JTI-Ökolab
Vienna, Austria

Determination of *N*-Nitrososarcosine (NSAR) in tobacco

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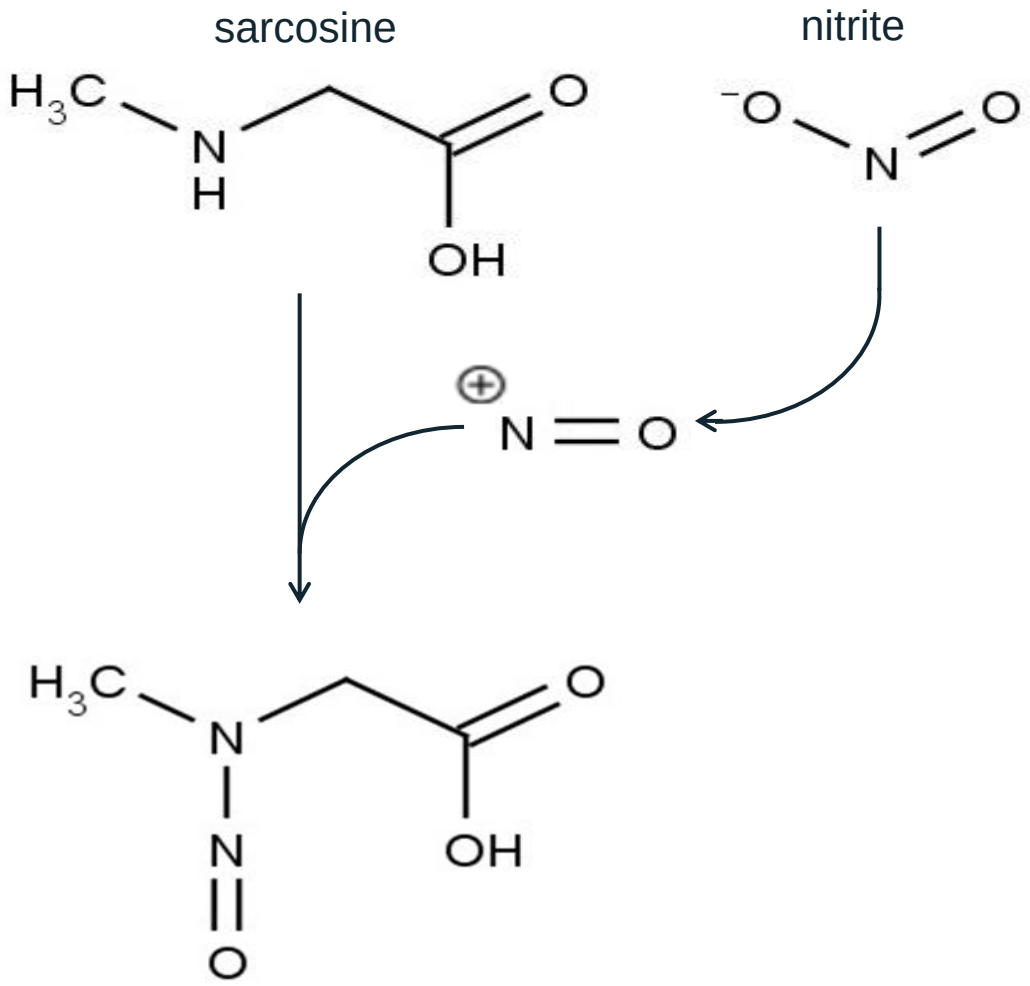
Background

What is N-Nitrososarcosine (NSAR)?

