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## Introduction

*Stevia rebaudiana* (Bertoni) (family Asteraceae (Compositae)) is a sweet herb native to South America. *S. rebaudiana* has been used by the Guarani Paraguayan Indians as a sweet substance for their traditional mate drink [1]. Products prepared from refined leaf extracts of *S. rebaudiana* or the pure diterpene glycosides stevioside and rebaudioside A are widely used as sucrose substitutes in many countries. The Joint FAO/WHO Expert Committee on Food Additives JECFA reviewed the safety of steviol glycosides and established an acceptable daily intake (ADI) for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day [2]. With wider commercial use of *S. rebaudiana* sweeteners and the regulatory approval for the use of stevia extracts in food and beverages by the European Food Safety Authority (EFSA) there is an increased need for methods for quantitating steviol glycosides in food containing Stevia [2,3]. The focus of this work was to analyze steviol glycosides in complex food matrices like jam, beverages, candies and yoghurt with LC-UV and LC-MS/MS. The analytes of interest for routine analysis were the sweet tasting main components rebaudioside A and stevioside of *S. rebaudiana*.

## Some food-uses and use-levels for steviol glycosides evaluated by the EFSA<sup>1</sup> and established by the European Commission [4]

Food matrix	Maximum Use Levels [mg/kg]		
	Steviol Equivalents	Rebaudioside A <sup>2</sup>	Stevioside <sup>2</sup>
Candies	270	821	684
Jam, jellies and marmalade	200	507	608
Flavored and fermented milk products	100	304	253
Flavored beverages	80	202	243

<sup>1</sup>EFSA= European Food Safety Authority

<sup>2</sup>calculated

## Sample preparation and instrumentation

5 g of samples were dissolved in 50 ml H<sub>2</sub>O and heated for 30 min at 60°C. The samples were then sonicated for 30 min and centrifuged. The supernatant was loaded on a C18 SPE cartridge (Mallinckrodt Baker, Philipsburg, US) [5]. The cartridge was washed first with H<sub>2</sub>O and then with 20/80 ACN/H<sub>2</sub>O (v/v). The steviol glycosides were eluted from the cartridge by using 80/20 ACN/H<sub>2</sub>O (v/v) and diluted with 80/20 ACN/H<sub>2</sub>O (v/v) prior to the analysis by LC-UV or LC-MS/MS.

The extracted steviol glycosides were separated and detected by an Agilent 1100 HPLC (Waldbronn,GE) coupled to a MS/MS 3200 Q Trap (AB SCIEX, Darmstadt, GE). Different HILIC columns were used.

