Preventing False Negatives with High Resolution Mass Spectrometry: The Benzophenone Case

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Abstract

Benzophenone (BP) is one of the many contaminants reported as present in foodstuff due to its migration from food packaging materials. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is acknowledged in the literature as the method of choice for this analysis. However, cases have been reported where the use of this methodology was not enough to unambiguously confirm the presence of a contaminant. In previous work performed by the authors, the unequivocal identification of BP in packaged foods was not possible even when monitoring two *m/z* transitions, since ion ratio errors higher than 20% were obtained. In order to overcome this analytical problem a fast, sensitive and selective liquid chromatography-high resolution-mass spectrometry (LC-HRMS) methodology has been developed and applied to the analysis of BP in packaged foods. A direct comparison between liquid chromatography high resolution mass spectrometry (LC-HRMS) and LC-MS/MS data indicated better selectivity when working with LC-HRMS at a resolving power of 50,000 FWHM than when monitoring two *m/z* transitions by LC-MS/MS. The resolving power used enabled the detection and identification of Harman as the compound impeding the confirmation of BP by LC-MS/MS. Similar quantitative results were obtained by an Orbitrap mass analyser (Exactive ™) and a triple quadrupole mass analyser (TSQ Quantum Ultra AM ™).

1. Introduction

Food matrices are complex mixtures consisting of naturally found compounds, such as carbohydrates, lipids, proteins, vitamins, phenolic compounds and organic acids. On the other hand, compounds such as pesticides, polycyclic aromatic hydrocarbons, chlorinated and brominated compounds, veterinary drugs, toxins, migrants from containers, metals and inorganic compounds may also be present and need to be monitored. Strict regulations apply for many of these compounds, expressed by maximum residues levels (MRLs) and specific migration levels (SMLs). In order to comply with these regulations, highly selective and sensitive analytical methods are required to identify, confirm and quantify the targeted compounds.

Photoinitiators are used as starters in the polymerization process to cure the ink by UV radiation. These inks are used to print packaging material such as multilayer laminates, rigid plastic, cardboard and paper. Although intermediate aluminum layers are commonly used to prevent the migration of ink components into food products, the unintentional transfer of print ink components from the outer printed surface onto the food contact surface can occur when the printed material is rolled on spools or stacked during storage. Benzophenone (BP) has a SML set at 600 μg L^{-1} [1] and is currently being analyzed by gas chromatography coupled to mass spectrometry (GC-MS)[2-5] or LC-MS/MS[2,6,7]. It has been reported the presence of BP at concentrations ranging from 2.9 ng L^{-1} to 39 ng L^{-1} in milk samples and between 5 μg L^{-1} and 217 ng L^{-1} in fruit juice samples. Nowadays, LC-MS/MS operating in the selective reaction monitoring (SRM) mode is the method of choice for food analysis due to its high sensitivity and selectivity. Such a performance helps the analyst to comply with the EU directive 2002/657/EC and to confidently report a positive or negative finding. The analytical criterion to report a

result is based mainly on the monitoring of two transitions, the deviation of the relative intensity of the recorded ions (must not exceed a certain percentage of the reference standard) and the retention time of the precursor ion (must not deviate more than 2.5%). However, the application of this criterion did not completely eradicate false positives or false negatives[8]. The occurrence of a false positive in LC-MS/MS using a triple quadrupole – QqQ - analyzer implies the presence of an interfering compound that is co-eluting with the monitored analyte. The maximum working resolution of this analyzer is sometimes not sufficient to completely resolve isobaric compounds. This problem has been discussed by several analysts and reported in the literature[8-11]. More problematic than reporting a false positive is the possibility of reporting a false negative because the presence of a possible harmful analyte would be ignored. Such cases have also been reported, for instance in the analysis of 2-hydroxy-terbutyazine in ground water[8]. Ion-ratio errors higher than 20% were obtained by the authors in the analysis of BP by liquid chromatography-tandem mass spectrometry, which prevented the confirmation of this compound in food samples[12]. A possible solution for this analytical problem is the monitoring of more than two transitions or the use of high resolution mass spectrometry (HRMS). Since the product ion scan of BP only shows two ions (*m/z* 77 and *m/z* 105) a LC-HRMS methodology, using an Orbitrap analyzer has been developed as an attempt to increase the selectivity of the analytical method. A comparison between LC-MS/MS and LC-HRMS results has also been performed.