- *N*-Nitrosamine
  - non-volatile nitrosamino acid
  - formed by nitrosation of amino acid sarcosine

- listed on a draft list by the FDA for harmful and potentially harmful constituents in tobacco products and tobacco smoke
  - IARC 2B: possibly carcinogenic to humans
  - present in smokeless tobacco (ng/g range)

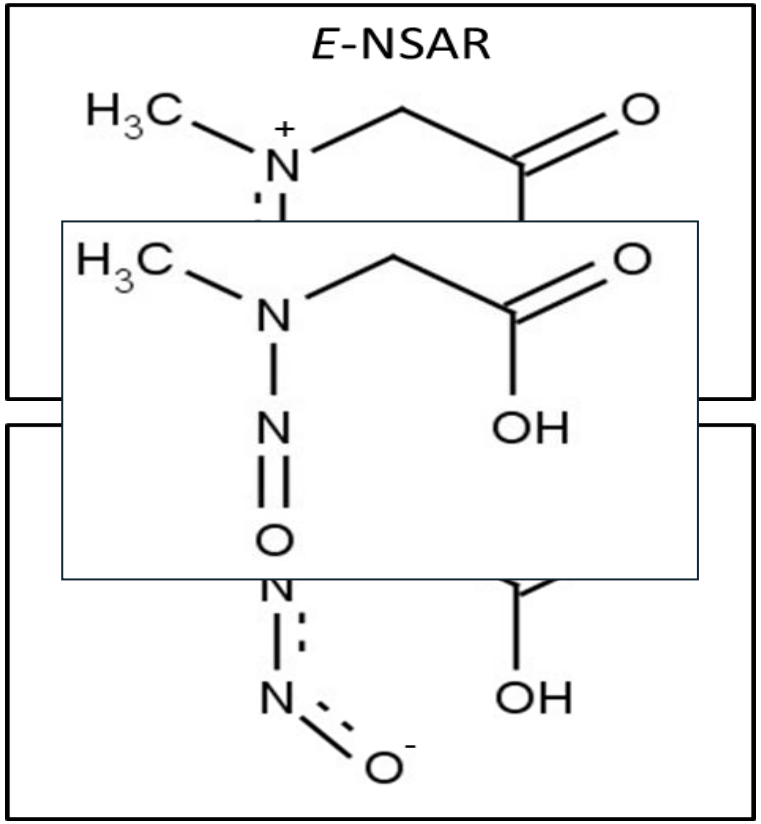


Background

What is N-Nitrososarcosine (NSAR)?

- exists as two stereoisomers
  - partial double bonds restrict rotation
  - solid state: *Z*-configuration
  - in solution: isomerization to *E*-configuration with an isomeric ratio of about 1:1 at equilibrium

[Chow et al., Organ. Magn. Reson. 1981, 15, 200]



Background

Options for NSAR analysis

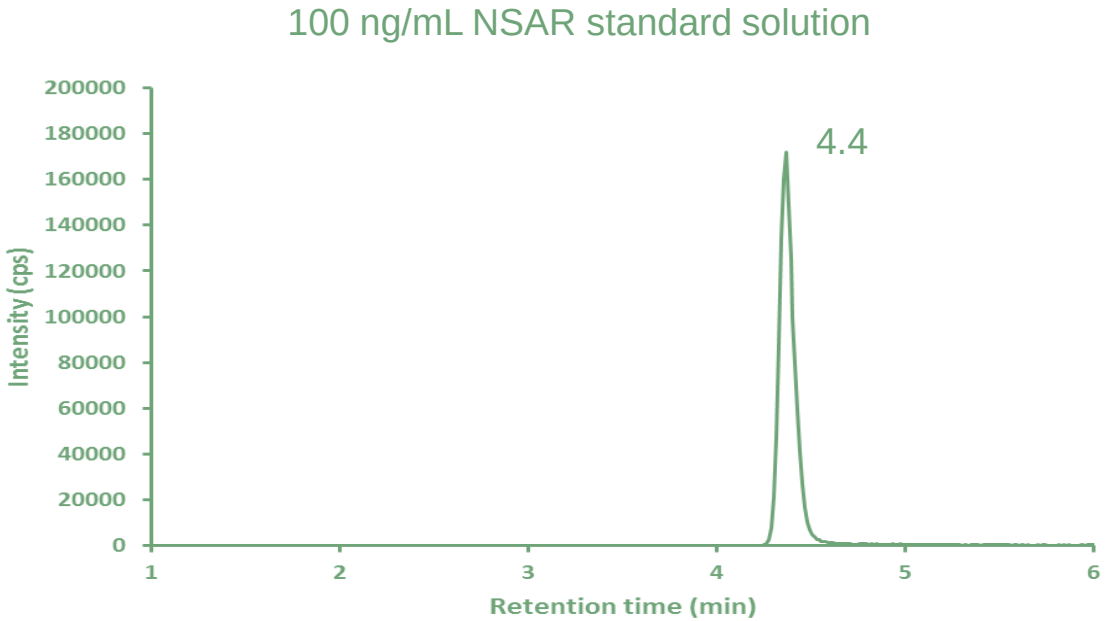
- regarding separation
  - gas chromatography: necessity of time-consuming derivatization
  - liquid chromatography: high polarity impedes retardation on conventional reversed phase columns
- regarding detection
  - TEA: low selectivity
  - ESI-MS
    - impedes addition of ion pairing reagents
    - low m/z range is susceptible to high noise

