

irm-LC/MS: Easy Referencing by Flow Injection ¹³C/¹²C Isotope Ratio Analysis

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Introduction

Referencing in isotope ratio MS is a basic principle, which takes into account that the relative isotopic abundance results from the comparison of the measured isotope ratio of the analyte with the measured isotope ratio of a reference. This allows expressing minute isotopic differences, due to normalization towards an international standard.

The isotope community has established the δ -notation, which is the difference in the ¹³C/¹²C isotope ratios of the sample and the standard normalized by the ¹³C/¹²C isotope ratio of the standard. The resulting $\delta^{13}\text{C}$ value is given in ‰.

$$\delta^{13}\text{C} = \frac{R_{\text{Sample}} - R_{\text{Reference}}}{R_{\text{Reference}}} * 1000 \text{ [‰]} \quad R: ^{13}\text{C}/^{12}\text{C}$$

All $\delta^{13}\text{C}$ data have to be reported versus Vienna PDB (VPDB), the international accepted primary standard (Figure 1). Two ways are established to compare isotope ratio data on an international basis. Either samples are determined versus a reference gas, which is calibrated against VPDB, or samples are compared versus international secondary reference materials.

Unfortunately, most reference materials are not applicable in helium based continuous flow techniques. Consequently, CO₂ reference gas pulses are admitted to the ion source within continuous flow applications. This has become common practice because of its user-friendliness and practicability.

There are only few organic secondary standards, for example, NBS 22, a hydrocarbon mixture, which can be only analyzed by elemental analyzer (EA). In 2006, glutamic acid, benzoic acid and caffeine have been additionally introduced as organic reference materials^[2].

For *irm*-LC/MS the Thermo Scientific LC IsoLink interface closes this gap in referencing. A direct flow injection mode for sub- μg bulk materials (μ -EA mode) is available in addition to the compound specific analysis using HPLC separation. This direct injection mode is a fast and precise analysis of all water soluble materials. It enables the analysis of reference material with unmatched sensitivity and speed. Reference material can be injected into the sample loop of a six-port valve which is located after the HPLC column. The reference can be introduced on trace before, in-between or after the eluting target compounds or just for a extremely fast and simple comparison of bulk materials.

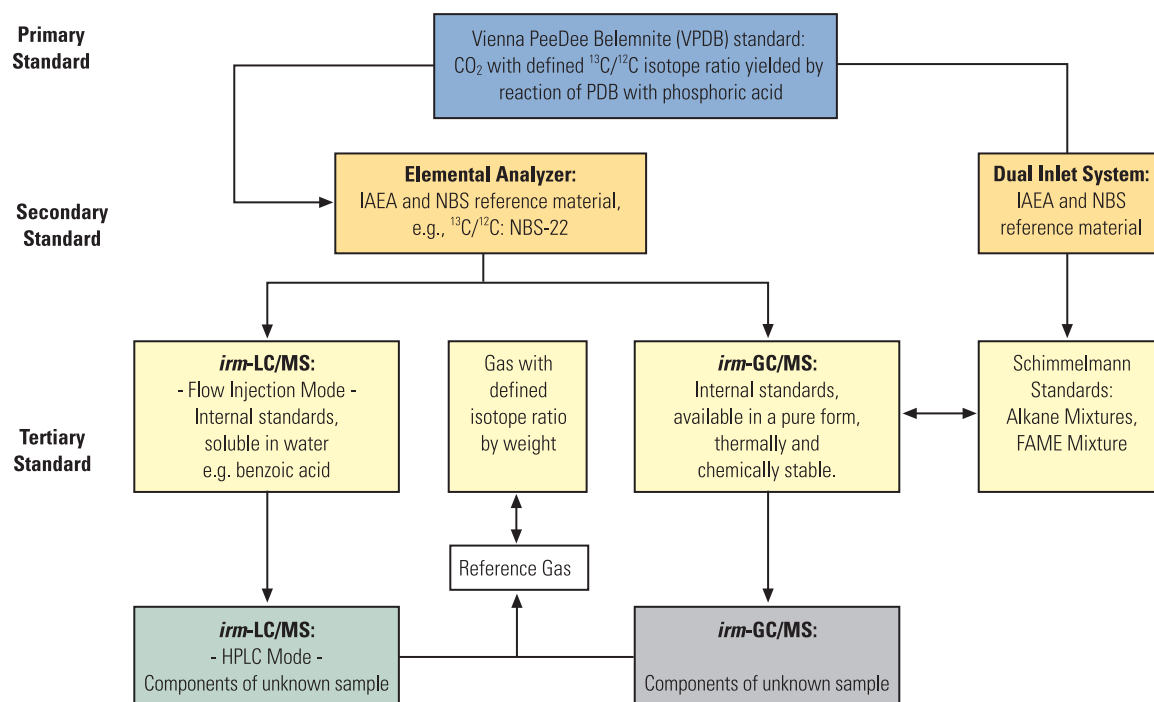


Figure 1: Referencing in isotope ratio mass spectrometry.