2. Experimental

2.1. Materials and chemicals

Benzophenone (99%, CAS No. 119-61-9) was purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (98-100%) was provided by Merck (Darmstadt, Germany). Anhydrous magnesium sulfate was obtained from Sigma-Aldrich (Steinheim, Germany), sodium chloride from Fluka (Steinheim, Sweden), and propylamino (PSA) bonded silica SPE bulk from Supelco (Gland, Switzerland). LC-MS grade methanol (MeOH), acetonitrile (ACN) and water were purchased from Riedel-de-Haën (Seelze, Germany).

Stock standard solution of BP (1,000 mg kg^{-1}) was prepared by weight in methanol and stored at 4ºC. Working standard solutions were prepared weekly by appropriate dilution in acetonitrile:water (1:1) of the stock standard solution. Mobile phases were filtered using 0.22 μm nylon membrane filters (Whatman, Clifton, NJ, US) and sample extracts were filtered through 0.22 μm pore size Ultrafree-MC centrifuge filters (Millipore, Bedford, US).

Nitrogen (99.98% pure) supplied by Claind Nitrogen Generator N_2 FLO (Lenno, Italy) was used for the API source; and high-purity Argon (Ar1), purchased from Air Liquide (Madrid, Spain), was used as a collision-induced gas (CID gas) in the triple quadrupole instrument.

2.2. Instrumentation

An ultra high performance liquid chromatography (UHPLC) system (Accela; Thermo Fisher Scientific, San José, CA, US) was used for the separation chromatography. The chromatographic separation was performed in a pentafluorophenyl propyl column, Kinetex PFPP (50 mm x 2.1 mm i.d., 2.6 µm particle size), from Phenomenex (Bellefonte, PA, US), using a gradient elution of methanol (solvent A) and 25 mM formic acid-ammonium formate buffer at pH 2.7 (solvent B): 60% solvent A for 0.8 min followed by a linear gradient up to 75% solvent A in 0.45 min, an isocratic step for 2 minutes at this latter percentage. The flow-rate was 500 µL min^{-1} and the column temperature was held at 25^{o}C.

LC-MS/MS (triple quadrupole mass analyzer)

The Accela UHPLC system was coupled to a triple quadrupole mass spectrometer TSQ Quantum Ultra AM (Thermo Fisher Scientific), equipped with a heated-electrospray ionization (HESI-I). Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow-rates of 60, 2 and 40 a.u. (arbitrary units), respectively. The ion transfer tube temperature was set at 375^{o}C and electrospray voltage at +4 kV. Selected reaction monitoring (SRM) acquisition mode was used operating both quadrupoles (Q1 and Q3) at 0.7 *m/z* FWHM and a scan width of 0.01 *m/z*. Argon was used as collision gas at 1.5 mTorr and the optimum collision energy (CE) for each transition monitored 34 eV, *m/z* 183 → 105 (quantitation) and *m/z* 183 → 77 (confirmation). The Xcalibur software version 2.0 (Thermo Fisher Scientific, San Jose, CA, US) was used to control the LC-MS system and to process data.

LC-HRMS (Orbitrap mass analyzer)

