Determination of Acrolein and Other Process Contaminants in Beer, Wine, and Potato Chip Matrices by Liquid Chromatography-Single Quadrupole Mass Spectrometry

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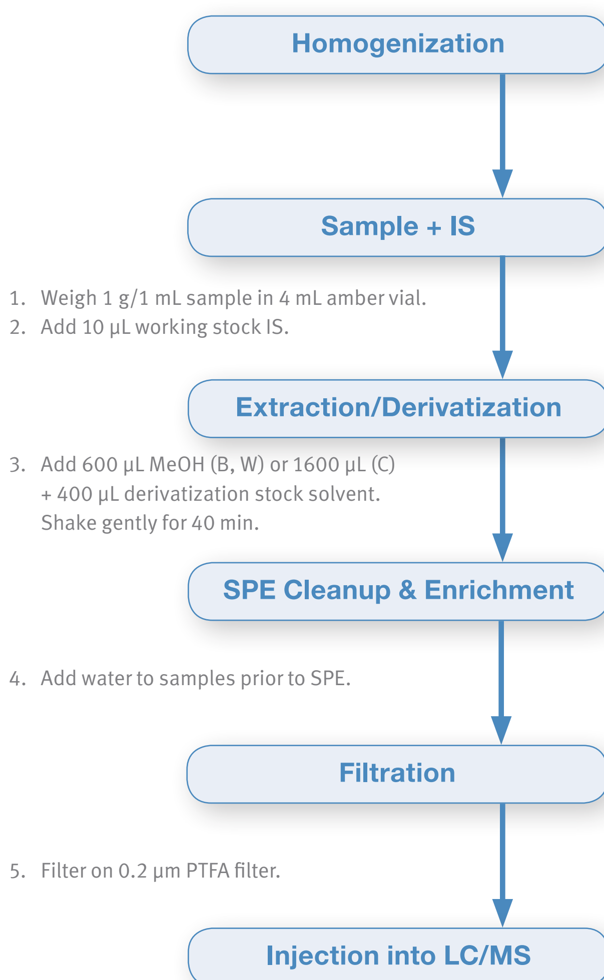
Key Words
Liquid chromatography, single quadrupole LC/MS, DNPH derivatization, reactive carbon compounds

1. Schematic of Method



2. Introduction

Carbonyl compounds are widely found in food products. They can originate from raw materials, alcoholic fermentation, or from a wide range of chemical reactions such as lipid oxidation, Maillard reactions, Strecker degradation, and aldol condensation. Acrolein is the α,β-unsaturated carbonyl compound also called prop-2-enal or acrylic aldehyde. Acrolein has a high volatility and very high reactivity.[1] To evaluate risk assessment, more information on its occurrence needs to be generated. The aim of this study was to develop an in-house validated method for the determination of a wide range of carbonyl compounds to quantify these process contaminants in beer, wine, and potato chips using LC/MS.

3. Scope

This method can be applied to screen for the presence of acrolein and its homologues and other process contaminants (5-hydroxymethylfurfural, acetoin, glyoxal, methylglyoxal, and nonenal) at levels above 0.2 mg/kg in wine, beer, and potato chip products.

4. Principle

The method involves simultaneous extraction and derivatization of the carbonyl compounds from foods to form the corresponding 2,4-dinitrophenylhydrazones. After cleanup on solid phase extraction cartridges, LC/MS analysis was performed for quantification. Non-naturally occurring hydroxyl-acetoin was used as an internal standard.

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5. Reagent List

5.1	Acetonitrile, LC/MS grade
5.2	Methanol, Fisher Chemical™ Optima™ LC/MS grade
5.3	Water, LC/MS grade
5.4	Hydrochloric acid, 37.5%
5.5	Dinitrophenylhydrazine

6. Standard List

6.1	Target compounds: acrolein (ACR), acetoin (ACET), glyoxal (GLX), methyl-glyoxal (MeGLX), 5-hydroxymethylfurfural (HMF), and 9-nonenal (NON) obtained from Sigma-Aldrich®
6.2	Internal standard: hydroxyl-acetoin (ACETOH) from Sigma-Aldrich

7. Standards & Reagent Preparation

7.1 Standard stock solutions (including IS) (1000 µg/mL)
Approximately 30.00 mg of the compound (the amount was re-calculated based on the actual purity of the standard) was weighed into a 40 mL screw cap amber vial and dissolved in 30 mL methanol. The real concentration of solution was calculated gravimetrically. Standard solutions were kept in a refrigerator and in the dark. Long-term exposure (0.5 hour or more) to room temperature or daylight during preparation of working standards was avoided.

