Journal of Chromatography A, 1217 (2010) 2206–2215

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Dynamic covalent hydrazine chemistry as a selective extraction and cleanup technique for the quantification of the *Fusarium* mycotoxin zearalenone in edible oils

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ARTICLE INFO

Article history:
Received 16 January 2010
Received in revised form 4 February 2010
Accepted 11 February 2010
Available online 18 February 2010

Keywords:
Zearalenone
Extraction
Edible oil
Maize
Dynamic covalent hydrazine chemistry
Hydrazone
SPE
Hydrodynamic radius
HPLC-FLD

ABSTRACT

A novel, cost-efficient method for the analytical extraction of the *Fusarium* mycotoxin zearalenone (ZON) from edible oils by dynamic covalent hydrazine chemistry (DCHC) was developed and validated for its application with high performance liquid chromatography-fluorescence detection (HPLC-FLD). ZON is extracted from the edible oil by hydrazone formation on a polymer resin functionalised with hydrazine groups and subsequently released by hydrolysis. Specifity and precision of this approach are superior to liquid partitioning or gel permeation chromatography (GPC). DCHC also extracts zearalanone (ZAN) but not α-/β-zearalenol or -zearalanol. The hydrodynamic properties of ZON, which were estimated using molecular simulation data, indicate that the compound is unaffected by nanofiltration through the resin pores and thus selectively extracted. The method's levels of detection and quantification are 10 and 30 μg/kg, using 0.2 g of sample. Linearity is given in the range of 10–20,000 μg/kg, the average recovery being 89%. Bias and relative standard deviations do not exceed 7%. In a sample survey of 44 commercial edible oils based on various agricultural commodities (maize, olives, nuts, seeds, etc.) ZON was detected in four maize oil samples, the average content in the positive samples being 99 μg/kg. The HPLC-FLD results were confirmed by HPLC–tandem mass spectrometry and compared to those obtained by a liquid partitioning based sample preparation procedure.

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1. Introduction

ZON (6-(10-hydroxy-6-oxo-trans-1-undecyl)-b-resorcylic acid lactone, **1**, Fig. 1) is a non-steroidal hyperestrogenic mycotoxin produced by a variety of *Fusarium* fungi, including *F. graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. rookwellense* and *F. semitectum* [1]. Although its acute toxicity is low, ZON was shown to be hyperestrogenic, hepatotoxic, haematotoxic, immunotoxic, genotoxic, teratogenic and carcinogenic in animal studies [1,2]. As ZON is commonly found on a variety of agricultural commodities like cereals, nuts, spices and mainly maize [1,2], it poses a food safety concern. For maize, studies on the redistribution of ZON during dry and wet milling [3,4] revealed high concentrations in the oil fraction (up to 4.6 mg/kg). Thus, an EU legal limit for ZON in refined maize oil was introduced in 2006 [5]. Due to increased levels of ZON in the maize harvests of 2005 and 2006, the initial limit of 200 μg/kg was raised to 400 μg/kg in 2007 [6] to attenuate negative economic impact. The acceptable daily intake (ADI) for ZON is 0.0005 mg/kg bw [7].

In the case of solid matrices like cereals, the analytical extraction of ZON is usually done with mixtures of organic solvents and water followed by immuno-affinity cleanup (IAC) [8]. Edible oils, on the other hand, are apolar, liquid matrices composed of 95–98% fatty acid triglycerides [9,10]. Hence, extraction with organic solvents (in this case termed liquid partitioning) is problematic, as a significant part of the matrix (about 10% [10]) is co-extracted. Liquid partitioning without further cleanup has thus been termed "far from adequate" for use with modern GC or LC instruments [11] and the analytical extraction of apolar contaminants from oils has gained predicates from "formidable" [9] over "challenging" [11] to "tedious" [10] in recent reviews. Despite of this, a range of liquid partitioning based methods without cleanup step were published [3,12,13] for ZON. Only in one case IAC was applied after liquid partitioning [14]. However, in some of the cited papers no chromatograms are shown and in the cases a chromatogram is given [12,13] the need for further cleanup can be inferred.

To date, the only published alternative to liquid partitioning is gel permeation chromatography (GPC) [15–17]. This instrumental

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doi:10.1016/j.chroma.2010.02.019

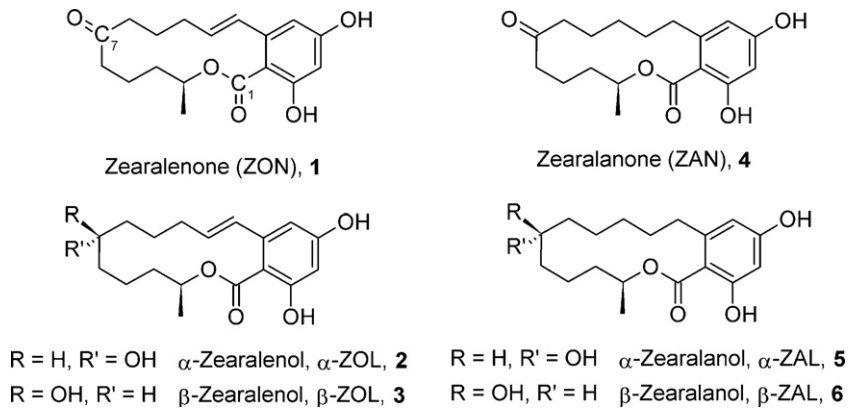

Fig. 1. Structures of ZON and its analogues.

technique separates substances by their hydrodynamic volumes and is well suited for the separation of ZON from the triglyceride matrix [17]. However, an additional IAC step is needed, as all molecules with hydrodynamic radii similar to ZON are co-extracted. The IAC step can be omitted, if the selective tandem mass spectrometry (MS/MS) detector is used instead of fluorescence detection (FLD) [17]. MS may also be used in multi-mycotoxin methods applicable to oily matrices [18], but generally isotope standards are required to achieve a performance comparable to FLD [17].

In summary it can be said that the involvement of GPC, IAC, MS/MS or isotope standards causes the methods for the quantification of ZON 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.

For the sum of these reasons, there is the need for a novel, alternative solid-phase principle for the extraction of ZON occurring in edible oils. The new technique should:

(i) show a sufficiently high specifity for ZON, 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 minimizing the handling efforts during sample preparation;
(v) meet the performance criteria required for monitoring the current EU legal limit for ZON in maize oil.

