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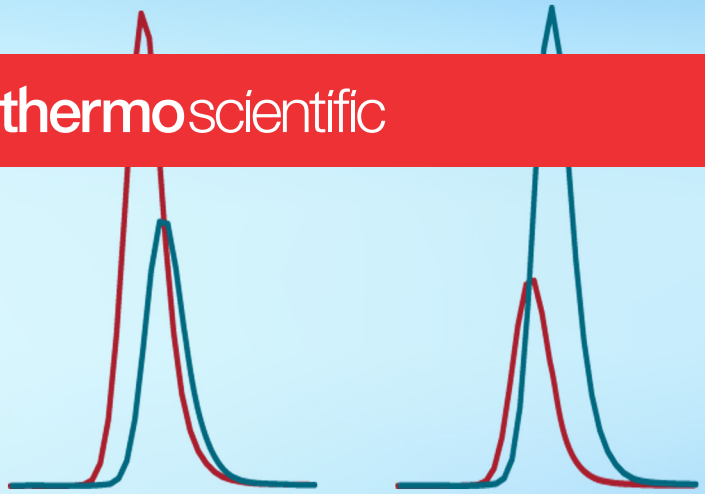

TECHNICAL NOTE 73152

Selective analysis of co-eluting isobaric compounds with single quadrupole mass detection

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Keywords

Single quadrupole mass spectrometer, ISQ EC, ISQ EM, Vanquish Flex Binary UHPLC, in-source fragmentation, desmedipham, phenmedipham

Goal

Demonstrate additional selectivity gained through in-source CID fragmentation for discriminating co-eluting, isobaric analytes.

Introduction

For confident analysis, unambiguous detection of sample compounds is required. In liquid chromatography, this is usually achieved by developing a method that sufficiently resolves the target compounds and identifies them based on their respective retention times. As a result, the detection itself (usually UV-based) does not need to be selective. However, if the analytes cannot be sufficiently resolved by chromatography, unambiguous detection can still be achieved by using selective mass spectrometric detection. It can measure the molecular mass, isotopic pattern, and fragmentation pattern of the analytes. This usually provides enough information to confirm the identity of small molecules.

Despite this advantage, mass spectrometry is not commonly used as a detector in routine chromatography as it is perceived to be too complex and difficult. The Thermo Scientific™ ISQ™ EC and EM single quadrupole mass spectrometers, however, are developed for operation by chromatographers. Their full integration into the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2 and the patented Thermo Scientific™ Autospray smart method set-up make LC-MS operation and data analysis straightforward and intuitive.

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The ISQ EC/EM mass detectors enable:

- Operation in Full Scan and Single Ion Monitoring (SIM) mode, to either scan a mass range for detectable analytes or to select for specific compounds.
- Adjustable source CID voltage for analyte declustering and adduct removal at low voltages, or analyte fragmentation at higher voltages.
- Scan rates suitable for fast UHPLC applications while delivering picogram detection limits.
- High levels of instrument robustness, even with challenging matrices, with a new orthogonal source design.

In the following application example, two isobaric, co-eluting analytes, desmedipham and phenmedipham, were selectively detected based on unique fragments generated by in-source CID fragmentation.

Experimental

Stock solutions (10 µg/µL in acetonitrile) of desmedipham and phenmedipham (Table 1) were prepared and stored at 4 °C. Unless otherwise stated, 100 ng/µL samples were used for analysis.

Chromatographic separation was performed using LC-MS grade solvents (Table 2) on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system (Table 3). For initial UV-based compound analysis and final method verification a 100 mm column was used; for method development a 50 mm column was used (Table 4). A 75 cm long MP35N capillary with 100 µm inner diameter (P/N 6042.2390) was used for connecting the UHPLC system to the ISQ EC mass detector.

The ISQ EC/EM mass detector is fully integrated into Chromeleon software 7.2, which was used for system operation and subsequent data analysis.

