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When Other Separation Techniques Fail: Compound-Specific Carbon Isotope Ratio Analysis of Sulfonamide Containing Pharmaceuticals by High-Temperature-Liquid Chromatography-Isotope Ratio Mass Spectrometry

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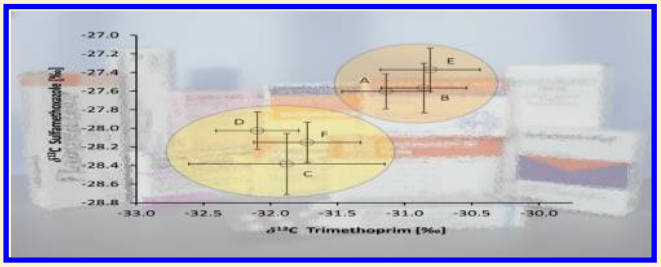
ABSTRACT: Compound-specific isotope analysis (CISA) of nonvolatile analytes has been enabled by the introduction of the first commercial interface to hyphenate liquid chromatography with an isotope ratio mass spectrometer (LC-IRMS) in 2004, yet carbon isotope analysis of unpolar and moderately polar compounds is still a challenging task since only water as the eluent and no organic modifiers can be used to drive the separation in LC. The only way to increase the elution strength of aqueous eluents in reversed phase LC is the application of high temperatures to the mobile and stationary phases (HT-LC-IRMS). In this context we present the first method to determine carbon isotope ratios of pharmaceuticals that cannot be separated by already existing separation techniques for LC-IRMS, such as reversed phase chromatography at normal temperatures, ion-chromatography, and mixed mode chomatography. The pharmaceutical group of sulfonamides, which is generally mixed with trimethoprim in pharmaceutical products, has been chosen as probe compounds. Substance amounts as low as 0.3 μg are sufficient to perform a precise analysis. The successful applicability and reproducibility of this method is shown by the analysis of real pharmaceutical samples. The method provides the first tool to study the pharmaceutical authenticity as well as degradation and mobility of such substances in the environment by using the stable isotopic signature of these compounds.



Compound-specific isotope analysis of nonvolatile organic compounds is often performed by gas chromatography–isotope ratio mass spectrometry (GC–IRMS) with prior derivatization.[1] Although these methods give accurate and reproducible results, they are not applicable for many nonvolatiles. The hyphenation of liquid chromatography (LC) to isotope ratio mass spectrometry enables the determination of carbon isotope ratios of very polar, thermolabile, and high molecular weight compounds without the need for derivatization.[2] Due to the design of the interface organic modifiers cannot be used in LC eluents.[3] In order to precisely measure carbon isotope ratios, the analytes need to be detected as CO_2. In the interface, the whole column effluent is nonselectively converted to CO_2 by peroxodisulfate in a heated capillary, extracted from the aqueous phase into a carrier gas stream via a membrane, and transported to the IRMS inlet. Consequently, any additional carbon compound (either as buffer or solvent) in the eluent besides analyte carbon would result in a severe decrease of sensitivity, wrong carbon isotope ratios, or a saturation of the isotope ratio mass spectrometer signal. This restricts the type of applicable separation techniques for LC-IRMS. Several methods have been reported for the determination of carbon isotope ratios of carbohydrates, amino sugars, and amino acids based on ion exchange chromatography.[4−6]

However, with ion exchange as the sole separation mechanism, it is difficult to obtain a full separation of proteinogenic amino acids.[2] The resolution of many amino acids can be substantially improved by adding the possibility for hydrophobic interactions by a further RP-column.[2] Almost all amino acids can be resolved by mixed-mode chromatography, i.e., the deliberate combination of RP and ion exchange interaction sites in one stationary phase, which is nowadays the most published method for LC-IRMS analysis of underivatized amino acids.[7] The stationary phase contains ion exchange groups bound to alkyl chains or phenyl rings, which also allow an operation with 100% aqueous eluents without the risk of a loss of retention due to particle pore dewetting.

There are no studies which use ion chromatography or mixed mode chromatography for compound-specific isotope analysis of pharmaceuticals. Solely, carbon isotope ratios of paracetamol and aspirin have been measured to show the applicability of the LC-IRMS interface system. In this study a reversed phase (RP) silica based column at ambient temperature was used to separate those two compounds.[3] However, it is obvious, that also ion chromatography as well as mixed mode chromatography can be used for those pharmaceuticals. In the following, we will discuss why reversed phase separation at ambient temperatures, HT-LC separation with porous graphitic carbon

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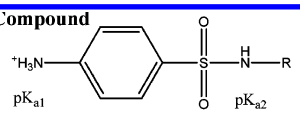
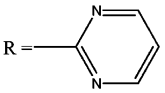
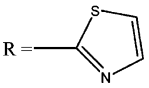
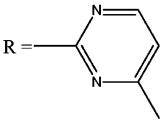
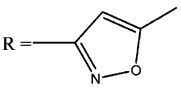
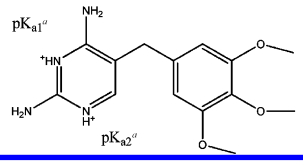
Table 1. Physico-Chemical Properties and Structures of the Investigated Substances

Compound	pK_{a1}[27]	pK_{a2}[27]	Molecular formula (MW/ g mol^{-1})	CAS-No.
				