7.2 Working standard solution (143 µg/mL)
The same volume of each individual stock standard solution (also from internal standard) was transferred into an amber vial. Working standard solutions were prepared fresh every time before using. All necessary dilutions were performed from this solvent.

7.3 Stock derivatization solvent (DNPH) (4000 mg/L)
600 mg of dinitrophenylhydrazine was weighed in a 250 mL bottle and 75 mL methanol (MeOH) and 37.5 mL water (H_2O) were added. The solution was shaken thoroughly before and while carefully adding 37.5 mL concentrated HCl in the fume hood. A subsequent volume of this solution was filtered on a 0.2 um PTFA filter into a smaller volume bottle and was used for derivatization purposes.

Note: Pellets and precipitates can be observed in this solution after a while. In this case, repeated filtration was applied.

8. Apparatus

8.1	Sartorius® analytical balance *(Sartorius GmbH, Germany)*
8.2	Thermo Scientific™ Barnstead™ Easypure II water
8.3	Horizontal shaker
8.4	Universal top frame for shaker
8.5	BRAND™ accu-jet® pipettor controller *(BRAND GmBh + Co. KG, Germany)*
8.6	pH meter
8.7	SPE vacuum manifold
8.8	Mortar
8.9	Thermo Scientific Heraeus Fresco™ 17 micro centrifuge
8.10	Thermo Scientific Accela™ UHPLC system
8.11	Thermo Scientific MSQ™ mass spectrometer

9. Consumables

9.1	LC vials
9.2	Thermo Scientific Finnpipette™ 100–1000 µL pipette
9.3	Finnpipette 10–100 µL pipette
9.4	Finnpipette 500–5000 µL pipette
9.5	Pipette holder
9.6	Pipette tips 0.5–250 µL, 500/box
9.7	Pipette tips 1–5 mL, 75/box
9.8	Pipette tips 100–1000 µL, 200/box
9.9	Spatula, 18/10 steel
9.10	Spatula, nylon
9.11	Tube holder
9.12	Wash bottle, PTFE
9.13	2 mL vial rack
9.14	15 mL centrifuge plastic tube
9.15	Syringe 1 mL
9.16	Syringe filter 0.2 µm
9.17	Thermo Scientific Accucore™ RP-MS 2.6 µm , 100 x 2.1 mm HPLC column
9.18	Thermo Scientific HyperSep™ C18 SPE cartridges, 3 mL, 200 mg
9.19	Thermo Scientific Uniguard™ holder
9.20	Thermo Scientific Hypersil™ GOLD 10 x 4 mm, 3 µm guard column

10. Glassware

10.1	1 mL glass pipette
10.2	1 L bottle
10.3	500 mL bottle
10.4	30 mL amber screw cap vials
10.5	Caps for vial
10.6	4 mL amber screw cap vials
10.7	Caps for vial

11. Procedure

11.1 Sample Preparation

Liquid samples (beer and wine)

A liquid sample of 1 mL was placed into a 4 mL amber vial and its weight was noted. Then, 600 µL MeOH, 100 µL IS, and 400 µL DNPH solutions were added. After shaking on a horizontal shaker at approximately 250 rpm for 30 min, 570 µL H_2O was added prior to application of solution onto the SPE cartridge.

Solid samples (chips)

Potato chip samples were manually homogenized in a mortar and 1g of the homogenate was placed in a 15 mL centrifuge tube. After recording the exact weight of the sample, 1600 µL MeOH, 100 µL IS, and 400 µL DNPH solutions were added and the tube was shaken on the horizontal shaker at approximately 250 rpm for 30 min. After finishing derivatization, the supernatant was decanted and 3200 µL H_2O was added prior to application of solution onto the SPE cartridge.

11.2 Sample Clean-Up, Enrichment (Solid Phase Extraction)

Solid phase extraction occurred on a Thermo Scientific HyperSep C18 SPE cartridges, 3 mL, 200 mg, as follows:

- Cartridge was conditioned with 2 mL MeOH.
- Cartridge was equilibrated with 2 mL 30% MeOH/H_2O.
- Sample was loaded.
- Cartridge was washed with 1 mL 50% MeOH/H_2O.
- Analytes were eluted with 2 mL MeOH. This fraction was used for further analysis.
- 1 mL eluate was filtered through a 0.2 µL PTFE syringe filter into a standard 2 mL HPLC vial and injected in the LC-MSQ™ instrument.