Table 1. Overview of analytes

Analyte	CAS	Chemical formula	Molecular weight	Monoisotopic mass [M]	Detected *m/z* $[M+H]^+$
Desmedipham	13684-56-5	$C_{16}H_{16}N_2O_4$	300.31	300.11	301.1
Phenmedipham	13684-63-4	$C_{16}H_{16}N_2O_4$	300.31	300.11	301.1

Table 2. Solvents and additives

Reagent	Grade	Supplier	Part number
Acetonitrile	Optima™ LC-MS	Fisher Chemical	A955-212
Formic Acid	Optima™ LC-MS	Fisher Chemical	A117-50
Methanol	Optima™ LC-MS	Fisher Chemical	A456-212
Water	Ultra-Pure, 18.2 MΩ at 25 °C	Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	

Table 3. Vanquish Flex Binary UHPLC system modules

Module	Part number
Vanquish System Base Horizon/Flex	VF-S01-A
Vanquish Binary Pump F (with 35 µL mixer set)	VF-P10-A (6044.3870)
Vanquish Split Sampler FT	VF-A10-A
Vanquish Column Compartment H	VH-C10-A
Vanquish Variable Wavelength Detector F (2.5 µL SST flow cell)	VF-D40-A (6074.0360)

Table 4. Thermo Scientific™ columns used for chromatographic separation

Stationary phase	Particle size	Dimensions	Part number
Hypersil GOLD™	1.9 µm	2.1 × 100 mm	25002-102130
Hypersil GOLD™	1.9 µm	2.1 × 50 mm	25002-052130

Table 5. LC-MS conditions for initial testing

Parameter	Value
Mobile Phase	A: Water with 0.1% formic acid B1: Acetonitrile with 0.1% formic acid B2: Methanol with 0.1% formic acid
Gradient	0–0.5 min: 2% B 0.5–10 min: 2–98% B 10–15 min: 2% B
Flow Rate	0.4 mL/min
Column Temperature	40 °C, forced air mode passive pre-heater
Injection Volume	0.1 µL
UV Detection	254 nm, 100 Hz
Source Settings	Default (easy mode)
Full Scan	
Time	0–15 min
Mass range	200–500 *m/z*
Dwell Time	0.2 s
Polarity	Positive
Source CID voltage	20 V

Table 6. LC-MS conditions for screening method

Parameter	Value
Mobile Phase	A: Water with 0.1% formic acid B1: Acetonitrile with 0.1% formic acid
Gradient	0–0.5 min: 40% B 0.5–2.5 min: 40–60% B 2.5–5 min: 40% B
Flow Rate	0.4 mL/min
Column Temperature	40 °C, forced air mode passive pre-heater
Injection Volume	1.0 µL
UV Detection	254 nm, 100 Hz
Source Settings	Default (easy mode)
Full Scan	
Time	0–5 min
Mass range	150–350 *m/z*
Dwell Time	0.2 s
Polarity	Positive
Source CID voltage	20–100 V in steps of 10 V

Table 7. LC-MS conditions for method development

Parameter	Value
Mobile Phase	A: Water with 0.1% formic acid B1: Acetonitrile with 0.1% formic acid
Gradient	0–0.5 min: 40% B 0.5–2.5 min: 40–60% B 2.5–5 min: 40% B
Flow Rate	0.4 mL/min
Column Temperature	40 °C, forced air mode passive pre-heater
Injection Volume	1.0 µL
UV Detection	254 nm, 100 Hz
Source Settings	Default (easy mode)
SIM Scan (Parent)	
Time	0–5 min
Mass	301.12 *m/z*
Dwell Time	0.1 s
SIM Width	0.4 Da
Polarity	Positive
Source CID voltage	0–100 V in steps of 10 V 20–40 V in steps of 2 V
SIM Scan (Desmedipham fragment)	
Time	0–5 min
Mass	182.08 *m/z*
Dwell Time	0.1 s
SIM Width	0.4 Da
Polarity	Positive
Source CID voltage	0–100 V in steps of 10 V 50–70 V in steps of 2 V
SIM Scan (Phenmedipham fragment)	
Time	0–5 min
Mass	168.07 *m/z*
Dwell Time	0.1 s
SIM Width	0.4 Da
Polarity	Positive
Source CID voltage	0–100 V in steps of 10 V 50–70 V in steps of 2 V