Key Words

- LC IsoLink™
- Bulk Analysis
- Flow Injection
- Isotope Ratio MS
- Referencing

Benzoic acid, the simplest aromatic carboxylic acid with a carboxyl group directly bonded to the benzene ring, has been selected as reference material for *irm*-LC/MS. One of the advantages is the long-term stability in water. It has good anti-microbial features. Sodium benzoate, which dissolves well in water (solubility: 290 mg /100 ml water), is often used. In acidic solutions it converts to benzoic acid. It's widely used as a preservative in foods, beverages and cosmetics, e.g. as a mild antiseptic in mouthwashes and toothpastes.

irm-LC/MS Technology

The LC IsoLink is the first high sensitivity interface connecting High Performance Liquid Chromatography (HPLC) with Isotope Ratio MS for the reproducible and accurate on-line determination of $^{13}\text{C}/^{12}\text{C}$ isotope ratios. All organic compounds eluting from an HPLC column are analyzed while maintaining the chromatographic resolution^[1].

In the LC IsoLink the sample is oxidized within the aqueous solvent eluting from the HPLC. The generated CO_2 is separated from the liquid phase and fed into the IRMS ion source.

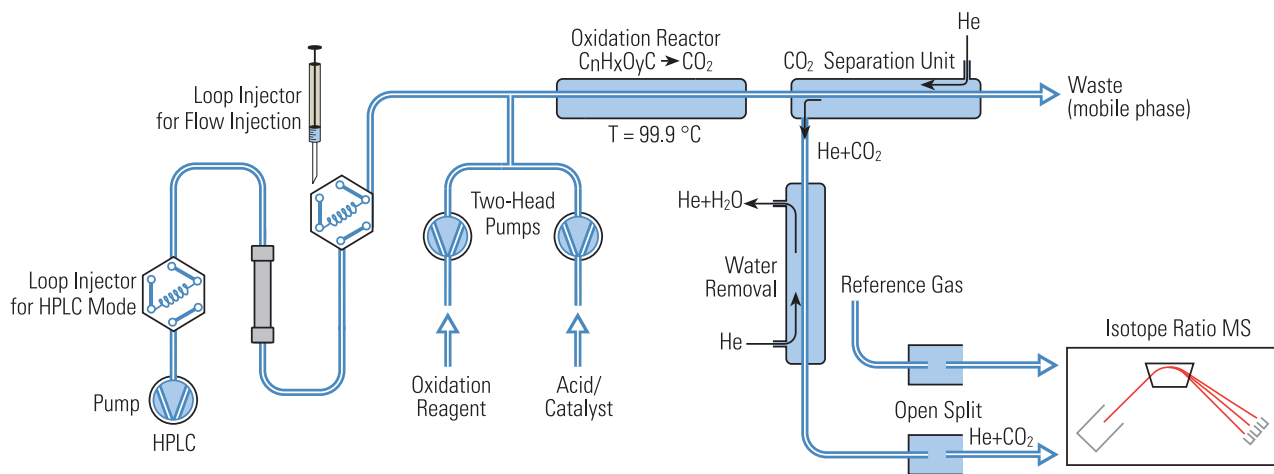
The oxidation reagent consists of two solutions, the oxidizing agent and an acid mixture. Both are pumped separately and added to the mobile LC phase. Within this mixture all individual organic compounds eluting from the HPLC column are oxidized quantitatively into CO_2 when passing through a heated reactor. In a downstream separation unit the CO_2 is removed from the liquid phase and entrenched into a stream of He. The individual CO_2 peaks in He are subsequently dried in an on-line gas drying unit (Nafion[®]) and then admitted to the Isotope Ratio MS via an open split interface.

Experimental Section

Figure 2 shows the technology of the LC IsoLink interface. The interface can be used in either of two operational modes, one for compound specific isotope analysis (*irm*-HPLC/MS mode) and one for bulk stable isotope analysis (μ -EA mode).



Figure 2: Scheme of the Thermo Scientific *irm*-LC/MS system with the LC IsoLink.