LC-ESI-MS/MS using a suitable stationary phase

Method development

Liquid chromatography

- Reversed phase
  - conventional C18 → no retardation
  - C18 + ion pairing reagent triethylamine → hampered ionization
- HILIC
  - Hydrophilic Interaction Liquid Chromatography
  - different stationary phases tested
  - best results with Obelisc N from SIELC
- Final mobile phase
  - 5 mM ammonium formate and 0.1% formic acid in 95/5 (5/95) water/acetonitrile
- Internal standard
  - NSAR-D_3



Method development

Sample preparation

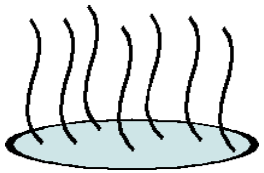
2 g sample

spiking with internal standard NSAR-D_3

extraction with 25 mL of 2% aqueous formic acid for 45 min under agitation

reconstitution with 1 mL of mobile phase B for subsequent LC-MS/MS analysis

evaporation to dryness under nitrogen at 50 °C



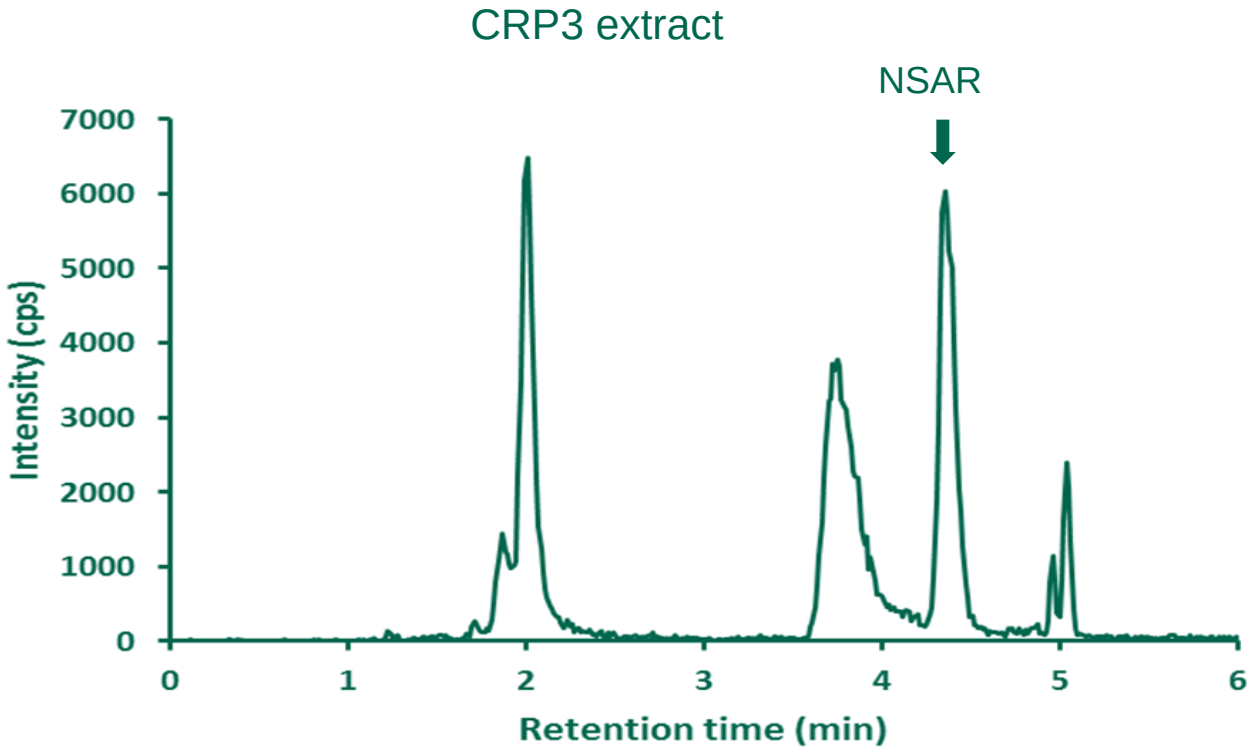
10 mL of supernatant loaded onto solid supported liquid extraction cartridge