Note: Precipitation of the derivatization agent still can occur in the LC vial after 24 hrs. For best practice and analysis, check the samples waiting for injection frequently (approximately every 8 hrs). To avoid unwanted precipitation, keep the autosampler temperature at 40 °C or filter unused samples again.

12. Analysis

12.1 LC Conditions

The LC condition were as follows:

LC column:	Accucore RP-MS 2.6 µm, 100 x 2.1 mm
Mobile phase A:	MeOH
Mobile phase B:	H_2O
Column oven temperature:	40 °C
Total measurement time:	7 min
Gradient:	Table 1

Table 1. Gradient program

Time [min]	A%	B%	Flow Rate [µL/min]
0.0	45	55	400
1.0	45	55	400
3.6	86	14	400
4.0	100	0	400
5.9	100	0	400
6.0	45	55	400
7.0	45	55	400

12.1.1 Injector settings

The injector settings were as follows:

Injector:	Accela autosampler
Sample holder temperature:	30 °C
Cleaning solvents:	Acetonitrile
Injection loop volume:	25 µL
Pre-clean solvent volume:	100 µL
Pre-clean solvent:	2 steps
Filling speed:	50 µL/s
Post-clean solvent volume:	100µL
Post-clean solvent:	1 steps
Injection volume:	2 µL

12.2 Mass Spectrometric Conditions

Mass spectrometric detection was carried out by the MSQ single quadrupole mass spectrometer in selected ion monitoring (SIM) mode with atmospheric pressure chemical ionization (APCI). All compounds were individually tuned for optimal cone voltage.

The MS conditions were as follows:

Ionization method:	APCI
Polarity:	Negative
Scan type:	Full scan 150–350 *m/z*
Scan time:	0.2 s
Probe temperature:	350 °C
Needle voltage:	3.2 kV
Time range:	1–7 min
Cone voltage:	15 V

13. Calculation of Results

Calibration by the internal standardization is applied for the determination of process contaminants. This quantification method requires determination of response factors R_f defined by the equation below. The calculation of the final result is performed by using the following equations.

Calculation of the response factor:

$$R_f = \frac{A_{St} \times C_{[IS]}}{A_{[IS]} \times C_{St}}$$

R_f – response factor

A_{St} – area of the target compound peak in the calibration standard

$A_{[IS]}$ – area of the internal standard peak of the calibration standard

c_{St} – target compound concentration of the calibration standard solution

$c_{[IS]}$ – internal standard concentration of the calibration standard solution

Calculations for each sample the absolute amount of analyte that was extracted from the sample:

$$X_{analyte} = \frac{A_{analyte} \times X_{IS}}{A_{IS} \times R_f}$$

$X_{analyte}$ – absolute amount of analyte that was extracted from the sample

$A_{analyte}$ – area of analyte peak in the sample

$A_{[IS]}$ – area of the internal standard peak in the sample

$X_{[IS]}$ – absolute amount of internal standard added to the sample

The concentration of analyte in the sample [µg/g]:

$$C = \frac{X_{analyte}}{m}$$

m – weight of sample [g]

$X_{analyte}$ – absolute analyte amount [µg]

14. Method Performance Characteristics

The method was in-house validated according to the criteria specified in the IUPAC/AOAC guideline for single laboratory validation.[2,3] Representative chromatograms are shown in Figures 1 and 2 for standard derivated carbon compounds in solvent and a spiked beer sample, respectively. Determined validation parameters were specificity, linear range, repeatability, accuracy, limit of detection (LOD) and limit of quantification (LOQ), and method robustness as listed below. Matrix samples purchased in local stores were used for establishing validation parameters after being checked for the presence of target compounds prior to the validation study according to 11.1. After it was concluded that a matrix sample was free of target compounds, it was able to be used as a blank matrix for spiked experiments and the determination of target compounds during method validation. Derivatization of methyl-substituted compounds (HMF and MeGLX) resulted in *cis* and *trans* isomers. Consequently, quantification of these compounds was based on the sum of the isomers.