Sulfadiazine 	1.6	6.8	$C_{10}H_{10}N_4O_2S$ (250.28)	68-35-9
Sulfathiazole 	2.01	7.11	$C_9H_9N_3O_2S_2$ (255.32)	144-74-1
Sulfamerazine 	2.07	6.9	$C_{11}H_{12}N_4O_2S$ (264.31)	127-58-2
Sulfamethoxazole 	1.85	5.60	$C_{10}H_{11}N_3O_3S$ (253.28)	723-46-6
Trimethoprim 	3.23	6.76	$C_{14}H_{18}N_4O_3$ (290.32)	738-70-5

[a]Single mesomeric structure showing the initial protonation sites.

materials, ion chromatography, and mixed-mode phases are not feasible for the investigated pharmaceutical compound classes of sulfonamides and trimethoprim.

Sulfonamides are amphoteric target compounds which are negatively charged at pH values greater than 8 and positively charged at very low pH. In theory, both anion as well as cation exchange chromatography may work under these circumstances. However, a change of the pH to 8 would result in negatively charged sulfonamides, but there is no anionic group at any pH for trimethoprim. To this end, a separation of the chosen target compounds would not be possible. Since trimethoprim is usually combined in pharmaceutical products containing sulfonamides, valuable information on its carbon isotope ratio would be lost. Another problem at pH 8 is an increased background caused by a much higher solubility of CO_2 from air in the employed eluents, which requires a sophisticated eluent preparation and conservation in order to maintain a low baseline in the chromatogram. Apart from the above-mentioned criteria against anion exchange, cation exchange is not a good option, since the pH of the mobile phase should be two pH units below pK_a for good peak shape and so a high concentration of acid would be necessary in the eluent. Despite these rather practical reasons, the retention of the target sulfonamides and trimethoprim on such columns will remain a mixture of ion exchange and reversed phase chromatography due to hydrophobic interactions with the polymeric support of the ion-exchange resin.

Such an intentional combination of retention mechanisms is utilized in the mixed-mode columns mentioned above. In the case of amino acids, it was shown that these stationary phases work well because the elution strength of water is high enough to elute hydrophobic amino acids from the mixed-mode phase without assistance of organic modifiers.[7] In contrast to this, using comparable mixed-mode phases for sulfonamides and trimethoprim a much higher retention than for a reversed phase separation is expected due electrostatic and strong hydrophobic interactions. Thus, this method would require an organic proportion in the mobile phase to aid elution, which is not compatible with LC-IRMS, or operate the chromatography at higher temperatures. However, the temperature stability of mixed-mode columns remains to be examined.

An alternative approach that overcomes these described restrictions is high-temperature-liquid chromatography (HT-LC) with reversed phases. HT-LC coupled to IRMS has already been used to determine carbon isotope ratios of small organic acids[8] and caffeine derivatives.[9] The elution strength of aqueous mobile phases without organic modifiers is often too low to elute compounds from nonpolar stationary phases at ambient temperatures. Especially for the investigated compounds here, elution times increase dramatically, which results in inadequate retention times and a loss in resolution and signal sensitivity. The application of temperatures notably higher than ambient to the mobile phase and the column has been proposed to substitute for organic solvents.[10] With increasing temperatures, the static permittivity and viscosity of water decreases. At 150 °C (at a pressure of 50 bar), the static permittivity of water is nearly the same as that of about 70% methanol or acetonitrile/water mixtures.[10] Thus, elution strength of pure aqueous

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mobile phases in RP chromatography at high temperatures is similar to solvent/water mixtures encountered in RP-LC. Temperature gradients instead of solvent gradients can be used to separate compounds on nonpolar stationary phases such as octadecyl silica or porous graphitized carbon (PGC). Temperature-programmed elution can even be predicted from two measurements with different temperature gradients.[11]