The Accela UHPLC system was also coupled to a single-stage Orbitrap instrument (Exactive; Thermo Fisher Scientific, Bremen, Germany) equipped with a HCD collision cell and a heated-electrospray ionization probe (HESI-II). Nitrogen (purity > 99.98%) was used as a sheath gas, ion sweep gas and auxiliary gas at flow-rates of 60, 2 and 40 a.u. (arbitrary units), respectively. The ion transfer tube temperature was set at 375^{o}C and electrospray voltage at +4 kV. The Exactive mass spectrometer was operated in positive ion mode, alternating full scan MS (*m/z* 50 – 1000) and "all ion fragmentation" (AIF) MS/MS scan (*m/z* 50 – 1000) using higher energy collision dissociation (HCD) at 22 eV. The system was operated at different resolving power settings of 10,000; 25,000 and 50,000 (*m/z* 200) at full width half maximum (FWHM) on both full scan and AIF scan modes. Full instrument calibration was performed using a MSCAL5 ProteoMassT LTQ/FT-Hybrid ESI Pos (Sigma-Aldrich). The external mass axis calibration without the use of the specific lock masses was employed. For the accurate mass measurements, mass at the average of the chromatographic peak was obtained. The Xcalibur software version 2.1 (Thermo Fisher Scientific, San Jose, CA, US) was used to control the LC/MS system and to process data. The online database Chemspider was also used.

To optimize both the ESI source and mass spectrometry working conditions, 1 mg L^{-1} stock standard methanol solution was infused at a flow-rate of 3 µL min^{-1} using the syringe pump and mixed with the mobile phase (500 µL min^{-1}, methanol:formic acid-ammonium formate buffer (70:30, *v/v*)), by means of a Valco zero dead volume tee piece (Supelco).

176 *2.3. Sample treatment*

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178 For the sample analysis a QuEChERS method developed in our laboratory for
179 the analysis of photoinitiators in packaged food was used[12]. 2.5 g of sample and 5 µL of
180 2-ITX-D_7 used as a surrogate (100 µg kg^{-1}) were extracted using acetonitrile. Then the
181 mixture was shaken for 1 min using a vortex (Stuart, Stone, UK). Then, 1.5 g of NaCl
182 and 4 g of $MgSO_4$ were added to the extract and shaken again. The extract was then
183 centrifuged (2,500 rpm) and 10 mL of the supernatant were clean-up using 250 mg of
184 PSA (propylamine bonded silica SPE bulk) and 750 mg of $MgSO_4$. The mixture was
185 energetically shaken and centrifuged again at 3,700 rpm for 1 min.. Finally, 8 mL of the
186 supernatant were evaporated to dryness under a nitrogen stream and reconstituted in 500
187 µL acetonitrile:water (1:1, *v/v*). Prior to analysis, the extract was filtered through 0.22
188 µm-pore Ultrafree-MC centrifugal filters and transferred into an amber vial to prevent
189 analyte photodegradation. Finally, 5 µL of this extract were injected into the LC-HRMS
190 and LC-MS/MS system.

191 A total of 28 packaged food samples, including baby food, fruit juices, milk and
192 soy milk, *sangria* and three blank samples (a pineapple juice sample and a milk sample
193 packaged in a plastic bottle, and a baby food sample in a glass bottle) obtained from
194 local supermarkets during July 2010 (Barcelona, Spain), were analyzed. Matrix matched
195 calibration curves for different matrices were prepared and used as quantification
196 method.

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3. Results and discussion

Liquid chromatography tandem mass spectrometry has been applied previously by the authors to the analysis of photoinitioators, BP included, in different food matrices[12]. Although, there was a strong indication of the presence of BP in the analysed samples, in some of the cases, this fact could not be completely confirmed. In this work, benzophenone was analyzed in twenty eight packaged food samples using a triple quadrupole, following the Directive 2002/657/EC in which two transitions (*m/z* precursor ion - *m/z* product ion) were monitored. The results obtained show an ion ratio error higher than 20% for half of the analyzed samples indicating the presence of BP (Table 1). These samples could not be confirmed due to this deviation. To overcome this problem, the monitoring of a third transition is recommended. However, this strategy could not be followed in this case because the fragmentation pattern of BP only reveals two product ions, *m/z* 105 and 77. Since the occurrence of false negatives is normally attributed to the presence of interfering compounds co-eluting with the analyte of interest, it was decided to investigate this analytical problem using high resolution mass spectrometry (HRMS) with an Orbitrap analyzer. Firstly, as an attempt to obtain good mass accuracies for the analysis of BP, different mass resolving powers (10,000 FWHM, 25,000 FWHM and 50,000 FWHM) were tested. For this purpose some of the unconfirmed samples analyzed by QqQ were injected at three mass resolving powers. Figure 1 illustrates the results obtained when analyzing sample baby food 1.