## Chromatography

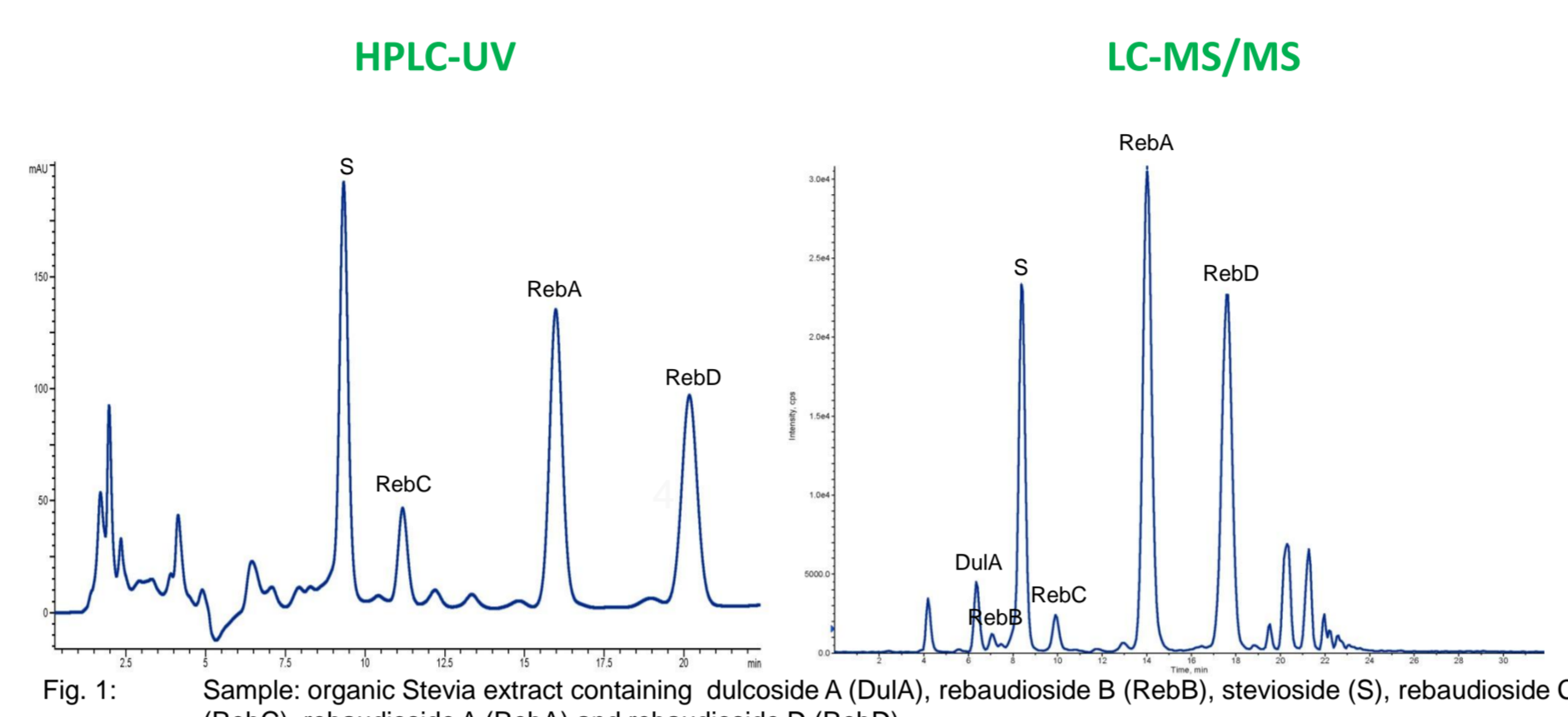


Fig. 1: Sample: organic Stevia extract containing dulcicoside A (DuIA), rebaudioside B (RebB), stevioside (S), rebaudioside C (RebC), rebaudioside A (RebA) and rebaudioside D (RebD)