As the LC-IRMS detector is very sensitive to any carbon containing compound, a major concern using high-temperature-liquid chromatography is the carbon feed from the column into the interface. At high temperature, column bleed is much higher due to faster hydrolysis of the support material and the bonded phase. Column bleed invisible for the most common LC detection techniques may overlay the analyte peak or produce elevated background levels. Thus, Zhang et al.[9] evaluated four stationary phases which have proven their temperature stability in previous studies.[12] Hybrid particles from ethylene bridged silica and zirconium dioxide based materials can be used with excellent results for LC-IRMS at HT-LC conditions. Up to column-specific maximum temperatures, the column bleed did not affect δ^{13}C-values of various probe compounds.[9] It also has to be considered that carry over or late elution of hydrophobic compounds retained on very nonpolar phases like PGC can alter δ^{13}C-values of the analytes due to ghost peaks or an occasional change in the background $^{13}C/^{12}C$-ratio. Additionally, δ^{13}C-values of thermolabile analytes may be influenced by isotopic fractionations resulting from thermal degradation on the column.[13] Porous graphitic carbon columns such as the Hypercarb possess very high retention at ambient temperatures.[13] Although the retention times are decreased at higher temperatures, an unacceptable column bleed can be observed during temperature gradient elution.[8]

The aim of this work was to show that a HT-LC can be used for the determination of carbon isotope ratios of sulfamerazine, sulfadiazine, sulfamethoxazole, sulfathiazole, and trimethoprim. These antibiotic substances have been widely used in veterinary and human medicine for some decades. Although they have been subject of many research campaigns in the environmental[14] and pharmaceutical sciences[15] so far, no determination of isotope ratios at natural abundance has been published. Several HT-LC methods for trimethoprim and sulfonamide measurements have been published in the literature[11,16,17] for quantitative analysis, but to our best knowledge no carbon-free eluents have been used and no stable isotope signatures of these compounds have been determined. Up until now, isotope ratios of polar active pharmaceutical ingredients can only be determined either by ^{13}C NMR or labor-intensive offline techniques like preparative LC followed by an elemental analyzer (EA-IRMS). Here, we demonstrate a pure aqueous separation of these compounds for its use in CSIA and proved its potential for verifying the authenticity of antibiotics containing sulfamethoxazole and trimethoprim via δ^{13}C-values.

EXPERIMENTAL SECTION

Chemicals and Reagents. Analytical standards of sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxazole, and trimethoprim were purchased from Sigma-Aldrich GmbH (Steinheim, Germany) in a purity of at least 98%. Sulfamethoxazole and trimethoprim were purchased from Sigma-Aldrich GmbH (Steinheim, Germany) as well but in >99% purity (VETRANAL). Structures and pK_a-values of these compounds are listed in Table 1. Sodium peroxodisulfate ($Na_2S_2O_8$), phosphoric acid (H_3PO_4), and sodium hydrogenphosphate (NaH_2PO_4) for eluent and reagent solutions were supplied by Fluka (Steinheim, Germany). Reagent solutions and eluents were degassed in an ultrasonic bath (Bandein Eletronic, Berlin, Germany) under vacuum (Vacuubrand, Wertheim, Germany). In order to avoid regassing, these solutions were continuously purged with helium of 99.999% purity (Air Liquide, Oberhausen, Germany) at a flow rate of approximately 30 mL min^{-1}.

Analytical Standard Solutions. Standard solutions of sulfonamides were freshly prepared prior to each set of analyses by dissolving a required amount of substance in 15 mL of HT-LC buffer solution at approximately 60 °C.

LC Conditions. HT-LC system consisted of a Rheos Allegro binary pump (Flux Instruments, Buchs, Switzerland) and a HT-HPLC 200 column oven (SIM Scientific Instruments Manufacturer GmbH, Oberhausen, Germany). The compounds were separated on an XBridge C_{18} column 100 mm × 2.1 mm, 3.5 μm particle size equipped with a 10 mm × 2.1 mm precolumn packed with the same material (Waters, Eschborn, Germany). A sodium phosphate buffer of 5 mM and pH 3 was used as the eluent at a flow rate of 500 μL min^{-1}. The temperature gradient was 60 °C for 3 min, then to 80 at 3 °C min^{-1}, held for 15 min, then to 100 at 3 °C min^{-1} and held for 5 min.

Flushing of the organic solvent from shipping or storage of the column prior to measurements was done while the column was installed in the HT-LC-IRMS system. In order to prevent precipitation, the column was first flushed for 2 h with pure water and afterward equilibrated with the phosphate buffer for 1 h at a flow rate of 500 μL min^{-1}.