While most solid-phase based cleanup methods rely on rather unspecific physisorption or ion–ion 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 specifity. In the case of ZON, the unconjugated carbonyl group at C_7 enables a range of chemical reactions, *inter alia* the formation of a hydrazone with a hydrazine. As hydrazone formation is known to be reversible [19–22], it was considered suitable for the extraction and cleanup of ZON. Also, we have previously shown that hydrazine chemistry works well in the presence of food matrices (i.e. cereals and beer) [23,24].

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. [25], introducing the concept of dynamic covalent chemistry (DCC). Recent applications of dynamic covalent hydrazine chemistry (DCHC) in particular can be found in the fields of profragrances [26,27], dynamic covalent polymers [28] and drug discovery [29]. We also know of one method for which a hydrazine moiety has been covalently fixed on a solid support to react reversibly with a solubilised carbonyl: Roe et al. reported a technique for enriching peptides modified by the lipid peroxidation product 4-hydroxynoneal (HNE) [30]. The peptides were fixed on hydrazine modified aminopropyl glass beads and released by hydrolysis after washing to be analysed by MS techniques. However, to our knowledge DCHC has not yet been used for quantitative instrumental analysis.

In the present paper, we would like to present a novel approach for the combined extraction and cleanup of the *Fusarium* mycotoxin zearalenone (ZON) occurring in edible oils, which is based on reversible hydrazone formation on a hydrazine-functionalised polymer resin. This is a new application of DCHC and a third alternative to liquid partitioning and GPC. The advantages of DCHC will be discussed and results from the analysis of oil samples will be shown.

2. Materials and methods

2.1. Materials

ZON (OEKANAL®, solid substance, certified purity: 99.3%), α- and β-zearalenol (α-/β-ZOL, no purity given), α-zearalanol (α-ZAL, 97%), β-zearalanol (β-ZAL, 98%), zearalanone (ZAN, no purity given) and sulfonylhydrazide (polymer bound, macroporous, pore size: 3–6 nm, typical loading: 1.5–3.0 mmol/g, synonym: MP-TsNHNH$_2$) were obtained from Sigma–Aldrich (Steinheim, Germany). All standard chemicals were of p.a. grade, all solvents HPLC-grade. Deionised water was sourced from a Milli-Q® Synthesis A10 system equipped with a Quantum® EX Ultrapure Organex cartridge (Millipore, Billerica, USA).

2.2. Terms and definitions

In the following passages the term 'elution solvent' is used with respect to a freshly prepared mixture of acetone:0.13 M HCl 70:30 (v/v). The term 'spiked blank matrix' refers to a single commercial edible maize oil sample, which showed no natural ZON contamination upon analysis and was spiked with varying amounts of ZON. All analytical results are given as $x \pm$ SD, except for the ZON levels of samples which are reported as $x \pm u$, with u being the expanded uncertainty ($k = 2$). All measurements were done using the HPLC-FLD method described below if no other indication is given.

2.3. Polymer resin pre-treatment (hydrochloride formation)

5 g of new or used polymer resin (sufficient for 50 sample preparations) were put into a glass column and washed with 50 mL

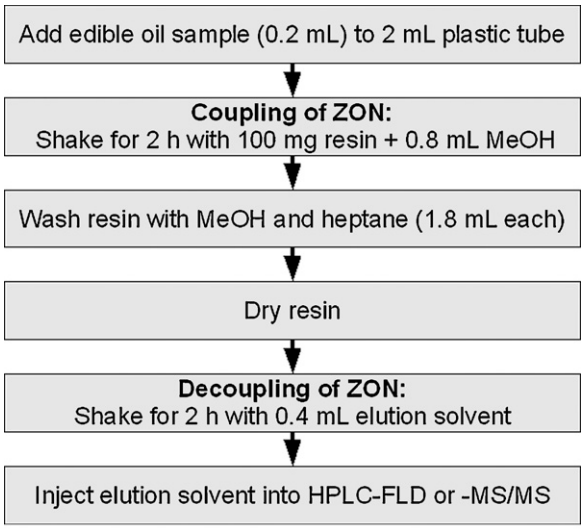

Fig. 2. Flowchart of the DCHC sample preparation procedure.

heptane. Subsequently, 500 mL of MeOH:0.4 M HCl 90:10 (v/v) were passed through the column overnight. The column was then washed twice with 50 mL of diethyl ether and dried in the gentle nitrogen stream to be stored at 4 °C. The stored resin was stable for at least 2 months.

2.4. DCHC sample preparation (Fig. 2)

100 ± 2 mg of the conditioned polymer resin were weighed into a 2 mL Eppendorf safelock tube. 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 then shaken for 2 h on a Promax 2020 horizontal shaker (Heidolph, Kelheim, Germany) at 400 rpm. 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 μL (approx. 0.32 g, exact weight recorded) of the elution solvent were added and the safelock tube was shaken for 2 h at 400 rpm. The supernatant was taken of and transferred to a HPLC vial through a Phenex 4 mm syringe filter (pore size: 0.2 μm, filter material: regenerated cellulose, Phenomenex, Aschaffenburg, Germany). Given the injection volumes stated below, equivalents of 6.9 mg/7.5 μL (FL detection) or 2.3 mg/2.5 μL (MS/MS detection) matrix were injected with each chromatographic run.

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 for further use.

2.5. HPLC-FLD method

HPLC-FLD analyses were done using an Agilent 1200 HPLC tower equipped with Agilent 1200 DAD and FLD detectors (Agilent, Böblingen, Germany). A Gemini NX C18 column (2 mm × 150 mm, 3 μm particle size, Phenomenex, Torrance, USA) was used in combination with the respective precolumn. The chromatographic parameters were as follows: oven temperature: 50 °C, injection volume: 15 μL, flow rate: 0.4 mL/min, solvent A: water + 0.1% (v) formic acid, solvent B: ACN + 0.1% (v) formic acid. The following linear gradient was used: 0–70% B in 14 min, followed by 100% B for 5 min and 100% A for 7 min (re-equilibration). FLD detection of ZON (t_R = 13.2 min) was done at λ = 464 nm after excitation at λ = 232 nm, the PMT-gain (photomultiplier-gain) was 15. The same parameters were used for the ZON analogues (Fig. 1).