Table 8. LC-MS conditions for method verification

Parameter	Value
Mobile Phase	A: Water with 0.1% formic acid B1: Acetonitrile with 0.1% formic acid
Gradient	0–0.5 min: 2% B 0.5–10 min: 2–98% B 10–15 min: 2% B
Flow Rate	0.4 mL/min
Column Temperature	40 °C, forced air mode passive pre-heater
Injection Volume	1.0 µL
UV Detection	254 nm, 100 Hz
Source Settings	Default (easy mode)
Full Scan	
Time	0–15 min
Mass range	150–350 *m/z*
Dwell Time	0.2 s
Polarity	Positive
Source CID voltage	28 V
SIM Scan (Parent)	
Time	0–15 min
Mass	301.12 *m/z*
Dwell Time	0.1 s
SIM Width	0.4 Da
Polarity	Positive
Source CID voltage	28 V
SIM Scan (Desmedipham fragment)	
Time	0–15 min
Mass	182.08 *m/z*
Dwell Time	0.1 s
SIM Width	0.4 Da
Polarity	Positive
Source CID voltage	58 V
SIM Scan (Phenmedipham fragment)	
Time	0–15 min
Mass	168.07 *m/z*
Dwell Time	0.1 s
SIM Width	0.4 Da
Polarity	Positive
Source CID voltage	58 V

Results and discussion

Initially, UV-based chromatographic separation of desmedipham and phenmedipham was attempted using a 10 cm Hypersil GOLD column (Table 4), with a generic 10 min linear gradient with either acetonitrile or methanol as organic phase (Table 5). In chromatography, a resolution of 1.5 is often considered the lower limit for acceptable chromatographic separation. For the two analytes, the separation method resulted in a resolution (according to EP) of 0.40 for acetonitrile and 0.36 for methanol (Figure 1). Hence, the chromatographic separation was considered insufficent. Moreover, UV-based analysis of the analyte mix resulted in a single peak only slightly wider than the individual peaks of neat desmedipham or phenmedipham sample analyses. Therefore, co-elution was undiscernible by UV detection.

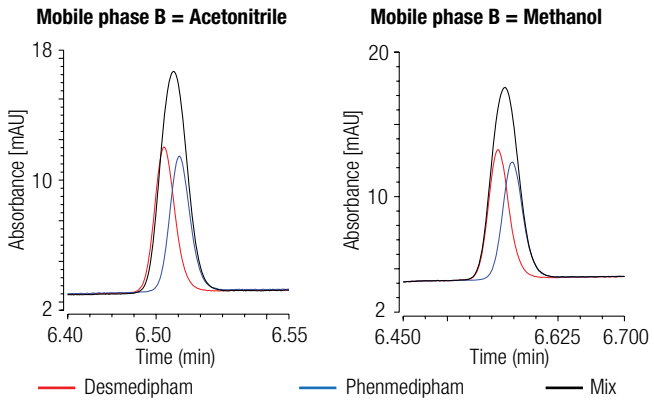

Figure 1. Chromatographic separation of desmedipham and phenmedipham. Peak detection was UV-based (254 nm). Using acetonitrile, a resolution of 0.40 was achieved; using methanol, a resolution of 0.36 was achieved (according to EP).

MS detection can usually overcome the problem of insufficient chromatographic resolution since analytes are selectively detected through their respective molecular masses. As a result, the analytes can be separated and analyzed individually by extracted ion chromatograms (EICs) using their specific molecular masses.

Unfortunately, desmedipham and phenmedipham have the same molecular mass (Table 1), so mass spectrometric detection cannot immediately distinguish the two compounds. However, they have different chemical structures (Figures 2A and 2B), which can be exploited for mass spectrometric separation. This is routinely done with triple quadrupole mass spectrometers, where the compounds are fragmented within the mass spectrometer and compound-specific fragments are subsequently detected.