LC-IRMS Interface Conditions. For the interface between the HPLC and IRMS, an LC-IsoLink (Thermo Scientific, Bremen, Germany) was used. The wet chemical oxidation of the compounds was realized by online mixing of HPLC column effluent with phosphoric acid (1.5 M) and sodium peroxodisulfate (200 g L^{-1}) at a reactor temperature of 99.9 °C. For all experiments, flow rates of both reagents were set to 50 μL min^{-1}. The formed CO_2 was separated from the aqueous phase by a gas exchange membrane and transferred to the open split in a helium stream (Air Liquide, Oberhausen, purity 99.999%) at a flow rate of 1.2 mL min^{-1}.

Without an HPLC column, this interface can be used for bulk carbon isotope ratio determination by flow injection analysis (FIA). The carrier flow rate to transport the bulk sample from the sample loop to the interface was set to 200 μL min^{-1}, 350 μL min^{-1}, and 500 μL min^{-1}, whereas reagent flow rates remained at 50 μL min^{-1} each.

The mass spectrometric detection was performed on a Delta V Advantage (Thermo Electron, Bremen, Germany) tuned on maximum linearity. Linearity and precision of the instrument were checked regularly by reference gas pulses of different amplitudes.

EA-IRMS. Elemental Analyzer CE 1110 (CE Instruments, Milano, Italy) coupled with a ConFlo IV Interface to an MAT 253 isotope ratio mass spectrometer (both Thermo Scientific, Bremen, Germany) was used to determine δ^{13}C-values of the analytical standards and bulk isotope ratios of the homogenized pharmaceutical pills. EA-IRMS values were obtained as described by Werner and Brand.[18] Reference materials for calibration of the working standard acetanilide to the IAEA scale were NBS-22 (oil; δ^{13}C = −30.031‰), IAEA-CH-6

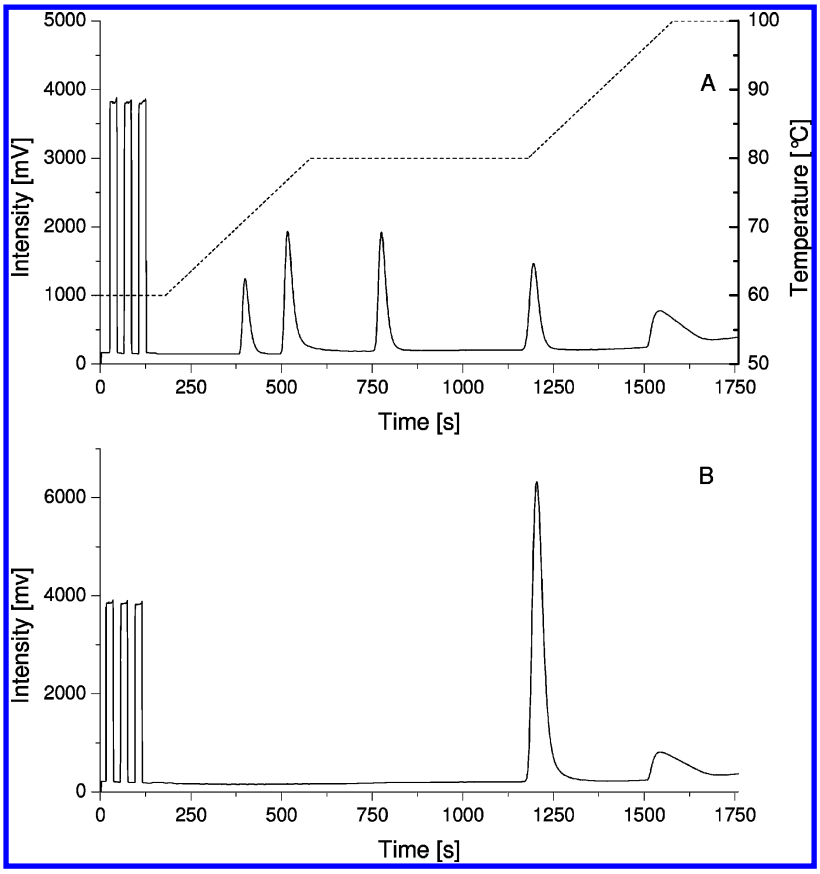

Figure 1. (A) Chromatogram (m/z 44) of a standard mixture separated by a temperature gradient indicated by the dashed line. The elution order is sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxazole, and trimethoprim. The concentration of each substance was 100 mg L^{-1}. (B) Chromatogram (m/z 44) of a pharmaceutical sample containing sulfamethoxazole and trimethoprim obtained by the same temperature gradient as above.

(sucrose; $\delta^{13}C = -10.449‰$), and IAEA 600 (caffeine; $\delta^{13}C = -27.771‰$).

Sample Preparation. Samples of pharmaceutical tablets (trade-names Cotrim and Cotrimoxazole) of six different manufacturers each containing 800 mg of sulfamethoxazole and 160 mg of trimethoprim were homogenized and dissolved with the HPLC eluent to 100 mg L^{-1} of the specific analyte according to the concentration declaration in the package inserts. Besides the active ingredients, the pills contained excipients like starch and stearate salts.