14.1 Selectivity

Using multiple single ion monitoring (SIM) the specificity was confirmed based on the presence of fragment ions at the correct retention time corresponding to the process contaminant standards in the solvent (Table 2). Acceptance criteria for retention time (less than 2.5% RSD) was set according to Bauer *et al.*[1]

Table 2. LC/MS parameters for selected reaction monitoring of analytes

Analyte	Rt [min]	M [g/mol]	Quantifier mass *m/z*	Ion 2 mass *m/z*	Dwell time [s]	APCI Polarity
ACR	3.79	56	236	237	0.2	negative
ACET	3.38	88	268	269	0.2	negative
ACETOH (IS)	2.56	74	254	255	0.2	negative
GLX	2.30	58	238	239	0.2	negative
MeGLX	2.65 & 3.14	72	252	253	0.2	negative
HMF	3.04 & 3.61	126	306	307	0.2	negative
NON	5.32	140	320	321	0.2	negative

14.2 Linearity and Calibration Curve

The linearity of the calibration curves was assessed by internal standardization over the range 0–100 mg/kg. The calibration curves were created at five levels (matrix-matched) by spiking cleaned-up extracts prior to LC injection. All levels were prepared and injected in duplicate. Calibration levels were 0, 5, 25, 50, 75, and 100 mg/kg. In all cases, the correlation coefficients of linear functions were better than 0.985. Rf values for internal standardization were determined from the calibration curves for each matrix and internal standards by calculating cumulative average response factor over the whole calibration range.

14.3 Accuracy

Method accuracy and precision was assessed by a recovery study using blank matrices spiked at three concentration levels and injected in six individually prepared replicates. Samples were spiked at 10, 50, and 100 mg/kg concentration levels prior to processing. All recovery samples were analyzed within 6 hrs after preparation to avoid or minimize further reaction of compounds. Found concentrations and relative standard deviation (% RSD) were calculated and expressed as recovery and precision (Table 3). The expectation of the method was to meet recovery values between 70%–120%, which was met for all compounds. An additional accuracy experiment was carried out by injection of Food Analysis Performance Assessment Scheme (FAPAS®) 2823 external quality control (QC) material (n=6). However, the QC contained only HMF in honey matrix, which was the only available test material with the target compound(s) and similar matrix. The measured average values for the samples were 54 mg/kg (±8.3%), which fell in the middle of the acceptable range (48–61.2 mg/kg) and corresponded well with the assigned values (55 mg/kg).

Table 3. Mean recovery (%RSD) of method

Compound	Recovery % (%RSD)								
	Wine			Beer			Chips		
	10 mg/kg	50 mg/kg	100 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg
ACR	79 (3)	119 (9)	93 (5)	79 (7)	103 (5)	93 (9)	106 (10)	103 (7)	111 (12)
ACET	119 (9)	79 (8)	87 (10)	119 (13)	93 (11)	87 (17)	116 (6)	84 (5)	107 (15)
GLX	<LOQ	114 (7)	101 (6)	<LOQ	117 (4)	101 (15)	<LOQ	111 (8)	100 (15)
MeGLX	95 (1)	107 (5)	106 (6)	95 (4)	110 (3)	106 (9)	<LOQ	87 (6)	97 (11)
HMF	100 (7)	113 (8)	85 (11)	100 (8)	115 (4)	85 (3)	87 (6)	104 (4)	92 (10)
NON	85 (6)	112 (6)	94 (2)	85 (7)	85 (11)	94 (10)	107 (9)	96 (8)	98 (13)

14.4 Precision

Method within-day precision and between-day precision values were determined with individually prepared samples for each matrix at the middle spiking level (50 mg/kg) each in six replicates and expressed as %RSD over three days. Measured values deemed to be acceptable (below 15%) and are shown in Table 4. All repeatability samples were analyzed within 6 hrs after preparation.

Table 4. Precision and intermediate precision at 50 mg/kg concentration level

Compounds	Repeatability			Intermediate Precision		
	Wine	Beer	Chips	Wine	Beer	Chips
ACR	9	5	7	15	12	9
ACET	8	11	5	9	16	7
GLX	7	4	8	12	10	10
MeGLX	5	3	6	6	4	8
HMF	8	4	4	11	5	10
NON	6	11	8	14	15	11

14.5 Limit of Detection, Limit of Quantification

Limits of detection and quantification were estimated following the IUPAC approach, which consisted of analyzing the blank sample to establish noise levels and then testing experimentally estimated LODs and LOQs for signal-to-noise ratios, 3 and 10 respectively. Due to the lack of legislation values, the expectation of the method was to achieve limits as low as possible. The resulted LOD and LOQ values are listed in Table 5.