elution with 2 × 20 mL of ethyl formate

based on [Wu et al., Anal. Methods 2012, 4, 3448]

Method development

Liquid chromatography



- Matrix peaks well separated
- 2 optimized MRM transitions required
  - Quantifier for quantification
  - Qualifier for identity confirmation

Method development

Mass spectrometry

- negative electrospray ionization
- multiple reaction monitoring mode
- 7 tested transitions

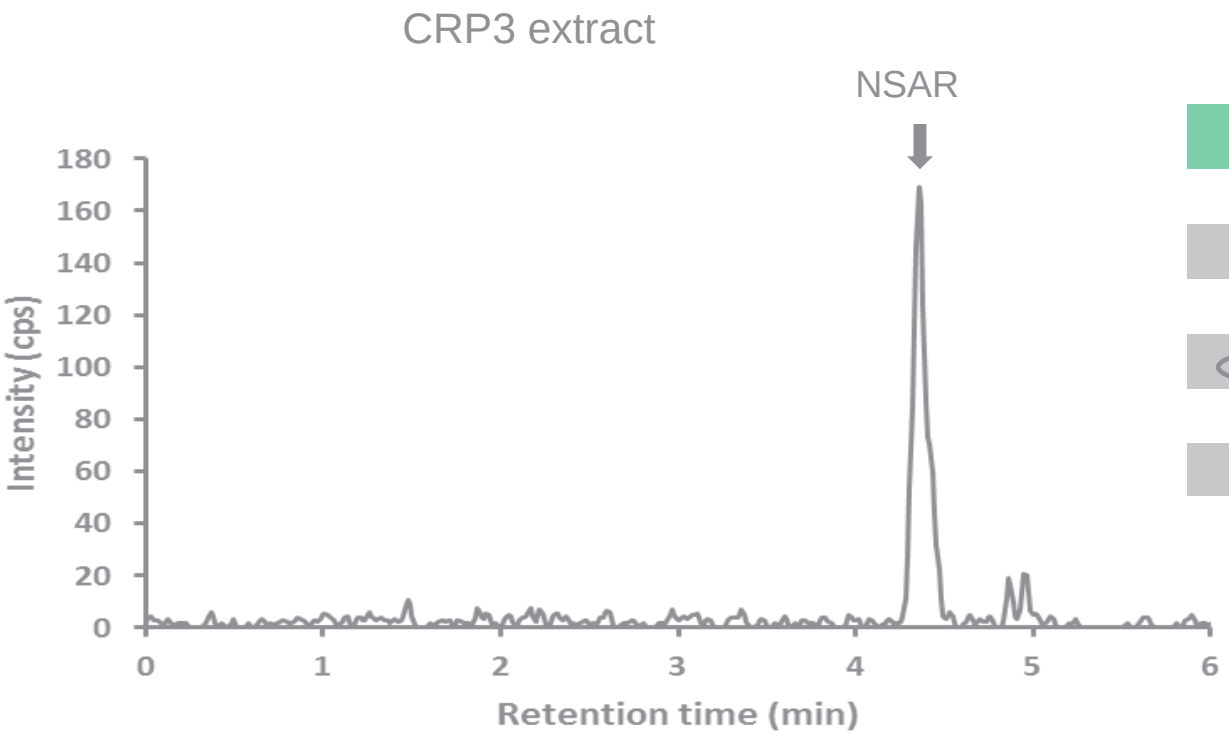
Precursor ion	Product ion	Relative intensity (%)
117.0	73.1	235
117.0	32.1	100
117.0	75.0	12
117.0	30.0	2.7
235.0	117.0	28
235.0	73.1	5.3
235.0	32.1	1.6