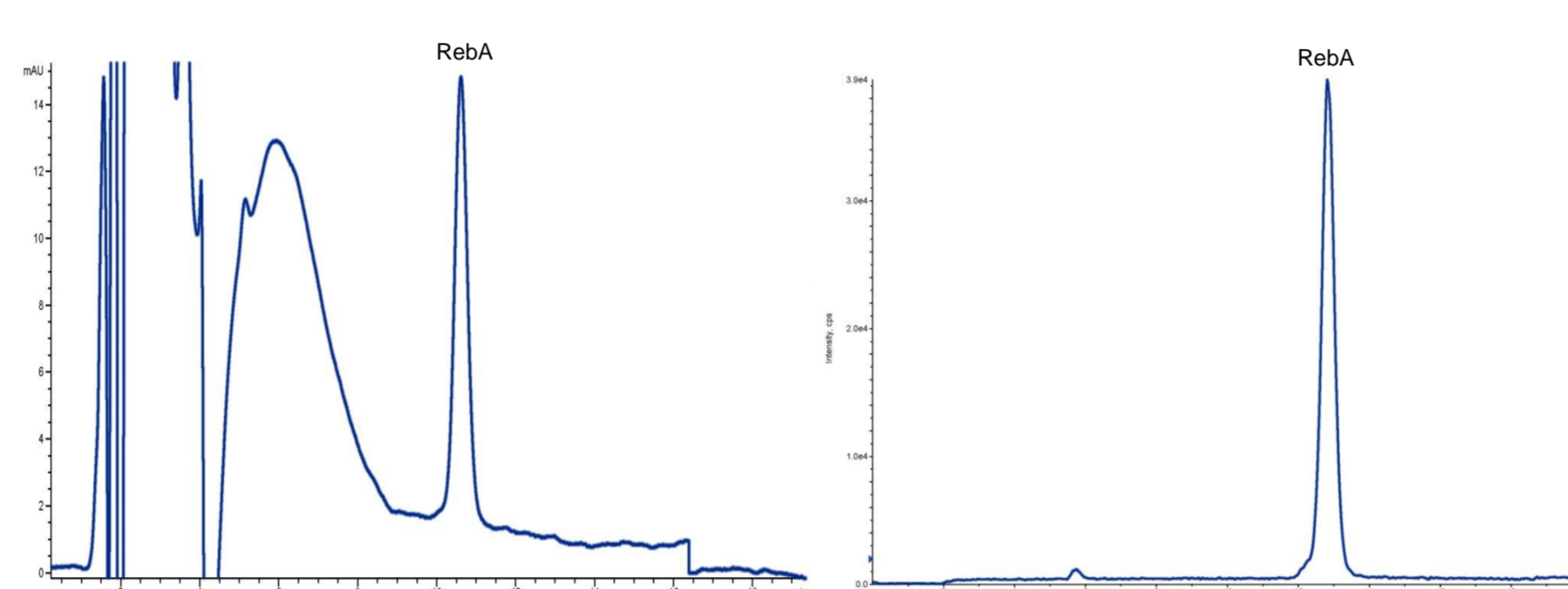


Fig. 2: Sample: strawberry jam containing 55 mg/kg rebaudioside A (RebA)

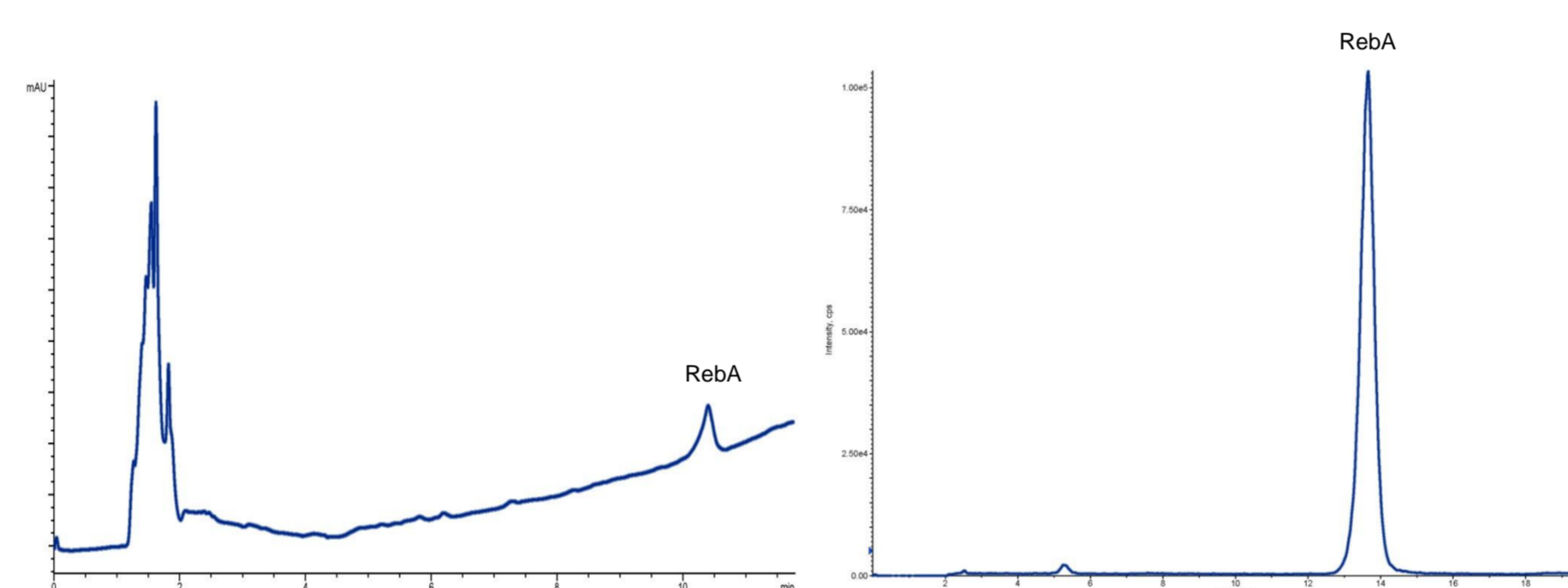


Fig. 3: Sample: caffeinated beverage containing 15 mg/kg rebaudioside A (RebA)