Data Acquisition and Handling. Acquisition and processing of data was performed by Isodat 2.5. The background subtraction algorithm used for these measurements was "individual background". All reported $\delta^{13}C$-values are normalized to the VPDB scale. All standard deviations refer to triplicate measurements if not stated otherwise. $\delta^{13}C$-values of the pharmaceutical samples were corrected with the difference of $\delta^{13}C$-values between EA-IRMS measurements of the particular compound and HT-LC-IRMS analyses. This corresponds to the principle of identical treatment as it was proposed by Werner and Brand.[18]

The reference gas pulses were used as an internal standard to calculate relative peak area per carbon. The pressure of the reference gas remained unchanged during the FIA measurements so that any variation of ionization between the runs is eliminated and peak areas can be compared.

Chromatographic resolution R_s was calculated according to equation:[19]

$$R_s = 2\left(\frac{t_{r(2)} - t_{r(1)}}{w_{h(2)} + w_{h(1)}}\right)$$

Here, t_r is the retention time of peak 1 or 2, and w_h is the peak width at half height for the two peaks.

RESULTS AND DISCUSSION

Chromatographic Conditions. All selected compounds can be baseline separated by the use of a temperature gradient. Figure 1 shows a chromatogram and the temperature program applied. The resolution between sulfadiazine and sulfathiazole was 3, which meets the requirement of a full separation ($R = 2$)[19] for compound-specific isotope analysis due to chromatographic isotope effects.[20]

A temperature gradient with a low steepness of 3 °C min^{-1} was chosen to keep the rising of the baseline as low as possible. Godin et al.[8] reported low precision and accuracy when high rising backgrounds were observed due to steep temperature gradients on a PGC column. In contrast, Zhang et al.[9] did not observe significant loss in precision or accuracy using gradients between 6 and 9 °C min^{-1} for hybrid particles and zirconium based stationary phases. These findings demonstrate the importance of background subtraction algorithms. Godin et al.[8] and Zhang et al.[9] used the "dynamic background" function in ISODAT. In this work the slope of the background was between 0.17 and 0.5 mV s^{-1} so that an individual background algorithm was used as suggested by Zhang et al.[9]

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Table 2. HT-LC-IRMS Performance Evaluation

	MDL[a] [μg]	$\delta^{13}C_{HT\text{-}LC}$ [‰][b]	$\delta^{13}C_{FIA}$[c] [‰][b]	$\delta^{13}C_{EA}$ [‰][b]	$\delta^{13}C_{EA}-\delta^{13}C_{HT\text{-}LC}$ [‰]
sulfadiazine	0.4	−30.8 ± 0.1	−31.6 ± 0.4	−29.2 ± 0.2	1.6
sulfathiazole	0.3	−28.5 ± 0.1	−29.6 ± 0.4	−26.64 ± 0.04	1.9
sulfamerazine	0.5	−31.3 ± 0.3	−31.2 ± 0.4	−28.90 ± 0.03	2.4
sulfamethoxazole	0.5	−30.7 ± 0.3	−30.3 ± 0.2	−27.91 ± 0.02	2.8
trimethoprim	0.4	−37.6 ± 0.3	−37.6 ± 0.2	−34.15 ± 0.04	3.4

[a]As absolute amount on column. [b]Standard deviations refer to at least triplicate measurement. [c]At a flow rate of 500 μL min^{-1}.

All sulfonamides have been thermally stable during the chromatographic run. Peak shape as well as comparable $\delta^{13}C$-values to FIA of single compounds showed no indications of degradation inside the column. Only trimethoprim showed a large tailing which can either be a sign of thermal decomposition in the column or column overload. These triangular peak shapes at low pH have been associated with column overload of protonated basic substances on silica-based columns due to charge repulsion between retained protonated molecules[21,22](see Figure 1).

As pointed out by Godin et al.,[8] thermal decomposition inside the column can influence $\delta^{13}C$-values. Since results obtained by FIA and HT-LC are comparable (see Table 2), we suggest that even if thermal degradation of trimethoprim takes place all metabolites elute as one peak and leave $\delta^{13}C$-values unaffected.

Method Detection Limits and Accuracy. Method detection limits (MDL) were calculated by the moving mean procedure[23] in order to account for any method-related offsets of $\delta^{13}C$-values. Here, the detection limit is defined as the lowest concentration which is necessary to achieve the measurement of a $\delta^{13}C$-value with a defined precision (±0.5‰) and accuracy with respect to the signal height-independent isotope ratio.[23]

MDLs of the selected sulfonamides and trimethoprim are given in Table 2. Sulfathiazole showed the lowest MDL of the selected compounds being 0.3 μg on column. If a lower precision of isotope data is acceptable, measurements of sulfadiazine, sulfamethoxazole, and sulfathiazole can be performed even below those MDLs given in Table 2 (see Figure 2).