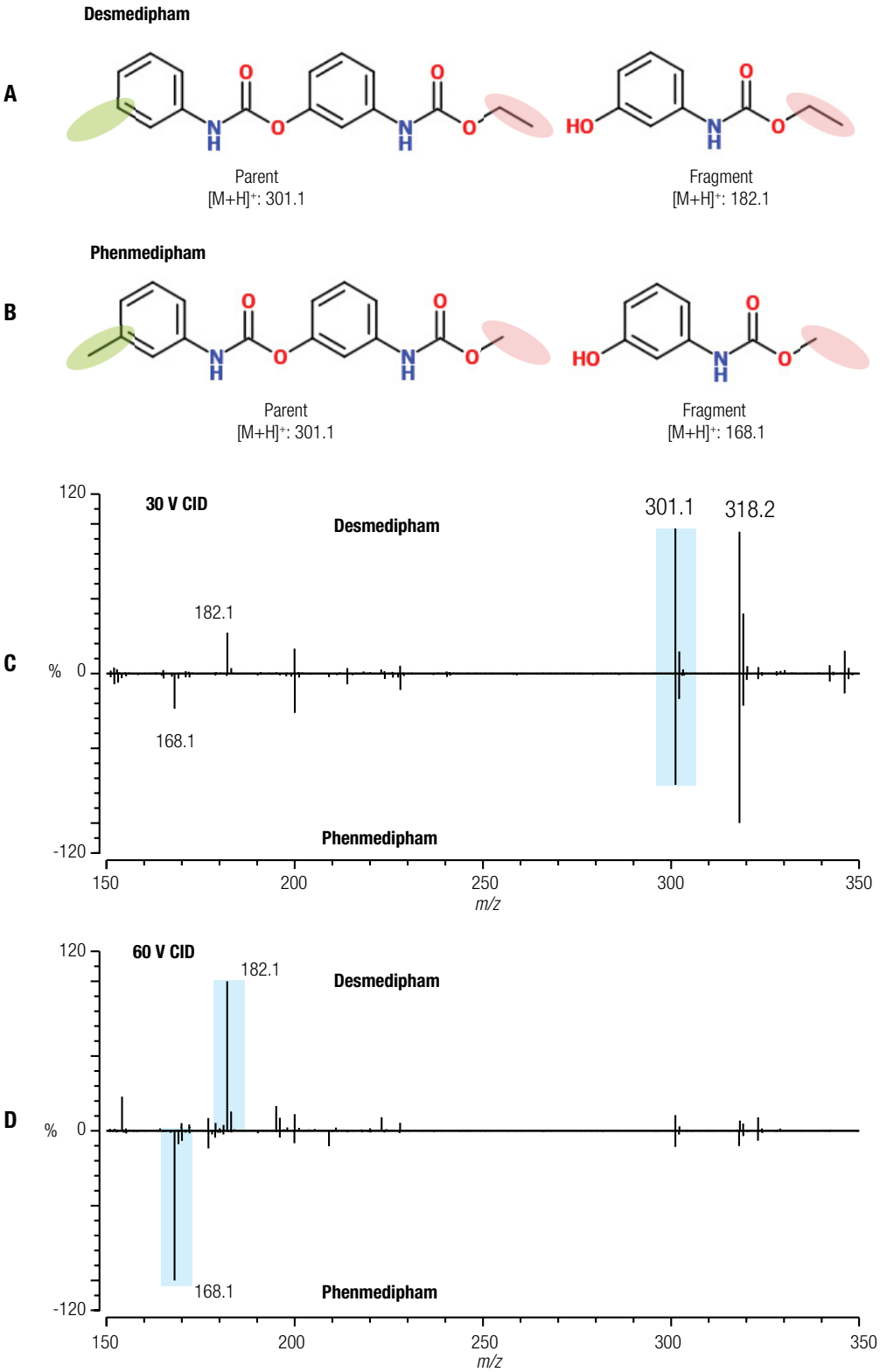

Figure 2 (A and B). Chemical structures of desmedipham and phenmedipham and their respective detectable fragments. The structural differences of the two compounds and their respective fragments are indicated in green and red. The monoisotopic masses of the protonated, singly charged species are stated below the chemical structures.

Figure 2 (C and D). Mirror plots of representative full scan mass spectra of desmedipham (top) and phenmedipham (bottom) with low (30 V) and high (60 V) source CID voltage. At 30 V CID voltages the protonated compounds (*m/z* 301.1) and their ammonia adducts (*m/z* 318.2) are the dominant species. At 60 V CID voltage the respective fragments, *m/z* 182.1 for desmedipham and m/z 168.1 for phenmedipham are the dominant species.