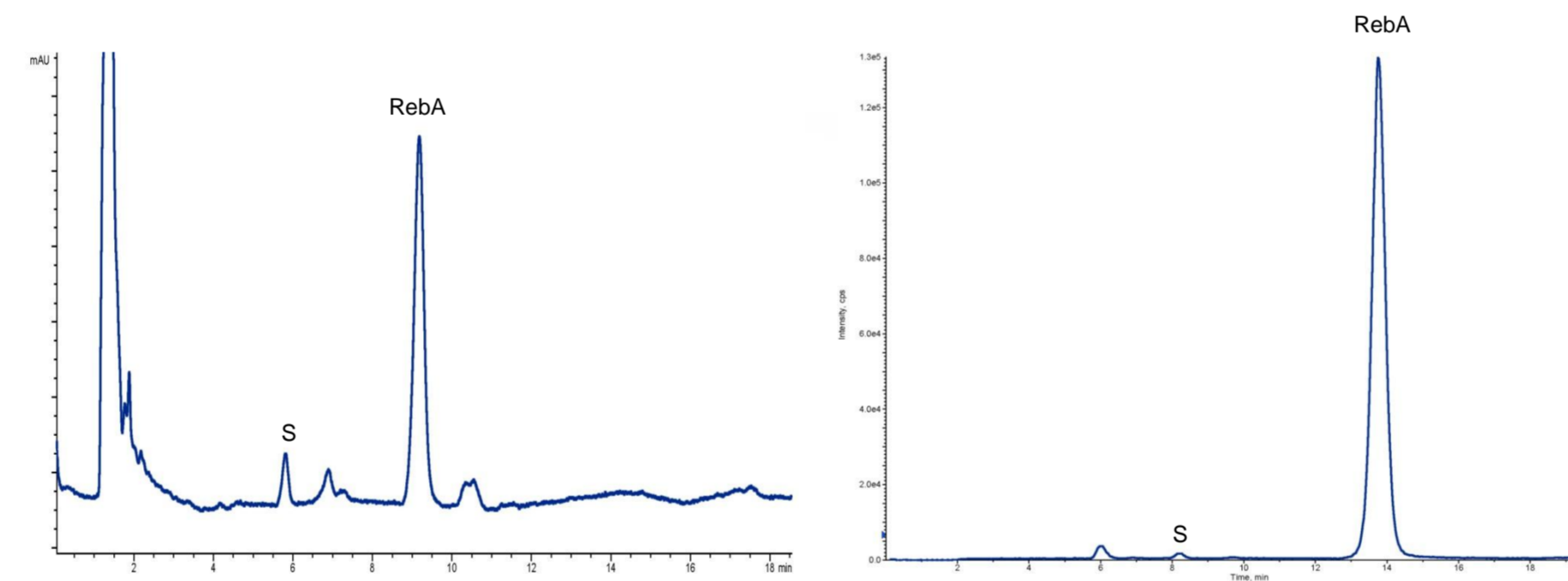


Fig. 4: Sample: candies containing 25 mg/kg stevioside (S) and 300 mg/kg rebaudioside A (RebA)