Comparing the results obtained by EA and both LC-IRMS measurement modes, it can be seen that there is a discrepancy of $\delta^{13}C$-values for all selected compounds (see Table 2). This is in agreement with other studies where substance-specific differences of more than 1‰ have been reported for various substances.[9,24,25] Although $\delta^{13}C$-values from EA measurements of the pure phase analytical standards might be biased by a contamination with other carbon compounds, the authors believe that even with a compound with a high $\delta^{13}C$-value, such as a carbonate, the error is smaller than the analytical precision.

FIA-IRMS analyses have been conducted at different liquid carrier flow rates but with constant reagent flow rates to simulate the influence of the HT-LC flow rate without compromising peak shape in order to investigate this offset. As can be seen from Figure 3, the lower the carrier flow rate in the interface, the more do the $\delta^{13}C$-values of FIA approach the $\delta^{13}C$-values of EA measurements. The peak area also shows a dependency on flow rate which confirms that oxidation is incomplete. Lower peak areas are observed in FIA when higher flow rates are applied because of reduced reaction time inside the oxidation reactor and lower reagent concentrations. The reagent is always delivered in far excess relative to the analyte so that concentration effects are irrelevant. In Figure 4 the same

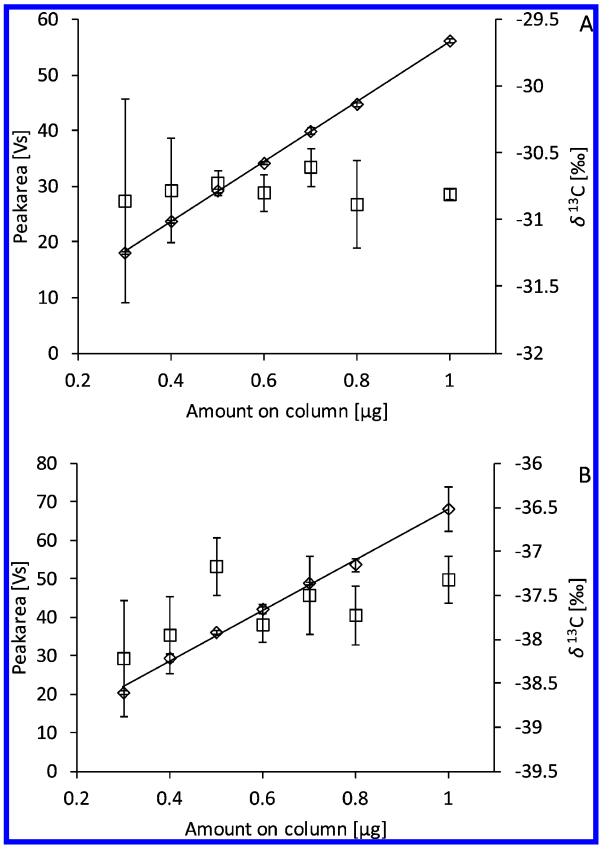

Figure 2. Representative calibration line of sulfadiazine (graph A) and trimethoprim (graph B). ◆ correspond to the peak area of the m/z 44 trace whereas □ relate to $\delta^{13}C$ values.

data were used to compare the difference between EA measurements and FIA measurements with the relative carbon sensitivity (peak area per nanomole of C relative to the reference gas peak area) from flow rate variation. It shows that the sulfonamides have a similar mineralization mechanism assuming that ^{13}C and ^{12}C are equally distributed in the standard compounds.

Incomplete extraction of formed CO_2 in the interface is not the reason for this bias since trimethoprim would follow the same trend as the sulfonamides and lead to a constant offset independent of the substance. For all analytes, $\delta^{13}C$-values were not dependent on concentration (see Figure 2). If incomplete gas extraction caused an isotopic fractionation, $\delta^{13}C$-values of all analytes would be dependent on concentration to the same degree. Thus, this reproducible offset obviously derives from incomplete oxidation.