When using a mass resolving power of 10,000 or 25,000 FWHM, mass errors higher than 16 ppm were obtained for BP - elemental composition ($C_{13}H_{11}O$) - not allowing the confirmation of this compound in the analyzed samples. However, when a mass resolving power of 50,000 FWHM was used, BP was detected with a mass error of 1.1

ppm. In addition, another compound with an assigned elemental composition of $C_{12}H_{11}N_2$ was detected with a mass error of 0.5 ppm.

The unknown compound (identified as $C_{12}H_{11}N_2$) was detected in all the analyzed samples with good mass accuracy, by means of a mass error below 3 ppm, with the exception of soy-milk 2, (Table 2). In order to identify this interfering substance, an online database search was performed using a database provided by the Royal Society of Chemistry - Chemspider. Several possible chemical structures were originated as possible matches, but only the ones providing a cation in liquid phase under positive electrospray ionization conditions were considered. The remain structures listed as possible matches, included 1-methyl-9*H*-pyrido[3,4-*b*]indole, also known as Harman. This compound is recognized as being present in foodstuff at concentrations ranging from 1 ng g^{-1} to 200 ng g^{-1} [13-16]. It is also acknowledged that β-carbolines are cyclization/oxidation products of the amino acid tryptophan[17], which explains its presence in foodstuff, more precisely in milk products, baby foods and juices.

To confirm the identity of Harman a standard solution of 0.6 mg/L was injected into the LC-QqQ-MS system in full scan mode using the same chromatographic method. Harman was found to elute around 1.2 minutes, the same retention time as BP. In addition, the product ion scan experiment showed an ion *m/z* 77 (Figure 2) at low relative abundance, confirming the suspicion that the concentration of Harman found in the analyzed samples is sufficient to interfere with the confirmatory transition of BP *m/z* 183 → 77. This concentration is estimated to range between 1- 10 µg/Kg, which indicates similar or higher concentration levels when comparing to the levels of BP detected in all samples. This fact may be an important contribution to the variability found when reporting ion ratio ratio values. It was observed that when the relative

abundance (%) of BP is below 50 % in relation to Harman (100%) the ion ratio calculation will fall outside the desired range (Figure 3). Furthermore by analyzing the product ion scan of BP, the phenyl cation *m/z* 77 represents less than 30% of the relative abundance. Nevertheless, a strict 20% window was selected in order to obtain good confirmatory results.

In a way to develop the LC-HRMS method, quality parameters such as limit of detection (LOD), limit of quantification (LOQ), run-to-run precision and linearity were estimated at a mass resolving power of 50,000 FWHM. LOD (7.5 pg injected) and LOQ (25 pg injected), based on a signal-to-noise ratio of 3 and 10 respectively, were estimated by the injection of 5 μL of the BP standard solution prepared at 10 μg L^{-1}. Calibration curve based on the peak area showed good linearity in the range studied with a coefficient of determination (r^2) > 0.995. Run-to-run precision was determined at 250 μg L^{-1} (n=5) obtaining a relative standard deviation lower than 7%.

To explore the feasibility of the method three blank samples, including fruit juice and baby food from a glass container and milk from plastic container, were spiked at different concentration levels and submitted to the sample treatment described in the experimental section. This method provided limits of detection (MLODs) of 0.6 μg kg^{-1} in fruit juice and baby food and of 1.3 μg kg^{-1} in milk. Furthermore good accurate mass measurements (< 5 ppm) were obtained for all the matrices studied. To evaluate run-to-run precision, six replicates of the three spiked samples (100 μg kg^{-1}) were analyzed by the developed method obtaining a relative standard deviation based on concentration lower than 10%. Finally, good linearity (r^2 > 0.994) was obtained for calibration curves prepared in the three matrices evaluated ranging from 1.0 μg kg^{-1} to 500 μg kg^{-1}.

In order to confirm the presence of BP, the 28 food samples were analyzed using LC-HRMS operating simultaneously in full scan and all ions fragmentation (AIF) mode at a resolving power of 50,000 FWHM (table 2).

