 Other methods

M18: Kinetic DCHC experiments

1 mL (~ 0.8 g) of a solution of ZON **10** or its analogues (Figure 8, c = 2 mg/L each) in various solvents was added to resins **17** or **23** (n = 3, respectively). The mixture was shaken and at t = 0, 5, 15, 30, 45, 60, 120 and 150 min, 50 µL of the supernatant were taken off and transferred to a vial for HPLC injection. For decoupling, the same procedure (using only resin **23**) was done again for 150 min without any supernatant being taken off. Then, the resin was washed and dried as described in {M4}. Subsequently, 1 mL of solvent **24** or **25** were added (n = 3, respectively). The mixture was shaken and at t = 0, 15, 30, 45, 60, 90, 120 and 150 min, 50 µL of the supernatant were taken off and transferred to a vial for HPLC injection. For the evaluation of the coupling and decoupling experiments, corresponding five-point calibration curves were constructed in the respective injection solvent. To obtain coupling rate constants (k [min⁻¹]) linear least squares regression of the expression ln (cc_0^{-1}) = -kt was performed, with t being the coupling time [min], c the ZON **10** concentration at t = 0 min.

M19: Isochronous kinetic study (stability of TA 5 in aq. buffers)

Experimental

Solutions of TA **5** in pH 3.5 and pH 7.0 phosphate/citrate buffer were prepared by weighing 14 mL of a TA **5** stock solution (c = 60 mg/L) into two 300 mL Erlenmeyer flasks, respectively. After removal of the solvent in a gentle nitrogen stream, 250 mL of the respective buffers were added and weighed. The solutions were filtered (filter material: regenerated cellulose, pore size: 0.2 µm, Whatman, Maidstone, United Kingdom) and the final TA **5** concentrations of the buffered, filtered solutions were determined by HPLC-UV to be $c = 16.5 \pm 0.1 \text{ µmol/kg}$ (pH 3.5 solution) and $c = 17.6 \pm 0.1 \text{ µmol/kg}$ (pH 7.0 solution).

The study period was 118 days / 17 weeks. The buffered TA **5** solutions were stored in HPLC vials (clear glass) in the dark at different pHs (3.5 and 7.0) and temperatures (4, 25 and 40 $^{\circ}$ C), resulting in a total of six datasets. Each dataset consisted of 17 datapoints, each datapoint was represented by two vials. This resulted in a total of 34 HPLC vials per dataset or 204 HPLC vials in total.

Initially, the HPLC vials were charged with 400 μ L buffered TA **5** solution (weights recorded). Subsequently, they were thermostated in the dark at the respective temperatures (4 °C, 25 °C or 40 °C). Each week, two vials (equalling one datapoint) were frozen at

T = -20 °C for each pH and storage temperature. At the end of the study period, the 204 vials were defrosted, ultrasonicated, weighed in order to account for solvent evaporation during the 17 week period and then analysed directly using {M5} and {M6}.

Kinetic evaluation

Kinetic and thermodynamic parameters were calculated using the pH 3.5 datasets only, as at pH 7.0 decay rates were not sufficient. The pseudo first order kinetic model resulted in the best regression fits for all considered processes. However, as at pH 3.5, T = 40 °C the LOQ of method was reached at week 9, only the first 8 datapoints (up to week 8) were used for curve fitting. In the following passage, c_1 and c_u refer to the concentrations of TA 5 and *u*-TA 19, respectively, which were obtained by HPLC-UV {M5}. c_1^0 and c_u^0 refer to the concentrations at t = 0. Other symbols used include (*cf.* Figure 32):

k_H rate constant for the non-epimerisation related decrease in c₁

 k_{H} rate constant for the non-epimerisation related decrease in c_{μ}

k' rate constant for the non-epimerisation related decrease of the sum $c_u + c_1$

 k_1 rate constant for the decrease in c_1 due to epimerisation of TA 5

k₂ rate constant for the increase in c₁ due to epimerisation of *u*-TA **19**

k" observable rate constant for the decrease in c_1 due to epimerisation (k" = $k_1 - k_2$)

 $k^{\prime\prime\prime}$ observable rate constant for the total decrease in c_{l}

In order to obtain the rate constants k_{H} and k_{H} ' the first order derivative

(1)
$$\frac{d(c_l + c_u)}{dt} = -c_l \cdot k_H - c_u \cdot k_H$$

was considered. The sum $(c_1 + c_u)$ was quantified experimentally and decreased following the pseudo first order kinetic model expressed by

(2)
$$c_1 + c_u = (c_1^0 + c_u^0) e^{-k' \cdot t}$$

Hence, after rearrangement of eq. 2, k' could be obtained by linear least squares regression $(R^2 = 0.9994, datapoints = 6 \text{ for } T = 25 \text{ }^{\circ}\text{C} \text{ and } R^2 = 0.99999, datapoints = 8 \text{ for } T = 40 \text{ }^{\circ}\text{C}).$ Next, eq. (2) was differentiated resulting in