This approach is referred to as single reaction monitoring (SRM). It requires a first quadrupole for selecting the compound, a second quadrupole acting as a fragmentation cell, and a third one for detecting the specific fragments. Thus, this cannot be done with a single quadrupole mass spectrometer. Nevertheless, the ISQ EC/EM mass detector offers an alternative. An additional voltage offset can be added to the ion transfer tube and the skimmer cone optics (Figure 3). This results in a faster acceleration of the ions into the Q00 octopole. When passing through it they may collide with residual nitrogen molecules and, due to their increased speed, fragment in a similar way as in the collision cell of a triple quadrupole mass spectrometer. Therefore, this process is called in-source collision induced dissociation or in-source CID. The voltage of the additional potential determines the speed of the analytes and thus, whether they fragment upon collision with nitrogen molecules. At low source CID voltages (up to approximately 30 V) mainly the loss of adducts, such as sodium, potassium, ammonia, or solvent, is detectable often resulting in higher signal intensities for the protonated analyte species. At higher voltages (above 30 V), the covalent bonds within molecules can break and actual fragmentation occurs. Increasing the voltage further (above 60 V) the fragments themselves may fragment. The fragmentation pattern and the optimal source CID voltages depend on the respective strength of the covalent bonds within the analyte molecules. Labile bonds tend to fragment at lower source CID voltages.

In order to test whether in-source CID fragmentation was feasible for our analytes, neat samples of desmedipham and phenmedipham were analyzed. A five-minute method (Table 6) using a 5 cm Hypersil GOLD column (Table 2) was used for testing whether the two compounds showed distinct fragmentation patterns. MS acquisition was performed in full scan mode (*m/z* 150–350) to screen for the unknown fragments. Source CID voltages between 20 and 100 V were tested. At low source CID voltages, the intact analytes (*m/z* 301.1) and their ammonia adducts (*m/z* 318.2) were clearly detectable (Figure 2C). Increasing the source CID voltage resulted in a reduction of the intact analyte signals while new signals appeared: for desmedipham at *m/z* 182.1, and for phenmedipham at *m/z* 168.1 (Figure 2D). Both fragment masses were specific for each analyte and not present for the other compound. This indicated that increasing the source CID voltage efficiently fragmented the analytes and that the unique fragments *m/z* 182.1 and *m/z* 168.1 could be used for selective detection. Considering the chemical structures of the analytes, the structure of the observed fragments could be deduced (Figures 2A and 2B). Desmedipham contains a methoxy group and phenmedipham, an ethoxy group, resulting in the mass difference of 14 Da for the two fragments, which was detected.