Quantifier transition

used for quantification

Method development

Mass spectrometry



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235.0	117.0	28
235.0	73.1	5.3
235.0	32.1	1.6

Qualifier transition

used for

1. **identification (relative intensities)**
2. **determination of LOQ/LOD**

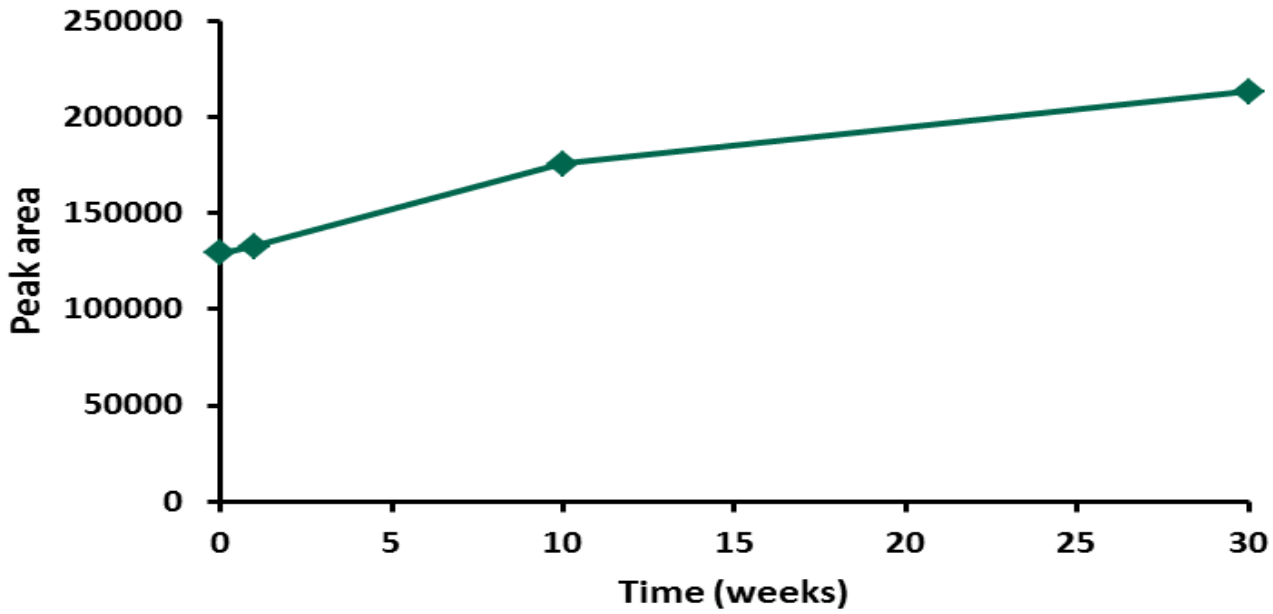
Method development

Stability of stock solutions

- Stock solutions in acetone
- Storage at -20 °C

? Why does peak area increase with the age of the standard?