2.6. HPLC–MS/MS method

HPLC–MS/MS analyses were done using an Agilent 1200 HPLC tower directly linked to an Applied Biosystems API 4000 QTRAP tandem mass spectrometer (Applied Biosystems, Foster City, USA) equipped with a TurboSpray ion source. A Dionex Polar Advantage II column (2 mm × 150 mm, 3 μm particle size, Dionex, Idstein, Germany) was used. The chromatographic parameters were as follows: oven temperature: 40 °C, injection volume: 5 μL, flow rate: 0.4 mL, solvents were identical to the FLD method, the following linear gradient was used: 0–100% B in 10 min followed by 100% B for 5 min and 100% A for 5 min (re-equilibration). ZON eluted at t_R = 10.4 min.

The ESI+ multiple reaction monitoring (MRM) transitions were m/z 319 → 301 (quantification) and m/z 319 → 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: curtain gas (CUR): 55, temperature (TEM): 500 °C, ion source gas 1 (GS1): 50, ion source gas 2 (GS2): 30, ion spray voltage (IS): 5500 kV, collision gas (CAD): medium, interface heater: on. The optimised compound specific parameters were (quantifier/qualifier): declustering potential (DP): 61/61 V, entrance potential (EP): 10/10 V, collision energy: 15/19 V, cell exit potential 8/8 V, dwell time: 50/50 ms.

2.7. Calibration

All stock solutions were prepared in ACN and stored at −20 °C. Calibration curves were constructed on the day of the analysis by weighing variable portions of the stock solution into HPLC vials. After removal of the ACN by a gentle nitrogen stream, the elution solvent was added gravimetrically. For example, 30, 60, 120, 240 and 480 mg of a ZON stock solution (c = 1.0 mg/kg) were taken up in 1 g elution solvent after removal of the ACN, to obtain a calibration curve with datapoints corresponding to 30, 60, 120, 240 and 480 μg ZON per kg elution solvent.

2.8. Quantification

Oil samples were screened by a single DCHC sample preparation. The approximate ZON content in the sample was evaluated semi-quantitatively (i.e. without recovery correction) using external calibration. If the approximate analyte content was > 0.5 × MLQ, five-point standard addition curves were constructed. The highest amount of added standard was equal to the approximate natural ZON content in the oil sample.

For an oil of the approximate natural ZON content of 500 μg/kg, 0, 22.5, 45.0, 67.5 and 90 mg of a ZON stock solution (c = 1.0 mg/kg) were added to safelock tubes with the ACN being removed. The oil was added, the tubes were ultrasonicated for 5 min and then shaken overnight. Subsequently, the DCHC sample was conducted, to obtain a standard addition curve with datapoints corresponding to 500, 600, 700, 800, 900 and 1000 μg ZON per kg oil. The same procedure was done for the standard addition curves to be measured by HPLC–MS/MS.

All liquids (solvent, oil) directly involved in the sample preparation processes were weighed and all calculations were done using exclusively gravimetric data. For each sample preparation the parameters m_{Oil} (weight of the used oil portion [g]), m_{solv} (weight of the used elution solvent portion [g]), c_{dot} (concentration change due to doting [μg/kg]) and PA (peak area [arbitrary units]) were recorded. Standard addition curve x-axis values corresponded to c_{Oil} and y-axis values to PA × m_{solv}/m_{Oil}. With a being the slope and

b the y-axis intercept of the resulting curve, the natural content of ZON in the sample (c_{ZON}) computes as $c_{ZON} = b/a$ [μg/kg]. The standard deviation of c_{ZON} was obtained from the standard deviations of a and b by error propagation.

For the calculation of the recovery from standard addition data another curve was drawn, omitting the datapoint corresponding to the unspiked oil. With this curve, the x-axis values corresponded to $c_{Oil} \times m_{Oil}/m_{solv}$ and the y-axis values to PA. With the obtained slope being a_2 and the calibration curve slope a_{CAL} (actual value: 0.786, see Section 3.7), the recovery calculates as $a_2/a_{CAL} \times 100$.

2.9. Validation parameters

The method's limit of detection (MLD) was defined as the concentration of ZON in the edible oil at which the signal to noise ratio was 3:1. The method's limit of quantification (MLQ) was defined as the MLD × 3.

For ZON positive samples, recoveries were obtained as described in Section 2.8. For further validation, spiked blank matrices (c_{ZON} = 30, 300 and 3000 μg/kg) were employed. All spiking was done as described in Section 2.8. The precision was characterised by the relative standard deviation (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 thus obtained ZON concentrations from the known, spiked concentration. RSD values were also obtained for all positive samples (n = 5, no standard addition).

2.10. Kinetic experiments

1 mL (~0.8 g) of a solution of ZON or its analogues (Fig. 1, c = 2 mg/L each) in MeOH or other solvents was added to 100 ± 2 mg conditioned or unconditioned resin (three replicates, 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 conditioned resin only) was done again for 150 min without any supernatant being taken off. Then, the resin was washed and dried as described in Section 2.4. Subsequently, 1 mL of elution solvent or methanol:0.13 M HCl 70:30 (v/v) were added (three replicates, 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 $\ln(c/c_0) = -k \times t$ was performed, with t being the coupling time [min], c the ZON concentration at t, and c_0 the ZON concentration at t = 0 min.

2.11. Molecular dynamics simulation

Molecular simulation data was generated with GROMACS (version 4.0.5) and the ffgmx force field [31]. The molecular dynamics simulations were performed with velocity rescaling thermostat [32] at a temperature of 298 K. One ZON molecule was placed in a 9.618 nm × 9.618 nm × 9.618 nm box filled with different solvents. For the simulation of water the standard single point charge (SPC) water model with a number of 29,860 water molecules was applied. For MeOH (13,214 molecules), THF (6602 molecules), EtOAc (5475 molecules), ACN (13,443 molecules) and hexane (4103 molecules) the simulation was performed on the basis of an ffgmx force field and the Dundee prodrug2 server [33]. The average number of H-bonds was calculated from a 1000 ps molecular dynamics simulation. A hydrogen bond between ZON 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 [34].