Benzophenone was detected in 20 of the 28 food samples at concentrations ranging from 0.7 μg kg^{-1} to 5.2 μg kg^{-1} in fruit juice samples, from 1.3 μg kg^{-1} to 4.5 μg kg^{-1} in milk based products, and from 0.6 μg kg^{-1} to 8.9 μg kg^{-1} in baby food. A statistical paired-sample comparison analysis between LC-HRMS and LC-QqQ-MS/MS quantification data was performed. For a 95% confidence level, a *p*-value of 0.33 was obtained, which indicates that the results were not significantly different. Furthermore, by making use of HRMS capabilities, the unequivocal identification of BP in the analyzed samples was possible since both precursor and quantifier product ions were detected at a resolution of 50,000 FWHM translated into a good mass accuracy, (error ≤ 5 ppm) with the exception of fruit milk 2, where the concentration of BP was found to be close to the MLOD. The concentration of BP in samples soy-milk 2, baby food 2 and 4 were below the MLOD.

3. Conclusions

A fast and sensitive LC-HRMS method has been evaluated in order to avoid the confirmatory problems experienced in the analysis of BP by LC-MS/MS in SRM mode. The unequivocal identification of BP was achieved by making use of an Orbitrap mass analyzer operating at mass resolving power of 50,000 FWHM. Moreover, the presence of BP in the analyzed samples was confirmed by operating simultaneously in AIF mode and full scan HRMS. The combination of high resolution and AIF mode helped overcome confirmatory problems experienced when using low resolution mass

spectrometry, as it provides good accurate masses measurements for both precursor and product ions of BP.

BP was detected in several packaged food samples at concentrations ranging from 0.6 μg kg^{-1} to 8.9 μg kg^{-1}. Harman has also been detected and identified as interference on the analysis of benzophenone by low resolution tandem mass spectrometry. In addition, no significant differences were obtained quantitatively when comparing both analyzers, confirming that the presence of Harman only affected the confirmatory transition of BP.

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Figure captions

Figure 1. Baby food 1 sample analyzed by LC-HRMS at three different mass resolving power A) 10,000 FWHM, B) 25,000 FWHM and C) 50,000 FWHM

Figure 2. A) LC-MS, B) LC-MS/MS and C) MS/MS spectrum at 22eV of a 600 µg/L Harman standard solution.

Figure 3. LC-HRMS chromatogram and spectra acquired at a mass resolving power of 50,000 FWHM of sample A) Soy-milk 4, B) Pineapple Juice 2 and C) Baby Food 3.

Table 1: Concentration of benzophenone (BP) found in 28 packaged food samples analyzed by LC-(LR)-MS/MS. Experimental ion ratio obtained between areas of quantifier and qualifier ions.

Sample	BP ($\mu g\ kg^{-1}$)	Ion ratio*
Pineapple juice 1	2.8	1.29 (confirmed)
Pineapple juice 2	1.1	0.94 (not confirmed)
Pineapple juice 3	n.d.	-
Orange juice 1	2.3	1.22 (confirmed)
Orange juice 2	n.d.	-
Orange juice 3	3.2	1.03 (not confirmed)
Peach juice 1	4.1	1.33 (confirmed)
Peach juice 2	n.d.	-
Sangria	3.4	1.19 (confirmed)
Fruit-milk 1	3.6	1.70 (not confirmed)
Fruit-milk 2	3.1	1.54 (confirmed)
Fruit-milk 3	3.9	1.58 (not confirmed)
Fruit-milk 4	3.8	1.36 (confirmed)
Fruit-milk 5	4.7	1.61 (not confirmed)
Milk 1	3.1	1.08 (confirmed)
Milk 2	4.8	1.93 (not confirmed)
Milk 3	3.2	2.71 (not confirmed)
Milk 4	3.0	0.88 (not confirmed)
Soy-milk 1	n.d.	-
Soy-milk 2	1.4	1.06 (confirmed)
Soy-milk 3	n.d.	-
Soy-milk 4	4.0	1.67 (not confirmed)
Baby food 1	8.8	1.52 (confirmed)
Baby food 2	~LOD	1.44 (confirmed)
Baby food 3	4.2	1.40 (confirmed)
Baby food 4	~LOD	1.00 (not confirmed)
Baby food 5	2.9	1.20 (confirmed)
Baby food 6	3.3	0.92 (not confirmed)

*Ion ratio confirmation range : 1.04-1.56

Table 2: Concentration of benzophenone (BP) found in 28 packaged food samples analyzed by LC-(HR)-MS/MS. Accurate mass measurements of BP, main product ion and the unknown identified as $C_{12}H_{11}N_2$.