Table 5. Method LOD and LOQ values

Analyte	SOLV		Beer		Wine		Chips	
	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)
ACR	0.15	0.5	0.25	0.8	0.25	0.8	0.25	0.8
ACET	0.24	0.8	0.6	2	0.6	2	0.6	2
ACETOH (IS)	7.5	25	7.5	25	7.5	25	7.5	25
GLX	0.5	1.7	6	20	4.5	15	4.5	15
MeGLX	0.9	3	5	17	4.5	15	7.5	25
HMF	0.1	0.3	0.36	1.2	0.3	1	0.3	1
NON	0.1	0.3	0.18	0.6	0.18	0.6	0.18	0.6

14.6 Survey Samples

To prove method applicability for real samples, different beer, wine and chip products were purchased (n=16) in local stores and analyzed with the method. Samples covered different types of beer, red wine, and potato chip samples with different flavors (onion, salted, pepperoni, cheese, ketchup). No traces of the main target compound (ACR) were found in any of samples. However traces of NON between 0.4–0.9 mg/kg and ACET between 0.8–1.3 mg/kg were found in three out of seven beer samples, while all beer samples contained HMF in concentrations between 0.4–6.1 mg/kg. In addition, ACET was measured in concentration between 4.8–5.5 mg/kg in two out of four red wine samples.

14.7 Robustness

The following parameters became evident during the robustness study as critical for a repeatable method:

- stability of working stock standard solutions is very limited at room temperature and during daylight
- concentration and amount of added derivatization solvent (analyte-to-derivatization-agent ratio)
- derivatization reaction time
- pH of derivatization solvent

Therefore to achieve comparable results, the instructions need to be followed very carefully.

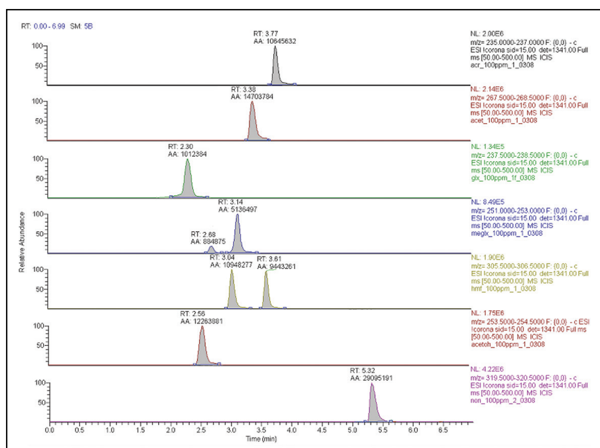

Figure 1. Chromatogram of 100 ppm standard derivated carbon compounds in solvent freshly after the derivatization reaction. (Traces from top: ACR, ACET, GLX, MeGLX, HMF, ACETOH and NON)

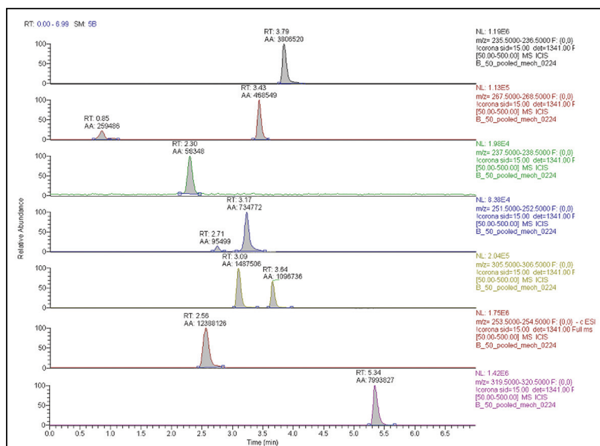

Figure 2. Chromatogram of a beer sample spiked at 50 ppm with carbon compounds and after the derivatization reaction. (Traces from top: ACR, ACET, GLX, MeGLX, HMF, ACETOH and NON)

15. Conclusion

The method presented describes determination of low molecular weight and very reactive food process contaminants (acrolein and other low molecular weight carbon components) in three different matrices by application of *in-situ* derivatization reaction and fast chromatographic determination by LC-MS instrumentation. Due to the very short half life of the target compounds, the derivatization reaction has to be carried out as fast as possible after sampling to be able to recover the maximum amount of analytes. The in-house validation of the method gave detection capability at the sub-ppm level and confirmed the reliability of the method for quantification under the described conditions: selectivity, recovery, and precision values were in accordance with the expectations of the latest method performance guidelines. Consequently, the method is applicable for determination of the target compounds in beer, wine and potato chip matrices by using LC/MS.

16. References

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