2.12. Comparative GPC method

GPC was done on a LC-Tech GPC Vario system, equipped with a FW-20 fixed wavelength detector, a GPC 1122 solvent delivery system and a GPC10011 column (dimensions 500 mm × 40 mm, Bio-Beads S-X3 filling, all LC-Tech, Dorfen, Germany). The GPC eluent was cyclohexane:ethylacetate 1:1 (v/v). The flow rate was 5 mL/min and the total runtime 40 min. 5 mL sample were injected. The ZON fraction was collected from t_R = 17 min to 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 fraction was removed and the fraction was reconstituted in 5 mL (approx. 4 g, exact weight recorded) elution solvent. This solution was injected into the HPLC-system (injected matrix equivalent identical to DCHC).

2.13. Comparative liquid-partitioning method

Liquid partitioning based sample preparations were done exactly as described in [12]. After the final evaporation step, the residue was taken up in 1.1 mL elution solvent and injected directly (injected matrix equivalent identical to DCHC). For each ZON positive sample, six sample preparations were done with the one increasing the resulting RSD most being omitted. The recovery was calculated as described in Section 2.8 and results were corrected accordingly.

3. Results and discussion

3.1. Initial considerations

The presented method is based on the reversible reaction of ZON (**1**) with a hydrazine moiety anchored to a polystyrene resin (**8**) (Fig. 3). This reaction yields the hydrazone species **9**, which may subsequently be hydrolysed to yield back ZON and **8**. The DCHC method thus consists of three major steps (Fig. 2):

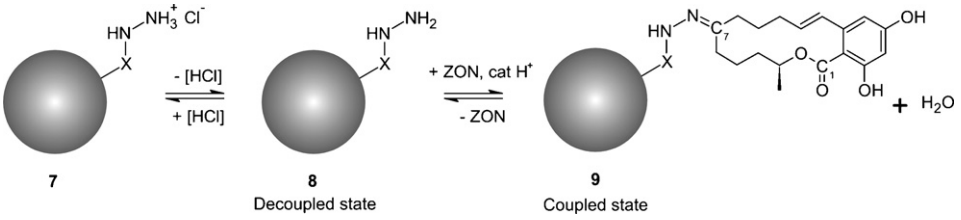

Fig. 3. Reversible hydrazone (**9**) formation by ZON (**1**) and a hydrazine moiety (**8**) covalently attached to a polymer resin, X = *p*-toluenesulfonyl linker.

(i) Coupling (i.e. covalent binding) of ZON, present in an diluted edible oil, to the polymer resin through hydrazone formation.
(ii) Washing of the polymer resin to remove the edible oil matrix.
(iii) Decoupling (i.e. hydrolytical release) of ZON for quantification.

This sequence should allow an overall recovery between 70% and 120% to meet the typical performance criteria for ZON requested by the European Commission [35,36]. In this respect, the inherent equilibrium character of hydrazone formation is disadvantageous: during coupling, a fraction of ZON will remain unreacted and is lost in the washing step. During decoupling, a fraction of ZON will remain coupled and is consequently not present in the solution to be analysed by HPLC. Also, hydrazone formation and hydrolysis are generally slower than physisorption or liquid partitioning.

Thus, conditions under which the hydrazone formation equilibrium (Fig. 3) allows pseudo-irreversible (i.e. quantitative) coupling and decoupling reactions, which proceed fast enough to be of practical use, need to be established.

3.2. DCHC catalysis by acids

Both hydrazone formation and hydrolysis are catalysed by acids or bases [19]. As ZON is unstable under alkaline conditions [12], only acidic catalysis is considered here. In the case of coupling, the acid may be introduced with the solvent. However, aqueous solvents (e.g. aq. HCl or aq. sulfuric acid) are shifting the hydrazone formation equilibrium to the undesirable product side. Carbonic acids (e.g. acetic acid) on the other hand, which may be used in water free mixtures, tend to cross-react with the hydrazone groups (acetylation) [37] and are thus also unsuited for coupling. Alternatively, the required catalytic protons may be introduced conveniently to the reaction mixture by converting the resin's hydrazine groups to their hydrochlorides before use. Hydrazine hydrochlorides liberate HCl in situ [37] and are compatible with non-aqueous solvents. It should be noted that the release of HCl from **7** prior to hydrazone formation with ZON is an essential mechanistic requirement, as in the hydrochloride species **7** no nucleophilic electron lone pair is available at N_1 [19] (Fig. 3).

A comparison of the coupling rates using (i) untreated resin and (ii) resin with hydrazine hydrochloride groups is shown in Fig. 4. In both cases, the concentration of ZON in the coupling solvent is described by an exponential decay curve, indicating pseudo first order coupling kinetics. The pseudo first order rate constants are $134(\pm2)\times10^{-4}\,min^{-1}$ (untreated resin, hydrazine) and $772(\pm14)\times10^{-4}\,min^{-1}$ (pre-treated resin, hydrazine hydrochloride). Hence, a sixfold increase in the reaction rate is achieved through pre-treatment of the resin. Under these conditions coupling is completed after 1 h at room temperature.

In the case of decoupling, the presence of water in the reaction mixture is desirable. Hence, rapid phenylhydrazone hydrolysis is usually done with aqueous solvents at high acid strengths, e.g., in sulphuric acid [38] or levulinic acid [39]. However, to protect analytes and resin, we intended to employ a milder pH and to use only completely volatile solvents. Initial tests with mixtures of MeOH and dilute HCl did not give acceptable results, i.e. approx. 98% of ZON remained coupled, even after 2.5 h decoupling time. However, if acetone was used instead of MeOH, ZON decoupled readily (approx. 93% in solution after 2.5 h). This is attributed to a displacement of the carbonyl part of the hydrazone species **9** by acetone. The pronounced effect of acetone on the rate of decoupling is shown in Fig. 5.

Contrary to coupling, the decoupling kinetics are not pseudo first order, but follow a rate expression *inter alia* involving the equilibrium between the free hydrazone sites (**8**), acetone and coupled acetone. This rather complicated kinetic problem is beyond the scope of this paper and will not be discussed here.