The oxidation is obtained by adding two reagents to the mobile phase. The two reagents consist of the oxidant, e.g. sodium peroxodisulfate and phosphoric acid. Additionally, silver nitrate as a catalyst can be added to the phosphoric acid (Table 1).

For preparation of the reagents water of HPLC-grade is used. The solutions are degassed in an ultrasonic bath under vacuum using a water jet pump. The reagents are pumped separately allowing different flow rates. The reaction is carried out in a stainless steel capillary reactor at 99.9 °C maintaining HPLC resolution.

Table 1 shows the analytical conditions, which were applied for bulk analysis in μ -EA mode (Flow Injection).

HPLC Parameters	
Pump:	Thermo Scientific Surveyor MS Pump
Mobile Phase:	Water, HPLC-grade, degassed with helium
Flow:	300 μ l/min
Interface Parameters	
Reagent Pump 1:	a.) 0.44 M Peroxodisulfate b.) 0.88 M Peroxodisulfate
	Flow: 30 μ l/min
Reagent Pump 2:	1.3 M H ₃ PO ₄ (density: 1.88 g/cm ³)
	Flow: 30 μ l/min
Reactor Temperature:	99.9 °C
He Carrier Gas:	2 ml/min
Sample Loop 2:	10 μ l

Table 1: HPLC and LC IsoLink parameters.

Evaluation Strategy

Two essential steps in the LC IsoLink interface are the conversion of organic compounds to CO₂ in the oxidation reactor and the separation of the produced CO₂ from the aqueous phase in the gas separation unit.

1. Quantitative Conversion

In isotope ratio MS quantitative conversion of organic components is mandatory in order to avoid fractionation. Complete conversion is achieved by adding two reagents to the mobile phase. The oxidation reagent consists of a salt of peroxodisulfate in water and the acid reagent. Both reagents are pumped separately. To avoid pulsations of the reagent flows a pulse damper is located behind the two reagent pumps. The mixture of reagents is added to the mobile phase at a T-piece.

Water-soluble peroxodisulfate, M₂S₂O₈, is available with three different cations (M⁺= NH₄⁺, Na⁺, K⁺). Potassium peroxodisulfate, K₂S₂O₈ cannot be used in higher concentration because of its low solubility in water (5.2 g in 100 ml, 20 °C) whereas the solubility of Na₂S₂O₈ (54.5 g in 100 ml, 20 °C) and (NH₄)₂S₂O₈ (62 g in 100 ml, 20 °C) is much higher.

Under oxidative conditions NO₂ can be produced by (NH₄)₂S₂O₈, which contributes to the m/z 46 background. Therefore, Na₂S₂O₈ is preferably used. In aqueous solution oxygen is produced by heating^[3]: $2 \text{Na}_2\text{S}_2\text{O}_8 + 2 \text{H}_2\text{O} \rightarrow 2 \text{Na}_2\text{SO}_4 + 2 \text{H}_2\text{SO}_4 + \text{O}_2$

The solid chemicals have to be stored under cool and dry conditions avoid production of O₂ and O₃.

Aqueous solutions of peroxodisulfate salts are quite stable for at least one week. The oxidation potential of the oxidant can be increased by adding a catalyst.

Ag⁺ ions act as a catalyst, which increases the oxidative power of peroxodisulfate. Silver ions cannot be used with saline samples. If a sample (or the mobile phase) contains chloride AgCl is precipitated and may plug the capillaries. This would damage the separation unit. Other ionic components having a small solubility product with Ag⁺ are known, e.g.: Br⁻, I⁻, S²⁻ (Table 2).

Formula	K _{sp}
Ag ₂ SO ₄	1.5 x 10 ⁻⁵
AgN ₃	2.8 x 10 ⁻⁹
AgCl	1.8 x 10 ⁻¹⁰
AgSCN	1.1 x 10 ⁻¹²
AgBr	5.0 x 10 ⁻¹³
AgCN	2.2 x 10 ⁻¹⁶
AgI	8.3 x 10 ⁻¹⁷
Ag ₂ S	8.0 x 10 ⁻⁵¹

Table 2: Solubility products of silver salts.

Samples which contain such ions should not be analyzed in presence of silver ions. Such anions could be precipitated before analysis.

Alternatively, the oxidant can be used in higher concentration without silver nitrate to ensure quantitative oxidation. For evaluation of the conversion in the interface different concentration of the oxidation reagents have been applied.