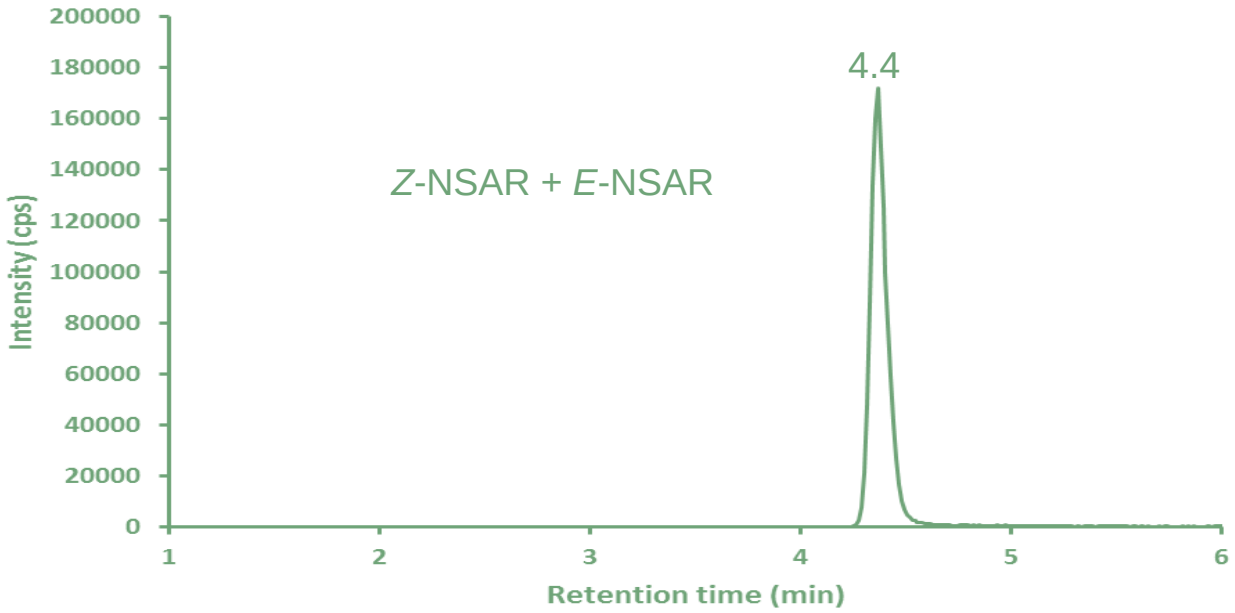
→ NSAR isomers!