3.3. Reactivity of ZON analogues

To better understand the reactivity of ZON, its analogues (Fig. 1) were tested under the optimised DCHC conditions. Only one analogue, ZAN, reacted with the resin, the pseudo first order coupling rate constant being $784(\pm33)\times10^{-4}\,min^{-1}$ (Fig. 4). As ZAN differs from ZON 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 (Fig. 5) is obtained. It should also be mentioned that ZAN (t_R = 13.1 min) partly coeluted with ZON (t_R = 13.2 min) under the employed HPLC conditions. This coelution is commonly observed and may be exploited by using ZAN, which is not occurring in food, as an internal standard in combination with MS or MS/MS detectors [40–42].

The lacking reactivity of α-/β-ZOL and α-/β-ZAL is due to an important chemical difference to ZON/ZAN: in the former compounds the carbonyl group at C_7 is reduced to the alcohol. As the lactone–carbonyl group at C_1 is unreactive due to conjuga-

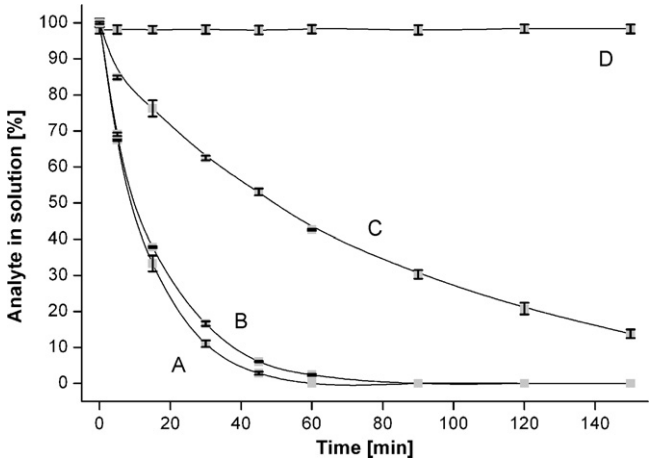

Fig. 4. Time-dependent percentage of ZON and its analogues in the solvent supernatant upon coupling, $n = 3$, see Section 2.1 for conditions. A: ZON, pre-treated resin; B: ZAN, pre-treated resin; C: ZON, untreated resin; D: α-ZOL, pre-treated resin (congruent curves were obtained for β-ZOL and α-/β-ZAL).

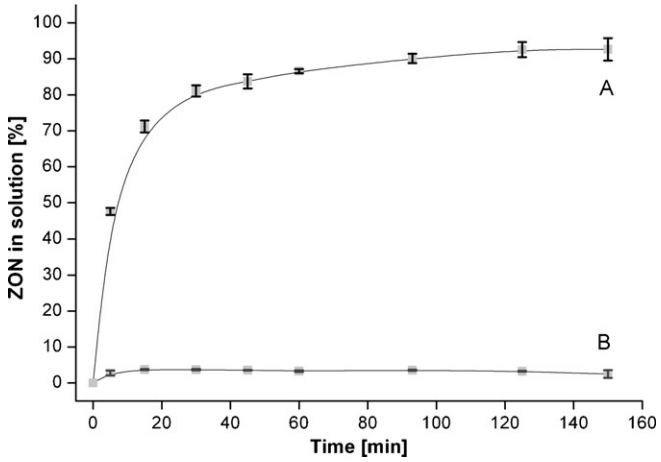

Fig. 5. Time-dependent percentage of ZON in the solvent supernatant upon decoupling, $n = 3$, see Section 2.1 for conditions. A: in acetone:0.13 M HCl 70:30 (v/v); B: in MeOH:0.13 M HCl 70:30 (v/v).

Table 1
Hydrodynamic parameters for ZON in various solvents as calculated from molecular simulation data (see Section 2.11) and experimental DCHC results.

Solvent		Molecular simulation results				Experimental DCHC results	
	Dipole moment [10^{-30} C m]	Diffusion constant [10^{-9} m^2 s^{-1}]	Viscosity [10^{-4} Pa s]	Average number of H-bonds	Hydrodynamic radius (R_H) [nm]	Coupling rate constant (see Section 2.10) [$\times 10^{-4}$ min^{-1}]	Recovery if used as coupling solvent (spiked blank matrix, c = 2 mg/kg) (see Section 2.4) [%]
EtOAc	6.27	0.2029	4.26	0.11	2.52	171 ± 9	73.5 ± 0.6
Water	6.07	0.1170	8.94	2.92	2.09	–	–
ACN	11.48	0.2816	3.80	0.21	2.04	573 ± 34	75.8 ± 0.6
Hexane	0.00	1.0602	4.70	0.01	0.44	–	–
MeOH	5.67	0.9281	5.44	1.47	0.43	772 ± 14	79.6 ± 0.6
THF	5.84	1.1022	4.70	0.06	0.42	70 ± 1	63.9 ± 0.8

tion, there is no potential site for hydrazone formation and hence no reaction with the resin. Consequently, in the experiment summarised in Fig. 5, no ZAL or ZOL is recovered. Hence, DCHC separates ZON and ZAN from α-/β-ZOL and α-/β-ZAL.

3.4. Molecular simulation data

To make use of all hydrazine sites (**8**) available on the resin, ZON needs to enter its macropores (diameter: 3–6 nm). Its crystal structure indicates that the ZON molecule is sufficiently small [43]. However, the hydrodynamic volume of a small molecule is not identical with its solvent accessible surface. In fact, hydrogen bonds with surrounding solvent molecules can increase the hydrodynamic radius (R_H). This may cause ZON 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 will also affect R_H. Solvents with high dipolar moments are more likely to be attached to ZON, causing a R_H increase. Hence, we estimated the R_H of ZON for different solvents on the basis of molecular simulation data (Table 1 and Fig. 6). 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 by the macropores likely. In ACN 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.

However, in hexane, MeOH and THF these effects are significantly less pronounced, resulting in fourfold lower R_H values. In summary, our molecular simulation data supports the hypothesis that ZON 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.