2. Separation of CO₂

The separation of CO₂ by the membrane exchanger should be also free of fractionation. The CO₂ is separated from the aqueous phase through thin membranes into a counter flow of helium. The almost quantitative degassing of the liquid phase is accomplished by the use of three membranes. The difference of CO₂ partial pressures inside and outside of the membranes contributes to the degassing. The separation efficiency has been evaluated by injection of different amounts of benzoic acids.

Results and Discussion

The LC IsoLink has been evaluated by flow injection (μ -EA mode) of benzoic acid. Different concentrations of benzoic acid were injected to evaluate the precision and linearity.

Figure 3 shows the repeated analysis of 188 ng and 2907 ng of benzoic acid demonstrating the speed of analysis. The analysis time is given by the transfer time through the interface, e.g. 1 min at a flow of 300 μ l/min of the mobile phase. Multiple injections can be performed within one data acquisition. Amounts between 188 ng and 1500 ng were injected 10 times, resulting in a standard deviation mostly of less than 0.1 % (Table 3).

For the first approach 0.44 M of peroxodisulfate has been used resulting in reproducible delta values up to 1500 ng. The injected amounts of 1875 ng and 2344 ng also show a good precision whereas the delta values become more negative. This is due to incomplete conversion. Consequently, a higher concentration of peroxodisulfate (0.88 M) has been applied.

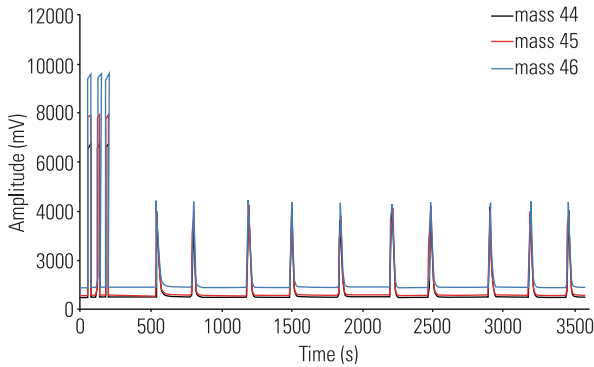


Figure 3a: *irm*-LC/MS traces (m/z 44, 45, 46) of benzoic acid analyzed by flow injection (μ -EA mode). Injection of 188 ng.

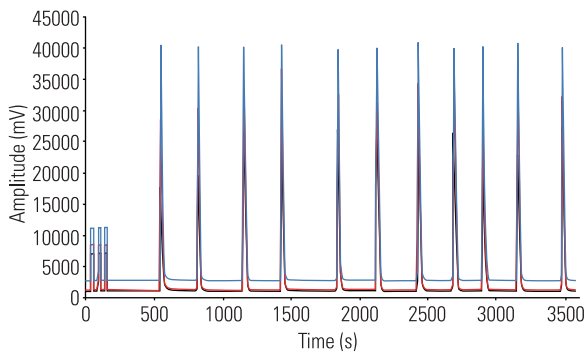


Figure 3b: *irm*-LC/MS traces (m/z 44, 45, 46) of benzoic acid analyzed by flow injection (μ -EA mode). Injection of 2907 ng.

Precision of $\delta^{13}\text{C}$ values of benzoic acid analyzed by flow injection *irm*-LC/MS (μ -EA mode)

	$\delta^{13}\text{C}$ (‰)	Std dev.	n
188 ng	-28.69	0.11	10
300 ng	-29.34	0.05	10
469 ng	-29.30	0.07	10
938 ng	-29.54	0.05	10
1500 ng	-29.60	0.04	10
1875 ng	-30.19	0.02	5
2344 ng	-31.04	0.02	5

Table 3a: Concentration of the oxidation reagent: 0.44 M $(\text{NH}_4)_2\text{S}_2\text{O}_8$.

Precision of $\delta^{13}\text{C}$ values of benzoic acid analyzed by flow injection *irm*-LC/MS (μ -EA mode)

	$\delta^{13}\text{C}$ (‰)	Std dev.	n
1875 ng	-29.38	0.05	10
2344 ng	-29.24	0.08	10
2907 ng	-29.29	0.12	10

Table 3b: Concentration of the oxidation reagent: 0.88 M $(\text{NH}_4)_2\text{S}_2\text{O}_8$.