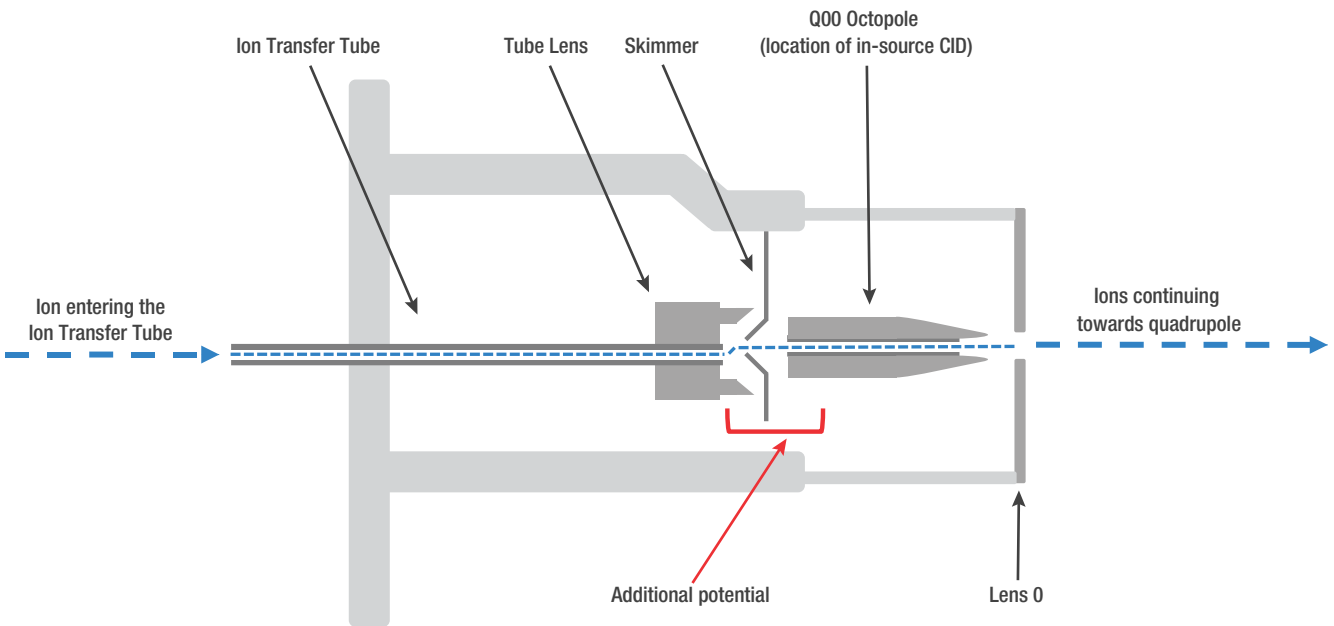

Figure 3. In-source collision induced dissociation (CID). The components involved in facilitating in-source CID are schematically depicted. A voltage offset is applied to ion transfer tube, tube lens, and skimmer. This results in an increased potential between them and the Q00 octopole further accelerating the ions travelling towards the Q00 octopole. Within the octopole the analyte molecules may collide with residual nitrogen molecules and fragment if the analyte molecules' kinetic energy is high enough. Fragments with the same charge state as the parent molecule continue towards the quadrupole.

After identifying the unique fragments, the detection of intact and fragmented compounds was optimized. Neat samples of desmedipham and phenmedipham were analyzed with the previously used 5-minute method but the MS detection settings were altered (Table 7). Instead of Full scans, SIM scans for the intact mass and fragments were performed. Different source CID voltages were tested to identify the values that give optimal signal intensity (Table 7):

- Increasing the source CID voltage from 0 to 30 V resulted in a signal increase for the intact masses of desmedipham and phenmedipham (Figures 4A and 4B). At the same time, analyte fragmentation becomes detectable at a 30 V source CID voltage. The increase of the intact mass intensity when increasing the source CID voltage from 0 to 30 V is due to adduct removal. The ammonia adduct (*m/z* 318.2) is the dominant mass at 0 V, and at 30 V it is less intense than *m/z* 301.1 (Figures 4C and 4D, page 8).
- Further increasing the source CID voltage results in a decrease of the intact mass signal and an increase of the fragment mass peak, until at 60 V both analyte fragments show highest intensity. Beyond 60 V the *m/z* 182.1 and 168.1 signals decrease because they themselves fragment.

Initial optimization was done with 10 V steps, which is usually sufficient for routine applications. In this case, a second optimization round was conducted where source CID voltage optimization was performed from 20 to 40 V (for the intact analytes) or 50 to 70 V (for the fragments) in steps of 2 V. This resulted in an optimum value of 28 V for the intact analytes and 58 V for the fragments (data not shown).

Finally, the optimized MS settings, in combination with a 10 cm Hypersil GOLD column and a 10-minute linear gradient (Table 8), were applied to samples containing various ratios for desmedipham and phenmedipham (Table 9, page 9).

With UV-based detection, the mixture of both analytes resulted in a single chromatographic peak that was slightly wider than the peaks observed for neat samples, but it did not show unusual peak shape, such as tailing or fronting (Figure 5 top, page 9). Thus, it would have been impossible to determine that the observed peak contained more than one analyte. The same was true for mass spectrometric detection of the parent ions (data not shown). Using SIM scans of the two fragments allowed selective detection of the two analytes (Figure 5 bottom, page 9). The neat samples showed high selectivity. No desmedipham fragment was detectable in the phenmedipham sample and vice versa. Analysis of the sample mixes resulted in selective detection of both compounds, clearly indicating that desmedipham and phenmedipham can be easily analyzed in parallel.

Conclusion

Mass spectrometry increases confidence in chromatographic data by providing the molecular mass of detected analytes. In combination with analyte retention time this is often enough for mass confirmation. However, in some cases additional information is needed. If both retention time and molecular mass are not distinctive then additional selectivity is needed. With the ISQ EC or EM mass detectors this can be achieved by using in-source CID fragmentation. Applying suitable source CID voltages results in parent ion fragmentation and, in most cases, distinctive fragment ions that can be used for selectively detecting co-eluting, isobaric analytes.

Acknowledgements

Desmedipham and phenmedipham were kindly provided by Dr. Kalle Uroic, Bayer AG, Monheim, Germany.