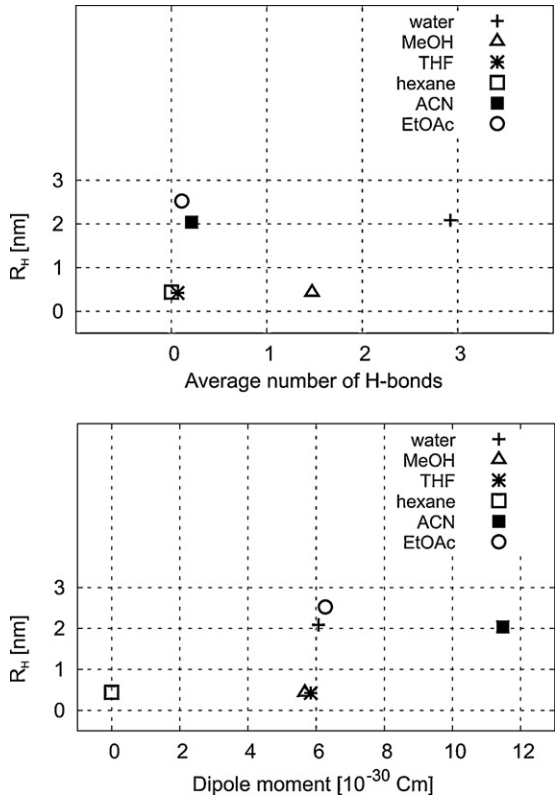

Fig. 6. Hydrodynamic properties of ZON calculated from molecular simulation data, see Section 2.11 for conditions.

3.5. Sample to resin ratio and choice of coupling solvent

To test the influence of the coupling solvent experimentally, the DCHC sample preparation was conducted with ACN, EtOAc or THF instead of MeOH and all corresponding coupling rate constants were determined (Table 1). Hexane and water could not be investigated experimentally due to the limited solubility of ZON in these solvents.

MeOH afforded the highest coupling rate and the highest recovery if applied with a sample matrix. The lower rate constants for ACN and EtOAc may be attributed to the significantly higher R_H of ZON in these solvents (Table 1), causing a lower accessibility of the resin pores and reactive hydrazine sites. However, this is cannot be considered the only factor of importance, as THF gave the lowest coupling rate, although the associated hydrodynamic radius was smallest.

The ideal sample to resin ratio was determined by evaluating the recoveries obtained by sample preparations with varying resin amounts (Fig. 7). The ideal ratio is 500 mg resin per mL oil or higher. 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.

3.6. Recycling of the polymer resin

As DCHC is reversible by principle, it seemed plausible to reuse the resin in order to lower analysis costs. Hence, recycling routines were evaluated. Best (i.e. most repeatable) results were obtained, if the resin was continuously washed overnight with the solvent also used for hydrochloride generation. Using this recycling approach, a spiked blank matrix (c_{ZON} = 5 mg/kg) was worked up on 12 separate days (10 replicates per day) starting with new resin. After each day, the used resin was recycled in bulk and used for the next day's determinations. Fig. 8 summarises the results of the trial. It can be seen that resin performance decreased gradually, start-

ing with the fourth day. For the first 3 days, the two-sided *t*-test ($f = 18$, $P = 95\%$) showed no statistically significant differences in the obtained recoveries. Also, the *F*-test ($f_1 = f2 = 9$, $P = 95\%$) detected no statistically significant inhomogenity if applied to the highest and lowest RSD obtained throughout the trial. Hence, it is concluded that the resin may be used up to three times without losses in recovery or precision.

3.7. Validation

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

- Curve A (solvent only):

$$y = (0.786 \pm 0.002)x - (3.911 \pm 5.268)$$
$$R^2 = 0.9999, \text{ residual standard error } s_y = 14.908$$

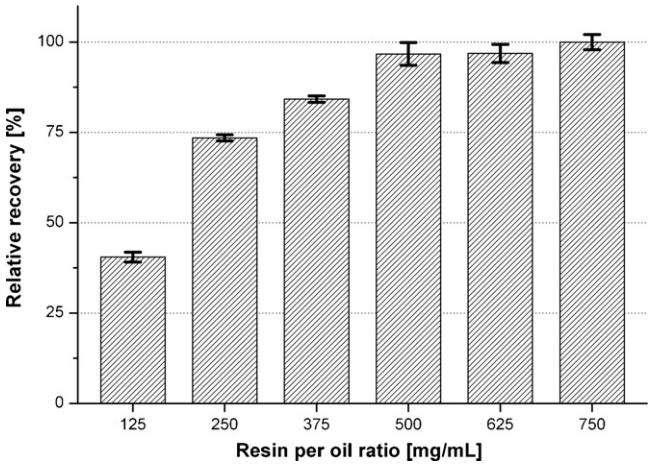

Fig. 7. Relative recoveries obtained from the analysis of a spiked blank matrix ($c_{ZON} = 5.0$ mg/kg), depending on the amount of polymer resin used, $n = 3$.

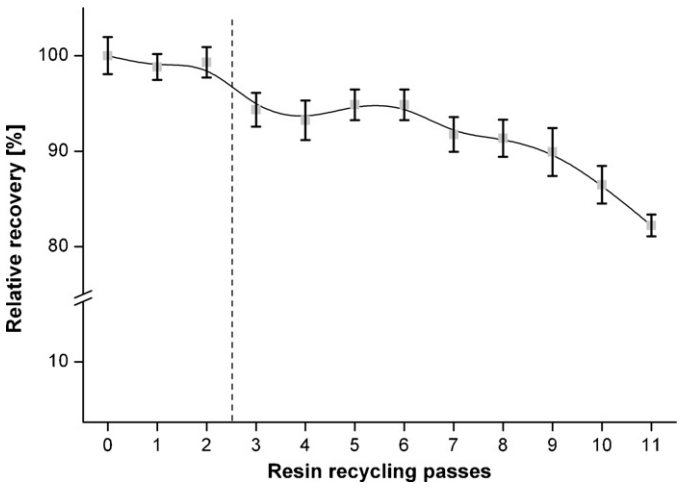

Fig. 8. Relative recoveries obtained from the analysis of a spiked blank matrix ($c_{ZON} = 5.0$ mg/kg), depending on the number of precedent resin recycling passes, $n = 10$.

- Curve B (spiked blank matrix, DCHC):

$$y = (0.626 \pm 0.004)x - (4.922 \pm 14.250)$$
$$R^2 = 0.9998, \quad s_y = 41.265$$

with $x = c_{ZON}$ [μg ZON per kg elution solvent], y = peak area [arbitrary units] and $n = 12$. From the two slopes, the method's recovery for the spiked blank matrix computes as 79.6(±0.6)%. The recovery without sample matrix was 92.6(±2.1)% (Fig. 5). Recoveries and RSDs for all contaminated edible oils are given in Table 2. The average recovery for the spiked blank matrix and the four positive samples was 89(±10)%. Using curve B, MLD and MLQ were determined to be 10 and 30 μg/kg, respectively. Since no certified reference material (CRM) was available for ZON in edible oil, the assessment of trueness had to be done by spiking a blank matrix. The thus obtained data are given in Table 3. To satisfy the official requirements (EU) set for ZON, RSDs should not exceed 25%. Also, the method's recovery should be in the range of 70–120% [35,36]. These criteria are well met by DCHC.