As shown in Figure 4 a higher concentrated reagent guarantees complete conversion resulting in consistent $\delta^{13}\text{C}$ values. Figure 4 exhibits an excellent linear behavior of the LC IsoLink. In the range from 300 ng to 2907 ng of benzoic acid (200 - 2000 ng carbon) the overall slope is 0.004 ‰ per 100 ng. The standard deviation is ± 0.14 ‰ (1 s). Hence, the conversion is demonstrated to be fractionation-free. Figure 5 shows the correlation between the peak area and the amount injected. A strong linear correlation has been found. The correlation is excellent with a coefficient of 0.999.

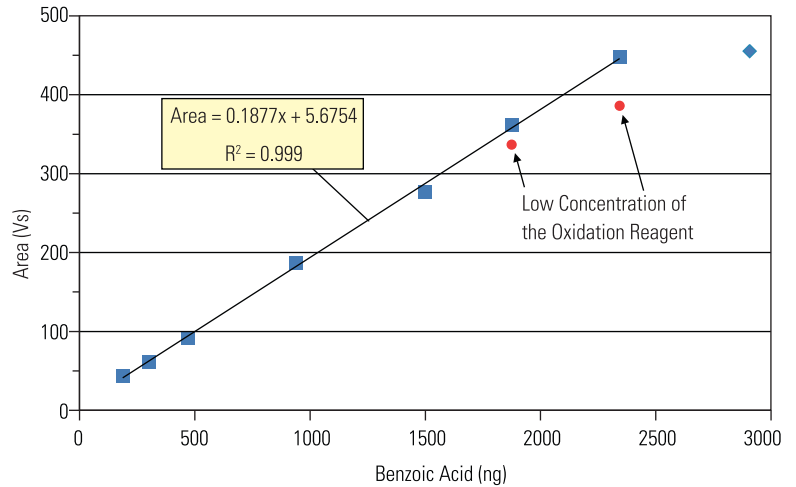


Figure 4: Linearity of benzoic acid analyzed by flow injection *irm*-LC/MS (μ -EA mode).

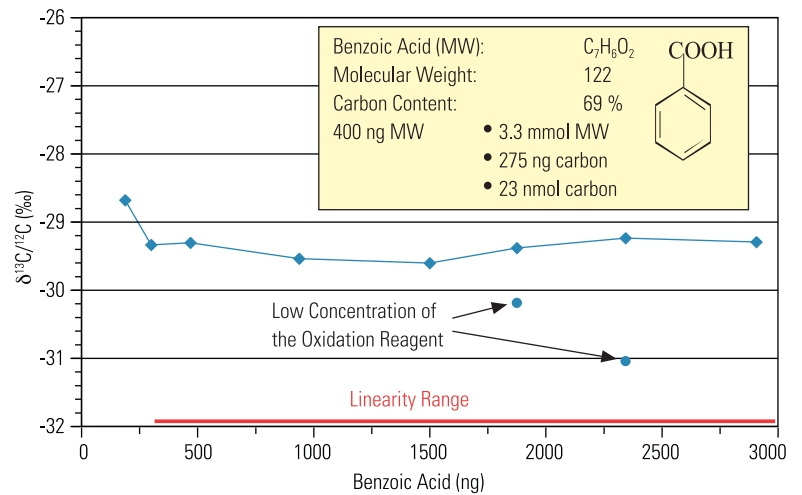


Figure 5: Direct injection *irm*-LC/MS (μ -EA mode) of benzoic acid, quantitation analysis by correlation of area [Vs] and amount injected [ng].

Although a high concentrated oxidant reagent was applied, the benzoic acid sample containing 2907 ng exhibits a peak area which is too small. This shows that the separation for such a high amount of CO_2 is no longer quantitative in the membrane exchange unit. Nevertheless, Figure 4 shows that higher amounts can still be analyzed fractionation-free. Under above conditions accurate quantification of amounts can be performed between 300 ng and 2344 ng.

Conclusions

The direct flow injection mode of the LC IsoLink enables a new approach for $\delta^{13}\text{C}$ referencing and bulk analysis in the sub- μg range. This outperforms the elemental analyzer for bulk $\delta^{13}\text{C}$ analysis by sample size and ease of use with typical sample amounts at 200 to 2000 ng of carbon.

Internal standards can be added during the run. Figure 6 shows the mass 44, 45, 46 traces of a standard mixture of different organic acids typical in fruits. Benzoic acid was introduced in-between and after the elution of the organic acids. This example illustrates the possibility for enhanced referencing to achieve unambiguous data.