(3)
$$\frac{d(c_l + c_u)}{dt} = -k! \cdot (c_l^0 + c_u^0) \cdot e^{-k' \cdot t}$$

This equation may be combined with eq. 1 to obtain
(4)
$$\frac{c_l}{c_u}k_H + k_H' = \frac{k' \cdot (c_l^0 + c_u^0) \cdot e^{-k' \cdot t}}{c_u}$$

With k' known, the latter equation was used for linear least squares regression giving k_H as the slope and k_H' as the y-axis intercept ($R^2 = 0.9999$, datapoints = 16 for T = 25 °C and $R^2 = 0.9999$, datapoints = 8 for T = 40 °C). *Ergo*, the individual rate constants for the non-epimerisation based decay of TA 5 and u-TA 19 were obtained. Considering the second process of interest, epimerisation of pure TA 5 yielding u-TA 19, the rate constant k" was obtained by linear least squares regression using

(5)
$$\frac{c_1}{c_1 + c_u} = \frac{c_1^0}{(c_1^0 + c_u^0)} e^{-k^n \cdot t}$$

after rearrangement ($R^2 = 0.9994$, datapoints = 16 for T = 25 °C and $R^2 = 0.9980$, datapoints = 8 for T = 40 °C). In order to obtain individual values for k_1 and k_2 the equation

$$(6) c_1 = c_1^0 e^{-k^{m} \cdot t}$$

was considered. As c_1 decreased following pseudo first order kinetics, k^{III} could be obtained by linear least squares regression ($R^2 = 0.9996$, datapoints = 16 for T = 25 °C and $R^2 = 0.9998$, datapoints = 8 for T = 40 °C). Subsequently, eq. 6 was differentiated and merged with

(7)
$$\frac{dc_{I}}{dt} = -k_{H}c_{I} - k_{1}c_{I} + k_{2}c_{u}$$

to obtain

(8)
$$\frac{c_1}{c_u}(k_H + k_1) - k_2 = \frac{k''' \cdot c_1^0 e^{-k''' \cdot t}}{c_u}$$

eq. 8 is evaluated by linear least squares regression to obtain $(k_H + k_I)$ as the slope and k_2 as the y-axis intercept ($R^2 = 0.9999$, datapoints = 16 for T = 25 °C and $R^2 = 0.9999$, datapoints = 8 for T = 40 °C). Finally, as k_H is known, k_I may be obtained. Because k_{-1} was found to be not significantly different from zero given the available precision, $k'' \approx k_I$. For this reason, an epimerisation equilibrium constant could not be obtained with reasonable precision. Activation energies were calculated using the Arrhenius plot and thermodynamic activation parameters were calculated according to:

(8)
$$dG^{\ddagger} = RT \left[\ln \left(\frac{k_B}{h} \right) - \ln \left(\frac{k}{T} \right) \right]$$

$$(9) \qquad dH^{\ddagger} = E_A - RT$$

(10)
$$dS^{\ddagger} = \frac{dH^{\ddagger} - dG^{\ddagger}}{T}$$

k = rate constant [s⁻¹] k_{B} = Boltzmann constant = 1.38065 × 10⁻²³ J K⁻¹ T = temperature [K] h = Planck constant = 6.62607 × 10⁻³⁴ J s R = gas constant = 8.3145 J mol⁻¹ K⁻¹

All uncertainties given correspond to SDs derived either directly or through error propagation where appropriate. SDs of temperatures were assumed to be \pm 0.5 K.

M20: Stability of TA 5 and DTA 20 in apple juice and beer

Clear pilsener beer and clear apple juice were obtained from a local supermarket and ultrasonicated for 10 min. 0.14 mL of DTA **20** stock solution in MeCN (c = 10 mg/L, respectively) were added to HPLC vials and the solvent was removed at RT by a gentle nitrogen stream. Subsequently, 1.4 mL of beer or apple juice (three replicates, respectively) were added to the vials through a Minisart RC 4 regenerated cellulose syringe filter (Sartorius, Göttingen, Germany), resulting in final analyte concentrations of 1 mg/L. After ultrasonication, the vials were placed in the HPLC autosampler and the first injection was performed. For three days, further injections were performed continuously in 5 h intervals using method {M6}. The samples were thermostated at 23 °C. For TA **5** the same procedure and matrices were used, however, the spiked concentration was lowered to 0.5 mg/L and only two datapoints (t = 0 and t = 3 d) were determined by method {M2}.