Table 2
Sample survey results (recovery corrected).

Sample	DCHC, HPLC-FLD			DCHC, HPLC–MS/MS			Liquid partitioning, HPLC-FLD, see Section 2.13		
	c_{ZON} [μg/kg], $k = 2$	Recovery [%]	RSD, $n = 5$ [%]	c_{ZON} [μg/kg], $k = 2$	Recovery [%]	RSD, $n = 5$ [%]	c_{ZON} [μg/kg], $k = 2$	Recovery [%]	RSD, $n = 5$ [%]
2	57 ± 2	96 ± 1	1.9	58 ± 4	107 ± 10	7.9	93 ± 70	69 ± 44	37.6
31	86 ± 2	102 ± 5	1.5	80 ± 10	89 ± 6	13.7	299 ± 126	36 ± 31	21.0
41	135 ± 6	84 ± 3	0.7	135 ± 14	102 ± 5	7.3	128 ± 8	80 ± 9	2.8
43	117 ± 6	82 ± 2	1.4	104 ± 10	75 ± 4	10.1	92 ± 10	131 ± 9	5.5
Mean	99	91 ± 10	1.4 ± 0.5	94	93 ± 14	9.7 ± 2.9	153	79 ± 40	16.7 ± 16.1

Table 3
Validation data obtained using a spiked blank matrix (maize oil).

Validation matrix spiking level	LOQ	LOQ × 10	LOQ × 100
c_{ZON} calculated [μg/kg]	30	301	3003
c_{ZON} measured (using external calibration, $n = 5$, recovery corrected, $k = 2$) [μg/kg]	32 ± 2	296 ± 16	3017 ± 16
RSD [%]	3.1	2.7	0.3
Bias [%]	6.7	1.7	0.5
c_{ZON} measured (using standard addition, $k = 2$) [μg/kg]	30 ± 2	299 ± 20	2950 ± 52
RSD [%]	3.3	3.3	0.9
Bias [%]	0	0.7	1.8
Injection RSD, $n = 5$ [%]	0.8	1.5	0.4

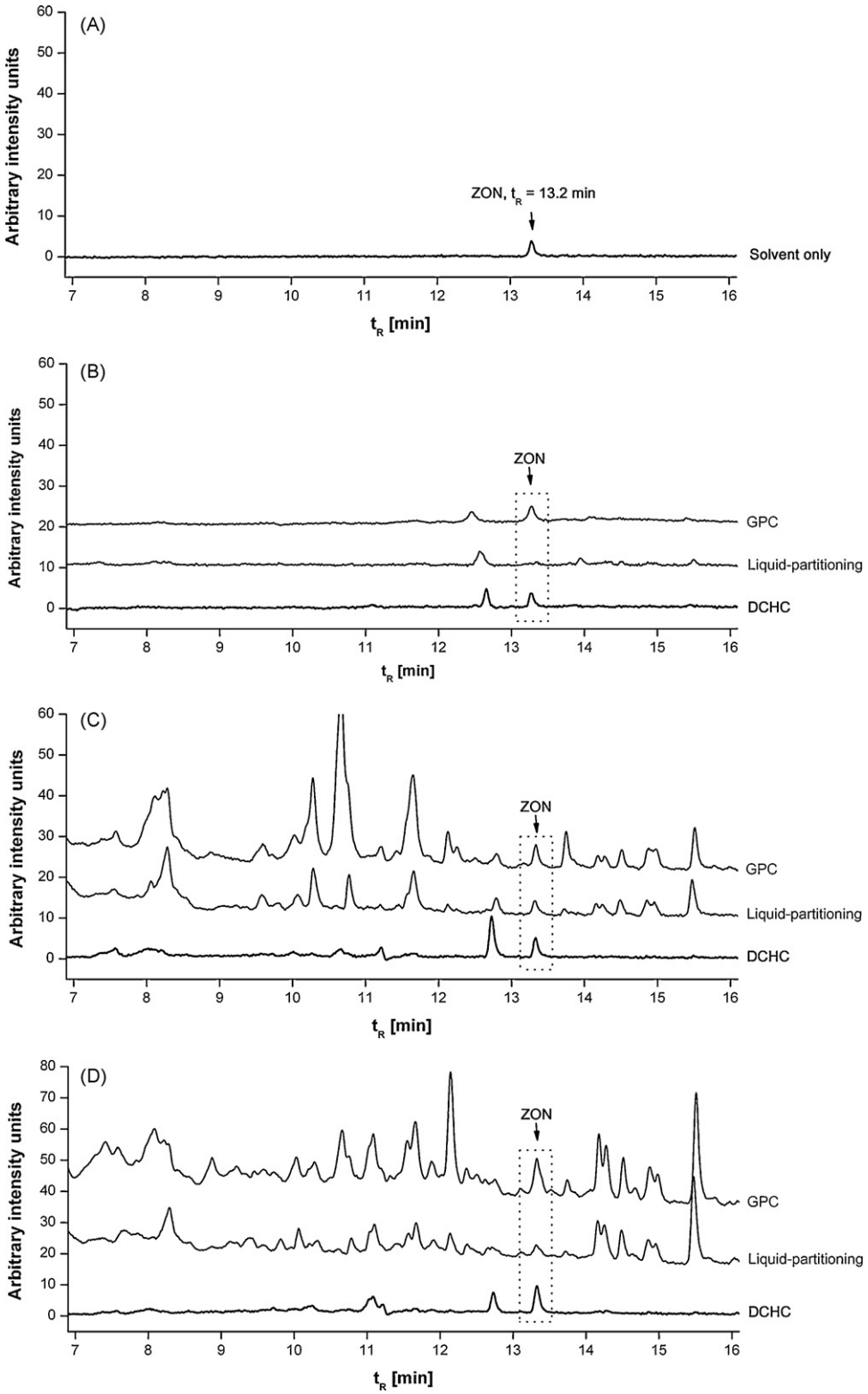

Fig. 9. Characteristic chromatograms. A: MLQ injection (30 μg/kg), ZON in elution solvent (no sample preparation, no sample); B: MLQ injection (30 μg/kg), sample preparation of spiked blank matrix; C: sample 31 (ZON content: 86 ± 2 μg/kg); D: sample 43 (ZON content: 117 ± 6 μg/kg).