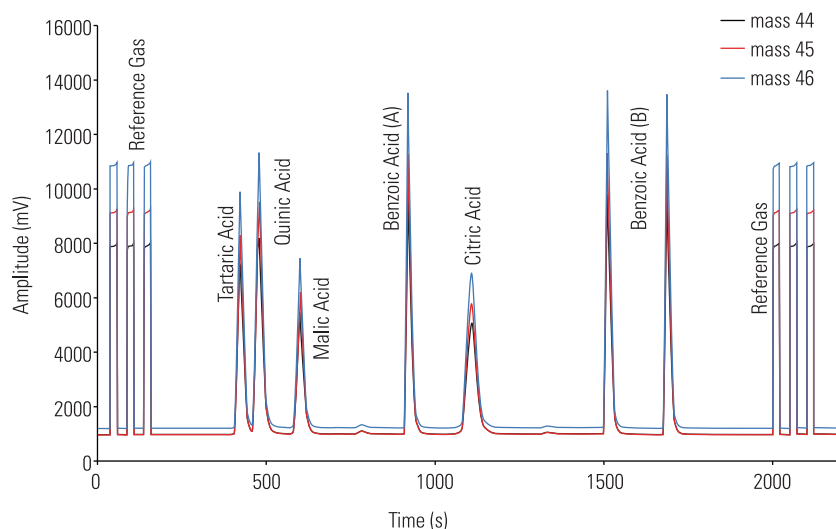


Figure 6: *irm*-HPLC/MS traces (m/z 44, 45, 46) of a fruit mix standard analyzed, benzoic acid was additionally introduced on trace as an internal standard by flow injection (μ -EA mode), A in-between the target components, B after the target components.

The LC IsoLink interface allows new studies for stable isotope research in pharmaceutical, biological, medical and environmental studies. Non-volatile components which are only accessible by liquid chromatography can be analyzed even in a complex matrix.

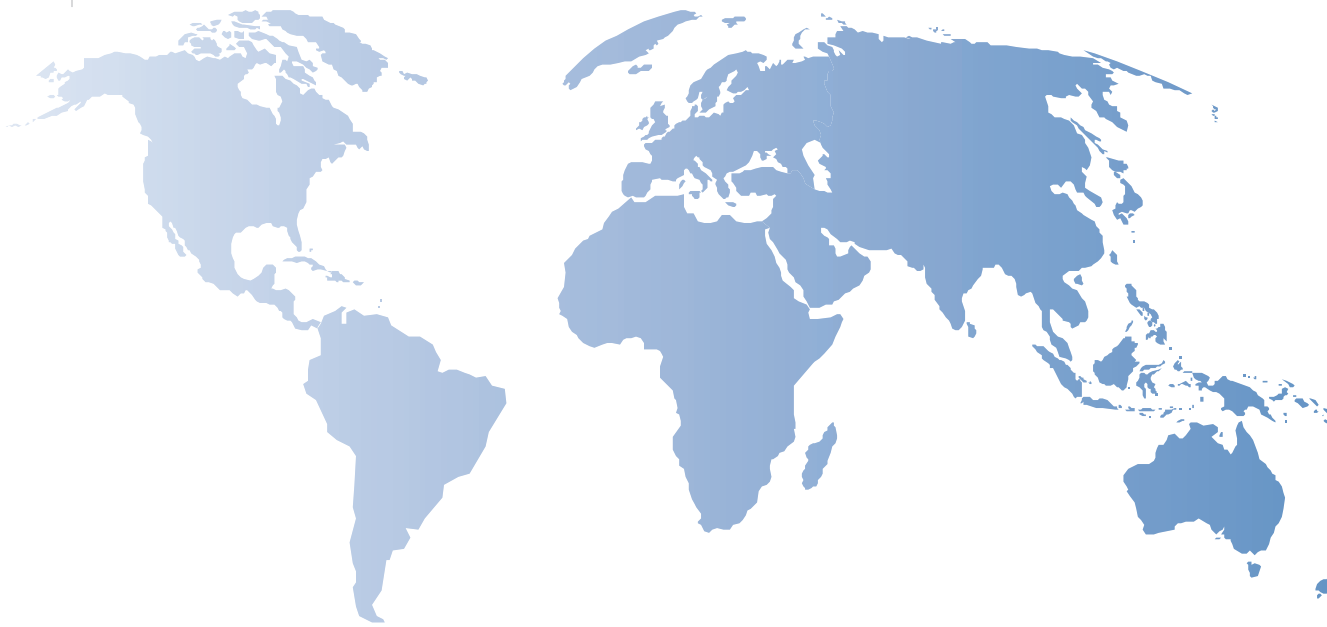
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