M21: Molecular dynamics simulation for ZON 10 in various solvents

Molecular simulation was entirely done by the group of Dr. Marcus Weber at the ZUSE Institut, Berlin, Germany. Data were generated with GROMACS (version 4.0.5) and the ffgmx force field [193]. The molecular dynamics simulations were performed with Velocity rescaling thermostat [194] at a temperature of 298 K. One ZON **10** molecule was placed in a 9.618 nm \times 9.618 nm \times 9.618 nm box filled with different solvents. For the simulation of water the standard single point charge water model with a number of 29,860 water molecules

was applied. For MeOH (13,214 molecules), THF (6,602 molecules), EtOAc (5,475 molecules), MeCN (13,443 molecules) and hexane (4,103 molecules) the simulation was performed on the basis of an ffgmx force field and the Dundee prodrug2 server [195]. The average number of H-bonds was calculated from a 1,000 picoseconds molecular dynamics simulation. A hydrogen bond between ZON **10** and a solvent molecule was defined by an H-O distance less than 0.35 nm and an O-H-R angle of less than 60°. Under the assumption that the viscosity of the solvent is equal to the viscosity of the free solvent, the hydrodynamic volume was computed from the viscosity and the estimated diffusion constant, using the Einstein-Stokes relation [196].

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Annex

The quote preceding this dissertation originates from the short story *Von Kempelen and his discovery* written by Edgar Allen Poe in 1850. The story portrays the hypothetical discovery of a method allowing the transformation of lead into gold by the German chemist Von Kempelen. Narrated in the language of an investigative, scientific journalist, it tails off with an anecdote on the seizure of Von Kempelen by the police of Bremen. Caught in the act of making gold, the scientist destroys his apparatus in order to hide its secret. The final sentences of the text then merely give the reactions of the European economic market to the looming Von Kempelen method: a drop in the price of gold, a rise in the price of lead and a rise in the price of silver, which was incidentally promoted in the ranking of noble metals. Today, some see *Von Kempelen and his discovery* as the foundation of scientific storytelling.

However, what fascinated me about this story is not its importance for subsequent cultural developments. Instead, I was rather intrigued by its beautiful perspective on the fundamentals of human nature. By picturing a fantastic discovery, Poe subjects the reader to a general moment of human fascination. At the same time, he soothes and structures the fantastic energy of his conception by using a highly objective, almost scientific prose. The result is a sublime interplay of imagination and ratio—of science and dream—which unambiguously points to the inner force that makes us, both feet firmly in the world we somehow rationalise, reach up and grasp the miraculous in order to make it manageable to the human intellect. This works as well today as it might have in 1850. And so, enchanted by the brilliance of Poe's efforts, we obtain an outstandingly vivid sensation of *human motivation*. A century after the publication of *Van Kempelen and his discovery*, Thomas Mann, who was indeed very fond of Poe, found a brief and appropriate term for this quality: "Form und Tiefe" (form and depth).

Reading *Van Kempelen and his discovery* showed me in a clear way, what fascinates me about the subjects of science, chemistry, the arts and—even more—how essential the power of imagination is in enjoying the human intellect. In view of this, I did not even find myself disturbed by the fact that Poe's subject, the chemical transformation of lead into gold, is virtually impossible.

Acknowledgements

A great many people have contributed to this dissertation by various ways and means. I am particularly grateful to my supervisors at the Federal Institute for Materials Research and Testing (BAM) in Berlin, Prof. Dr. Ulrich Panne, Prof. Dr. Irene Nehls and Dr. Matthias Koch. By granting me a maximum in academic freedom and support, they made working on this dissertation a genuine pleasure. I am also indebted to my co-workers at the BAM workgroup "Analytics of Food and Commodities", who always offered their help in an unconditional and friendly way. The same is true for my former co-doctorands at BAM, Dr. Robert Köppen, Susanne Esslinger, Sebastian Schmidt, Paul Kuhlich and Stefan Merkel.

Furthermore, I would like to acknowledge Dr. Franziska Emmerling (BAM 1.3) for her expertise in the field of X-ray crystallography, Dr. Christian Piechotta and Robert Rothe (BAM 1.2) for their kind collaboration as well as Prof. Dr. Michael Rychlik (Technical University München) for valuable discussions and for granting me my first "invited talk" at an international conference.

Several interns and diploma students have contributed "full-time" to my work and deserve my profound thanks. These were: Franziska Blaske (two month research internship), Sven Brehme (one month research internship), Cindy Kochan (diploma thesis, six months, topic: "Development of a solid phase extraction method for the quantification of the mycotoxin zearalenone in edible oils"), Max Krägerman (two month practical internship) and Stefan Merkel (diploma thesis, six months, topic: "Investigations on the occurrence and degradation of *Alternaria* mycotoxins").

My sincere gratitude moreover goes to Justin Baker (The University of Texas at Austin, USA), Dr. Matthias Koch (BAM 1.2), Dr. Robert Köppen (BAM 1.2) and Jesper Mølgaard Mogensen (Technical University of Denmark) for proofreading the dissertation and helping me with those final touches.

Lastly, I would like to extend my thanks to Dr. Teresa Babuscio (COCERAL, Brussels). That is for providing valuable insights to the world of the European mycotoxin legislation, for showing me what can be achieved by emphatic persistence and for a most charming illustration of the difference between the terms "feet number" and "shoe size" (\rightarrow "what is your feet number?").