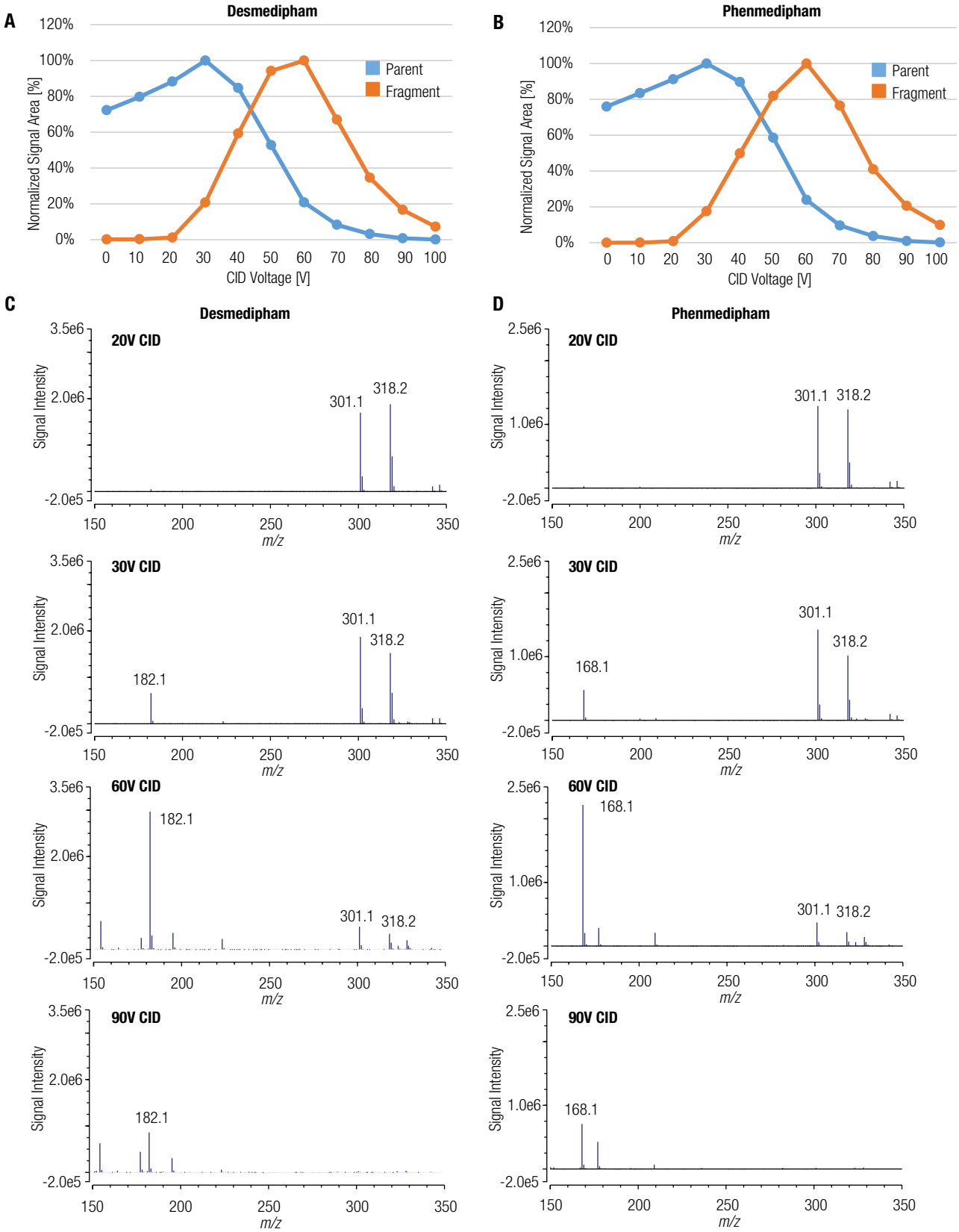

Figure 4. Optimization of fragmentation efficiency. A and B. Signal intensities of SIM scans for parent and fragment ions at different CID voltages. C and D. Representative full scan mass spectra at 20, 30, 60, and 90 V CID voltage showing the decrease of parent ions with increasing CID voltage, and the initial increase of fragment ions with increasing CID voltage and subsequent decrease at 90 V CID voltage.