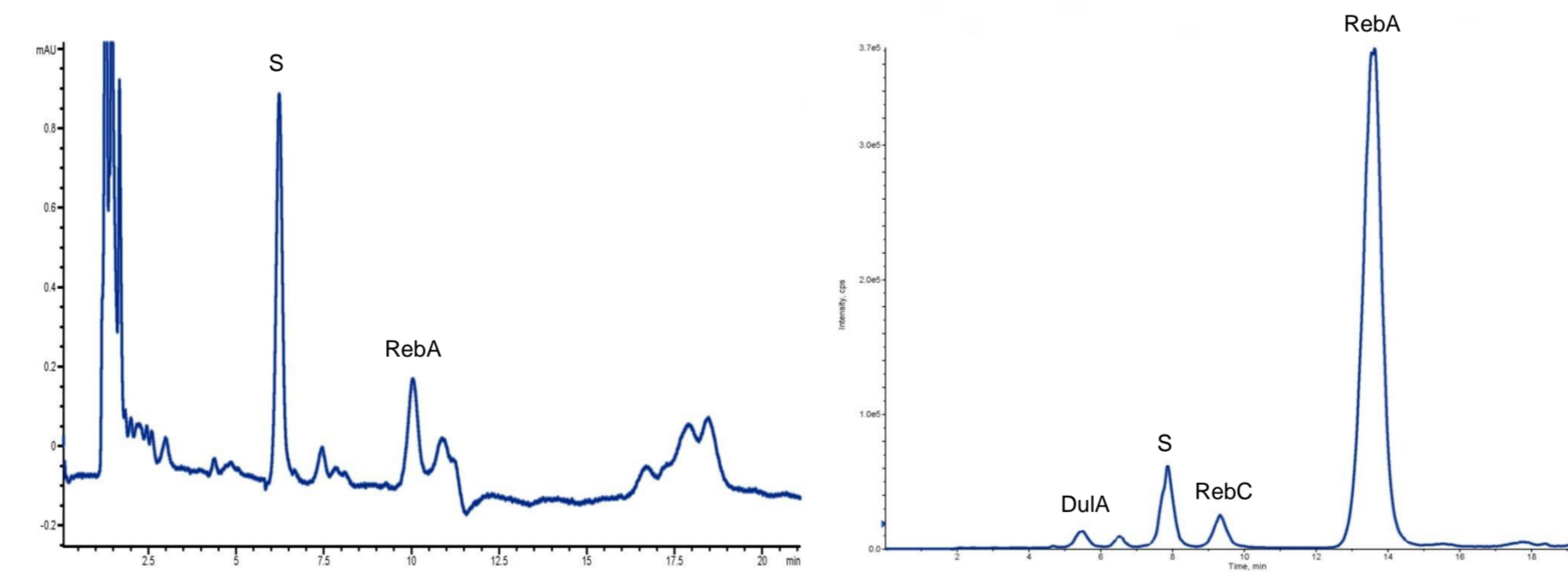


Fig. 5: Sample: yoghurt containing dulcicoside A (DuIA), rebaudioside C (RebC), 45 mg/kg stevioside (S) and 20 mg/kg rebaudioside A (RebA)

## Quantitation

Ion suppression or ion enhancement is a wide known drawback of LC-MS/MS. Therefore a strategy to minimize matrix effects is necessary. Isotopically labeled stevioside and rebaudioside A were not commercially available and custom synthesis of these compounds would have been very expensive. Therefore we used matrix-matched calibration to reduce matrix effects in quantitation. Matrix-matched calibration curves at five concentration levels were obtained from stevioside and rebaudioside A stock solution.

Regression analysis was employed to determine the linearity of the calibration graphs. The calibration curves were linear within the given concentration levels.

## Validation

Linearity, intra-day precision and accuracy were determined by adding 20 - 500 mg/kg of stevioside and rebaudioside A to analyte-free samples of jam, beverages, candies and yoghurt.

The method was linear for the added amounts of stevioside and rebaudioside A for all matrices.

Intra-day precision ranged between 3.5 and 9%.

Accuracy ranged between 90 and 106%.

## Conclusions

The sample clean-up prior to the analysis significantly reduces matrix effects and is applicable for many food matrices.

However, for the analysis of more complex matrices, enhanced selectivity and sensitivity of MS-MS-detection is needed.

## References

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- [3] EFSA Panel on Food Additives and Nutrient Sources <http://www.efsa.europa.eu/de/efsajournal/pub/1537.htm>
- [4] Commission Regulation (EU) No 1131/2011 of 11 November 2011
- [5] Wölwer-Rieck, U., Wawrzun, A., Wüst, M. (2010) Eur Food Res Technol. 231, 581–588