Sample	Calculated amount (µg kg^{-1})	Accurate mass precursor ion (error, ppm)	Accurate mass precursor ion (error, ppm)	Accurate mass interference (error, ppm)
Pineapple juice 1	2.1	183.0807 (1.6)	105.0338 (2.9)	183.0915 (-1.1)
Pineapple juice 2	~LOD	183.0813 (5.0)	105.0334 (-1.0)	183.0914 (-1.6)
Pineapple juice 3	n.d.	-	-	183.0920 (1.6)
Orange juice 1	3.4	183.0810 (3.3)	105.0335 (0.1)	183.0916 (-0.5)
Orange juice 2	n.d.	-	-	183.0915 (-1.1)
Orange juice 3	5.2	183.0805 (0.5)	105.0336 (1.0)	183.0916 (-0.5)
Peach juice 1	3.5	183.0808 (.22)	105.0338 (2.9)	183.0916 (-0.5)
Peach juice 2	n.d.	-	-	183.0920 (1.6)
Sangria	2.48	183.0813 (4.9)	105.0338 (2.9)	183.0916 (-0.5)
Fruit-milk 1	~LOD	183.0813 (4.9)	105.0337 (1.9)	183.0917 (0.2)
Fruit-milk 2	~LOD	183.0815 (5.0)	105.0342 (4.8)	183.0922 (2.7)
Fruit-milk 3	3.5	183.0810 (3.3)	105.0336 (1.0)	183.0914 (-1.6)
Fruit-milk 4	3.0	183.0812 (4.4)	105.0339 (3.8)	183.0919 (1.1)
Fruit-milk 5	4.1	183.0810 (3.3)	105.0337 (1.9)	183.0915 (-1.1)
Milk 1	3.6	183.0809 (2.7)	105.0335 (0.1)	183.0916 (-0.5)
Milk 2	4.5	183.0808 (2.2)	105.0338 (2.9)	183.0914 (-1.6)
Milk 3	~LOD	183.0814 (4.9)	105.0331 (-3.8)	183.0917 (0.2)
Milk 4	2.9	183.0812 (4.4)	105.0339 (3.8)	183.0918 (0.5)
Soy-milk 1	n.d.	-	-	183.0914 (-1.6)
Soy-milk 2	n.d.	-	-	n.d.
Soy-milk 3	n.d.	-	-	183.0920 (1.6)
Soy-milk 4	3.4	183.0813 (4.9)	105.0332 (-2.9)	183.0916 (-0.5)
Baby food 1	8.9	183.0806 (1.1)	105.0337 (1.9)	183.0916 (-0.5)
Baby food 2	n.d.	-	-	183.0918 (0.5)
Baby food 3	2.3	183.0812 (4.4)	105.0336 (1.0)	183.0914 (-1.6)
Baby food 4	n.d.	-	-	183.0922 (2.7)
Baby food 5	3.4	183.0808 (2.2)	105.0335 (0.1)	183.0915 (-1.1)
Baby food 6	2.3	183.0811 (3.8)	105.0340 (4.8	183.0920 (1.6)