Smaller peak areas from incomplete conversion are unfavorable because of the loss in sensitivity resulting in the need to use lower flow rates in the HT-LC. On the other hand, a reduction of the HT-LC flow rate could lead to poor peak

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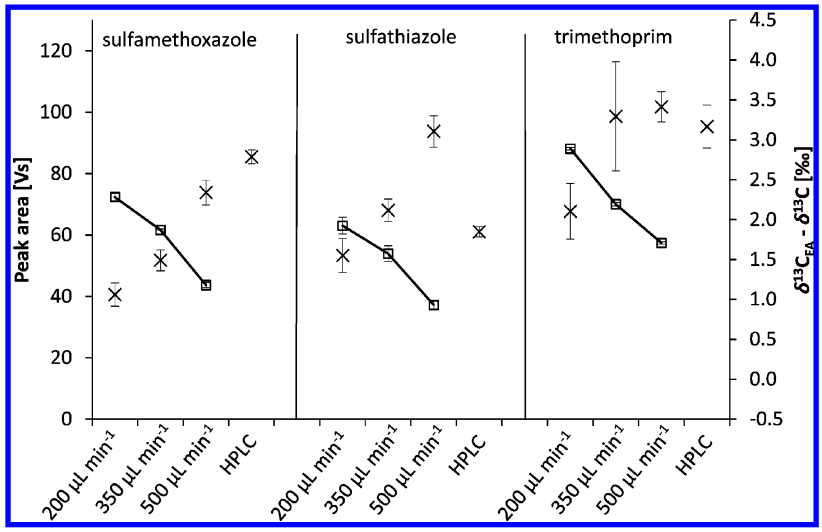

Figure 3. Peak areas (□) and δ^{13}C values (×) determined by FIA at various carrier flow rates and HT-LC-IRMS at 500 µL min^{-1}. No peak areas are given for HT-LC measurements due to incomparable ionization conditions with respect to FIA. Similar trends can be observed for sulfadiazine and sulfamerazine.

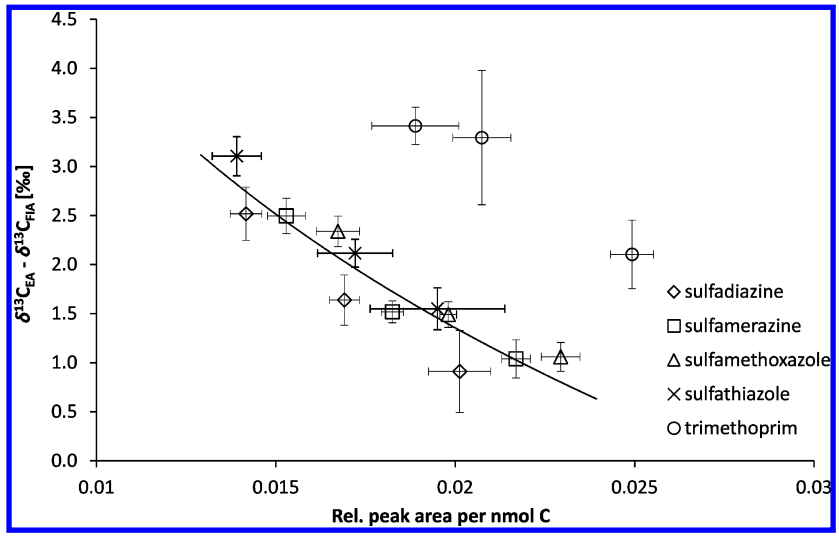

Figure 4. Dependency of the difference between EA-IRMS and FIA-IRMS derived δ^{13}C-values on the relative peak area per nanomole of carbon obtained by different carrier flow rates. Peak areas per carbon amount were referenced to the reference gas peak in order to account for differences in ionization between the single measurements. The solid line represents a logarithmic fit of the sulfonamide results.

Table 3. Bulk and Corrected Compound-Specific δ^{13}C-Values of the Measured Pharmaceutical Samples

brand	$\delta^{13}C_{bulk}$ [‰]	sulfamethoxazole δ^{13}C [‰] column 1[a]	sulfamethoxazole δ^{13}C [‰] column 2[b]	trimethoprim δ^{13}C [‰] column 2
A	−29.6 ± 0.1	−27.6 ± 0.2	−27.7 ± 0.4	−31.1 ± 0.3
B	−29.7 ± 0.4	−27.6 ± 0.3	−28.6 ± 0.4	−30.9 ± 0.3
C	−28.46 ± 0.09	−28.4 ± 0.3	−28.4 ± 0.2	−31.9 ± 0.7
D	−28.5 ± 0.1	−28.0 ± 0.2	−28.1 ± 0.2	−32.1 ± 0.3
E	−30.0 ± 0.4	−27.4 ± 0.2	−27.2 ± 0.4	−30.8 ± 0.4
F	−29.67 ± 0.04	−28.2 ± 0.2	−28.4 ± 0.1	−31.7 ± 0.4

[a]Old column >300 measurements. [b]New column.

shape especially at the end of the chromatographic run where higher temperatures are employed and actually higher flow rates should be used in order not to affect efficiency.[26] Columns with a smaller inner diameter can be used due to their lower optimum flow rate but they might be overloaded by the injected sample amount required for LC-IRMS.[8]

For sulfamethoxazole, sulfamerazine, and trimethoprim, $\delta^{13}C_{HT\text{-}LC}$ values fit $\delta^{13}C_{FIA}$ values obtained at a flow rate of 500 µL min^{-1}. Sulfadiazine and sulfathiazole show a small difference of about 0.9‰. Therefore, δ^{13}C values from samples have to be corrected by external standards if absolute values are needed.