3.8. Sample survey

44 edible oil samples based on various agricultural commodities were sourced from local supermarkets as well as online-shops and analysed by HPLC-FLD. A standard addition strategy was chosen for the quantification of ZON, since it afforded a lower bias at low ZON concentrations compared to external calibration (Table 3). Also, standard addition provides additional specifity and the obtained results are recovery corrected as a matter of principle. For further confirmation HPLC–MS/MS was employed. The MS/MS detector afforded an MLD and MLQ of 5 and 15 μg/kg, respectively, and was linear in the range of 5–1000 μg/kg.

Table 4
Comparison of DCHC to other published methods for the quantification of ZON in edible oil (all data taken from the respective publication).

Reference	Extraction	Cleanup	Quantification	Sample amount [g]	MLD [μg/kg]	Recovery [%]	Organic solvent consumed per sample preparation [mL]	Average RSD (samples evaluated)
This article	DCHC	DCHC	HPLC-FLD	0.2	10	89	17	1.4% (4)[b] 2.0% (3)[c]
This article	DCHC	DCHC	HPLC-MS/MS	0.2	5	93	17	9.7% (4)[b]
[12]	Liquid partitioning	–	HPLC-FLD	2	10	87	20	3.1% (1)[b] 2.8% (1)[c]
[13]	Liquid partitioning	–	HPLC-FLD	5	3	86	140	5.7% (2)[c]
[18][a]	Liquid partioning	SPE	HPLC-MS	5	13	70	99	Not available
[17]	GPC	IAC	HPLC-FLD	4	3	85	202	3.1% (3)[c]
[17]	GPC	–	HPLC-MS/MS (internal standard: α-d4-ZOL)	4	0.3	91	196	2.3% (3)[c]

[a] Method for oilseed cakes.
[b] Naturally contaminated samples.
[c] Spiked samples.

ZON was detected and quantified in four samples. All contaminated samples were maize oils. The sample survey data are summarised in Table 2. FLD and MS/MS results were in good agreement. ZON 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 in consumer maize oils reported average contents of 170 μg/kg (total samples: 38, pos. samples: 38) [17] and 505 μg/kg (total samples: 17, pos. samples: 9) [13]. Thus, the mean ZON content found in the present study is comparatively low. Also, in contrast to the previous studies, a violation of the current EU legal limit (400 μg/kg) for ZON was not observed. This may be attributed to a higher awareness for the problem of ZON in maize oil amongst producers due to the introduction of the EU legal limit in 2005.

3.9. Specifity

The DCHC method is based on extraction by chemical reaction on a solid phase. This reaction is of general nature and compounds other than ZON will be coupled to the resin as well. However, a potential contaminant, critically interfering with the quantification of ZON, has to meet the following selectivity criteria:

- Ability to pass the resin pores
- Presence of a carbonyl group
- Sufficient reactivity of the carbonyl group during coupling
- Sufficient reactivity of the hydrazone during decoupling
- Same RP-HPLC retention window as ZON
- Fluorescence properties similar to those of ZON

Carbonyl groups, which are conjugated to heteroatoms (as in lactones, carbonic acid esters and amides, etc.) show a reduced reactivity towards phenylhydrazines [44–47] and are thus of no major concern under the mild reaction conditions employed here. Consequently, a distinctive cleanup effect is achieved by DCHC (Fig. 9).

3.10. Comparison to pre-existing methods

To our knowledge, there is no reference method for the determination of ZON in edible oils. However, the DCHC method was compared to the most recently published liquid-partitioning method [12]. For this purpose, the ZON contents in the positive maize oil samples were determined by both methods, including the determination of RSDs and recoveries. For qualitative comparison of the resulting chromatograms, GPC was performed as well. The results are summarised in Fig. 9 and Table 2, while Table 4 gives a general overview of previous methods, including the performance characteristics given in the respective publications.

Upon applying liquid partitioning to the four ZON positive maize oil samples we experienced a series of problems which were highly sample dependant. 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 [12]). Secondly, the RSDs were unacceptable for two samples and thirdly, recoveries were outside the 70–120% range in three cases (Table 2). For the blank matrix spiked to 30 μg/kg, no ZON was recovered at all (Fig. 9). 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, we believe that 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 12-fold lower organic solvent consumption and avoids the GPC system. Also, as GPC does not provide cleanup (Fig. 9), it has to be used in combination with IAC (€5–20 per single use column) or MS/MS and isotope standards. By choosing the specific 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. Also, the use of small, disposable reaction vessels minimizes the need for laboratory glassware and allows a lab 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 4). This good repeatability is attributed to the summary of extraction and cleanup to one straightforward procedure. As the complete DCHC sample preparation is carried out in a 2 mL Eppendorf-tube, uncertainties due to handling, solvent transfer etc. are minimized. When post DCHC detection was done with MS/MS instead of FLD, the RSDs increased significantly (Table 2). Kappenstein et al. [17] achieved lower MS/MS RSDs by employing a deuterated internal standard (Table 4), however, due to its high specifity, DCHC allows the application of the reliable and widely available FL detector, avoiding MS/MS and expensive isotope standards.

3.11. Conclusion

It was shown that DCHC is well suited as a sample preparation technique for purposes of quantitative instrumental analysis. As far as ZON in edible oils is concerned, DCHC can be a valuable alternative to the extraction by liquid partitioning or GPC because it minimizes the need for laboratory equipment, being highly specific and accurate at the same time. For these reasons, DCHC is considered to be well suited for the monitoring of the current EU legal limit for ZON in refined maize oil as well as for the cost-efficient analysis of other oils or fatty matrices.

Acknowledgements

The authors would like to thank Sebastian Hein, Paul Kuhlich and Stefan Merkel (all BAM, Berlin) for valuable discussion and S. Blechschmidt (BAM, Berlin) for supervising a patent submission related to the DCHC method.

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