n.d. Not detected

Figure 1

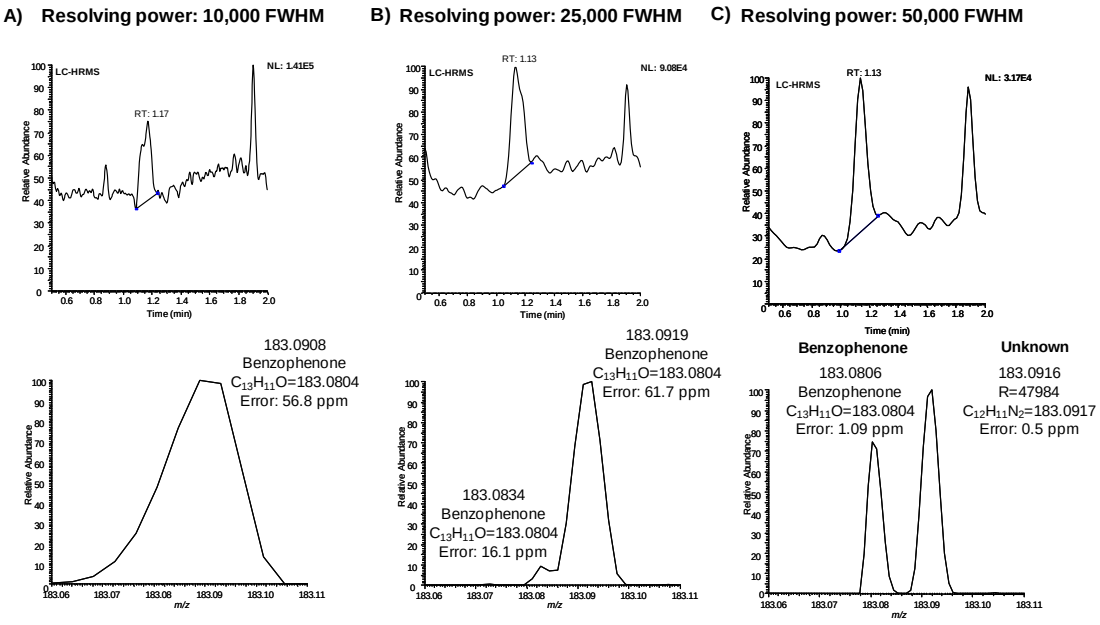
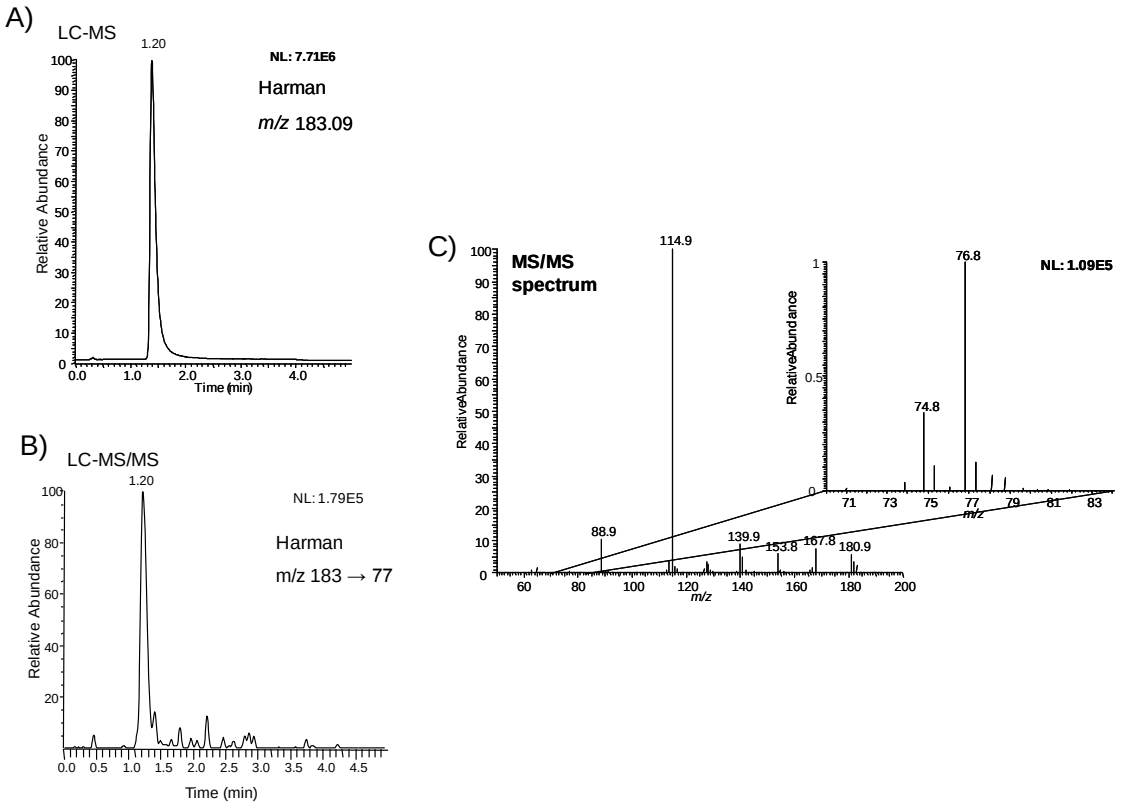
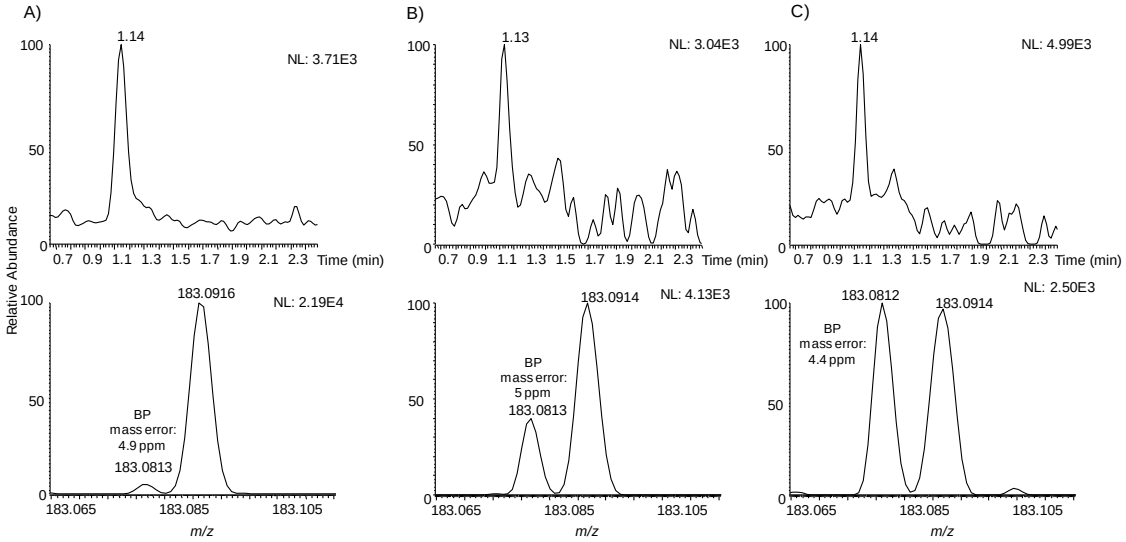

A) Resolving power: 10,000 FWHM
B) Resolving power: 25,000 FWHM
C) Resolving power: 50,000 FWHM
LC-HRMS
NL: 1.41E5
RT: 1.17
NL: 9.08E4
RT: 1.13
NL: 3.17E4
Relative Abundance
Time (min)
m/z
183.0908
Benzophenone
C13H11O=183.0804
Error: 56.8 ppm
183.0919
Benzophenone
C13H11O=183.0804
Error: 61.7 ppm
183.0834
Benzophenone
C13H11O=183.0804
Error: 16.1 ppm
Benzophenone
183.0806
Benzophenone
C13H11O=183.0804
Error: 1.09 ppm
Unknown
183.0916
R=47984
C12H11N2=183.0917
Error: 0.5 ppm

Figure 2


A)
LC-MS
1.20
NL: 7.71E6
Harman
m/z 183.09
Relative Abundance
Time (min)
B)
LC-MS/MS
1.20
NL: 1.79E5
Harman
m/z 183 → 77
Relative Abundance
Time (min)
C)
MS/MS spectrum
NL: 1.09E5
114.9
76.8
74.8
88.9
139.9
153.8
167.8
180.9
Relative Abundance
m/z

473 Figure 3

474



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