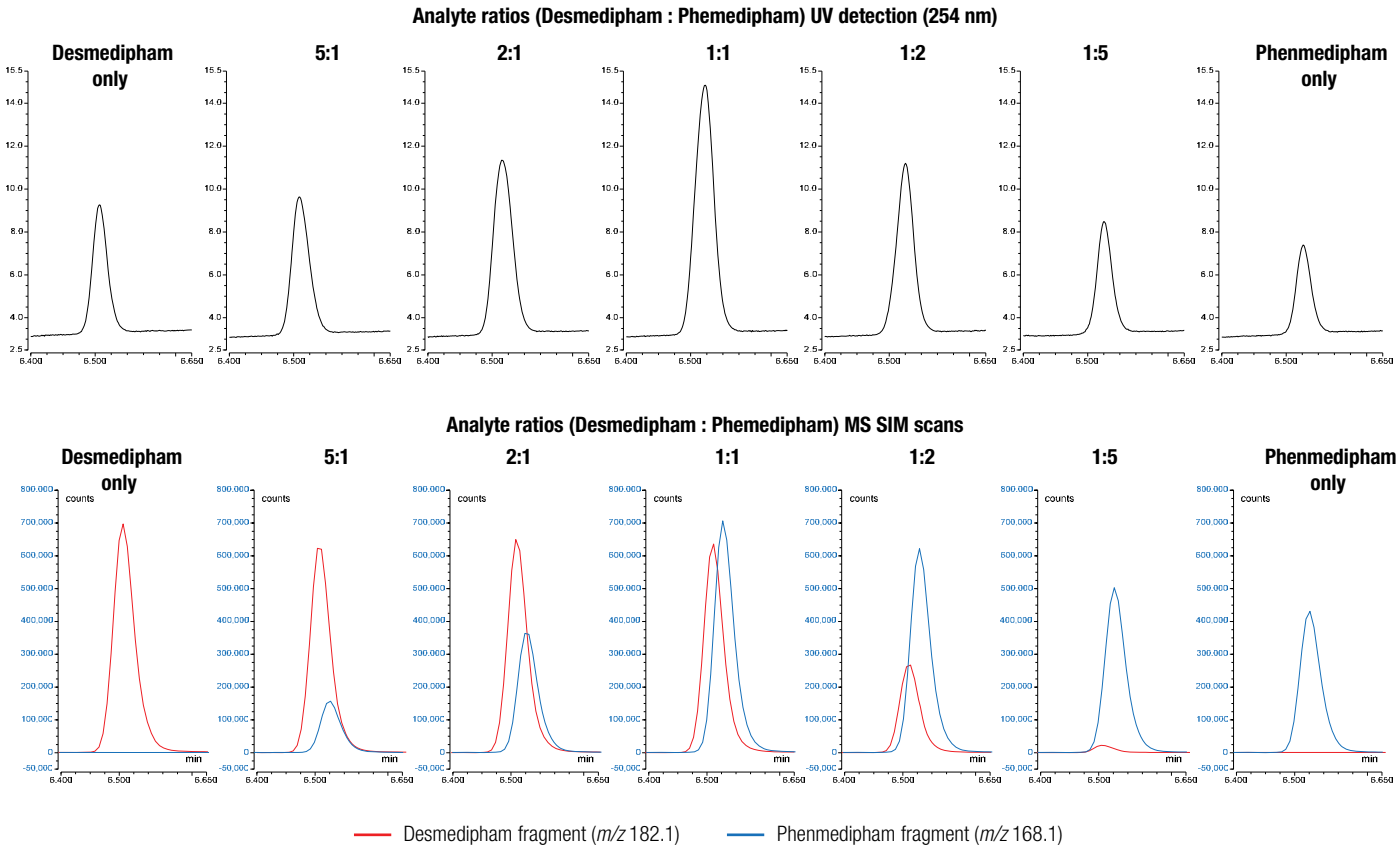

Figure 5. Analysis of sample mixes with different ratios of desmedipham and phenmedipham. The sample mix ratios are stated at the top, the UV chromatograms are shown in the middle, and the overlaid SIM scans of the fragments are shown at the bottom.

Table 9. Analyte ratio and sample amounts used for verification of selective analyte detection

Ratio	Amount on column	
(Desmedipham : Phenmedipham)	Desmedipham	Phenmedipham
Neat	10 ng	-
5:1	10 ng	2 ng
2:1	10 ng	5 ng
1:1	10 ng	10 ng
1:2	5 ng	10 ng
1:5	2 ng	10 ng
Neat	-	10 ng

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