Sample Analysis. Using common detection methods such as UV−vis or organic mass spectrometry, many carbon-bearing substances are mostly not interfering with detection of the target analytes, in particular if analyte-specific wavelengths or

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mass-to-charge ratios are selected. Here, unexpected matrix compounds can cause errors in the analyte peak area, e.g., if they coelute or suppress ionization of the target analyte. However, a full baseline is not a prerequisite for an accurate concentration measurement; coelutions are accepted for the sake of analysis time. In contrast, in LC-IRMS, any carbon-containing compound is detected making baseline separation of the matrix from the target compounds a crucial step in compound-specific LC-IRMS measurements. Overlapping peaks often cause inaccurate isotopic results as pointed out by McCullagh.[7]

Pharmaceutical samples gave no noteworthy contribution of matrix peaks to the chromatogram (see Figure 1B). In the small set of samples analyzed in this work, the variation of the corrected δ^{13}C values of sulfamethoxazole and trimethoprim from different manufacturers was small. However, there was a substantial difference between bulk sample δ^{13}C values and compound-specific δ^{13}C values (see Table 3).

It was observed that an aged column (column 1 in Table 3; >300 injections) showed an increased background signal due to column bleeding. To investigate the effect of an aged column on the measurement of the isotopic signature of the compounds, we compared the results with a second new column (see column 2 in Table 3). As shown in Table 3, repeated analysis of the pharmaceutical samples also proved a good repeatability of this method for most of the compounds. The same δ^{13}C-values were obtained for five of the six measured samples. An exception was observed for sulfamethoxazole of brand B between the old (column 1) and the new column (column 2) and trimethoprim, which gave no reliable results. The difference of about 1‰ can be explained by a steep increase of the background caused by column bleeding.

A two-dimensional plot of the δ^{13}C-values of trimethoprim and sulfamethoxazole suggests that the combined analysis could identify falsified antibiotics containing these compounds (see Figure 5). Together with bulk δ^{13}C-values, the individual samples can be distinguished clearly from each other. However, the analyzed set of samples is too small to draw statistically confirmed conclusions.

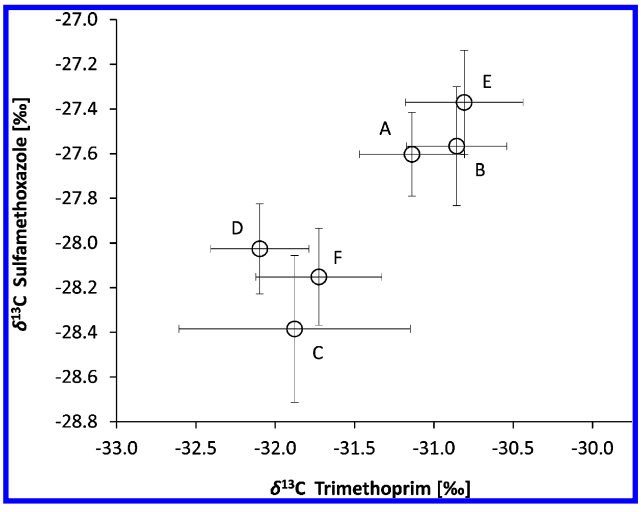

Figure 5. Corrected δ^{13}C-values of trimethoprim and sulfamethoxazole from pharmaceutical products.

CONCLUSIONS

In this work we present the first method for compound-specific carbon isotope analysis of pharmaceuticals, which cannot be separated by conventionally used techniques for LC-IRMS. Substance amounts as low as 0.3 μg are sufficient to perform a precise analysis. The applicability of this method for pharmaceutical research and product authentication was demonstrated. It is also the first step toward a study of the degradation and mobility of these substances in the environment on an isotopic level. Following appropriate sample enrichment compound-specific δ^{13}C-values of highly contaminated samples can be easily measured but need to be corrected by external standards.

There are also some indications that the frequent offset of δ^{13}C-values from LC-IRMS and EA-IRMS measurements derives from incomplete oxidation suggesting the need for improvements of the interface for higher sensitivity and accuracy.

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Notes

The authors declare no competing financial interest.

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