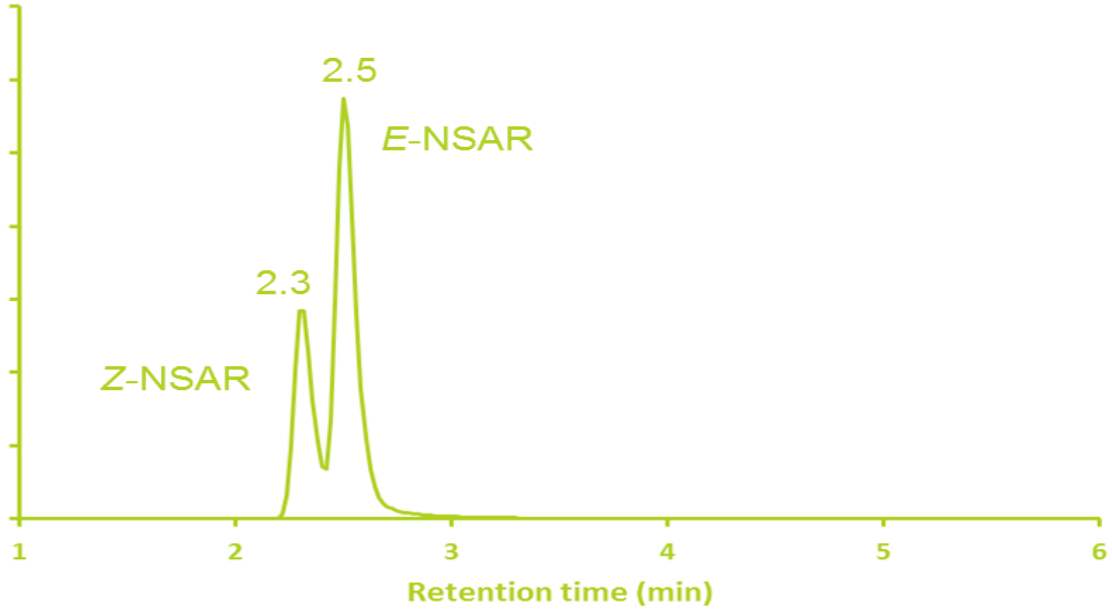
Method development

Isomer separation

co-elution: 30 min of re-equilibration

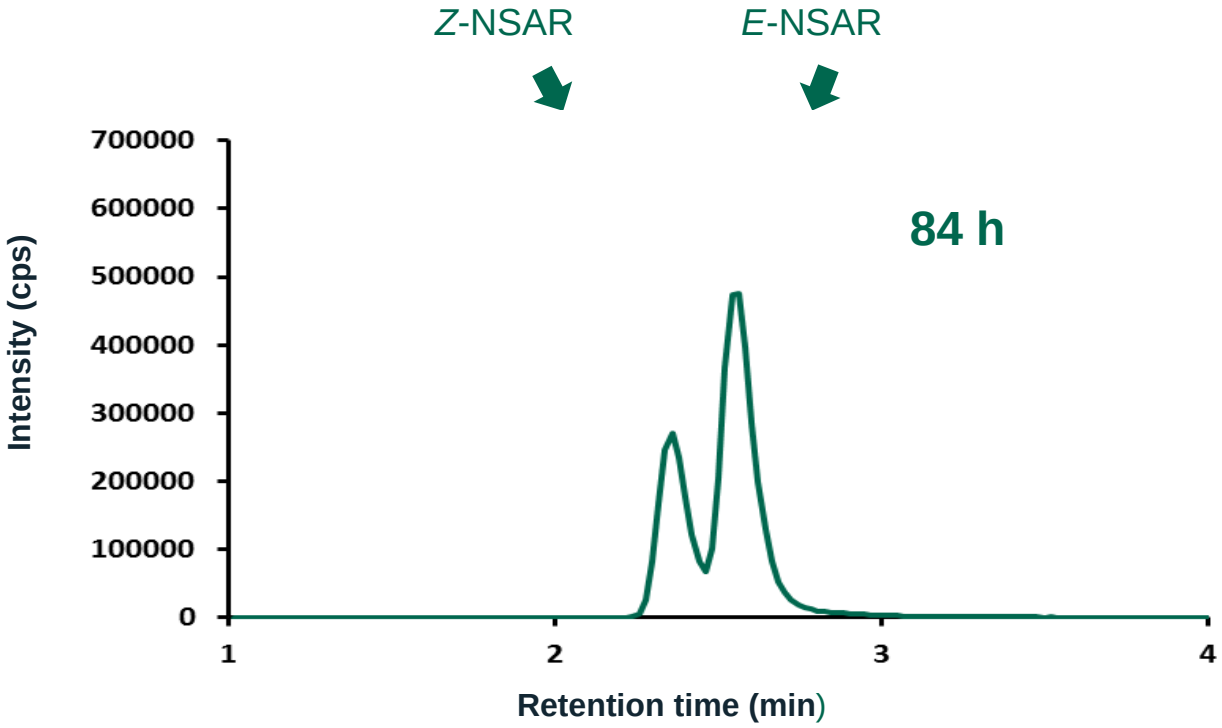


separation: 5 min of re-equilibration



Method development

Isomeric ratio investigations



- isomeric ratio is unstable
- changes with the age of the standard
- *Z*-NSAR peak decreases and *E*-NSAR peak increases
- *E*-NSAR peak increases twice as fast as *Z*-NSAR peak decreases
- peak area sum increases

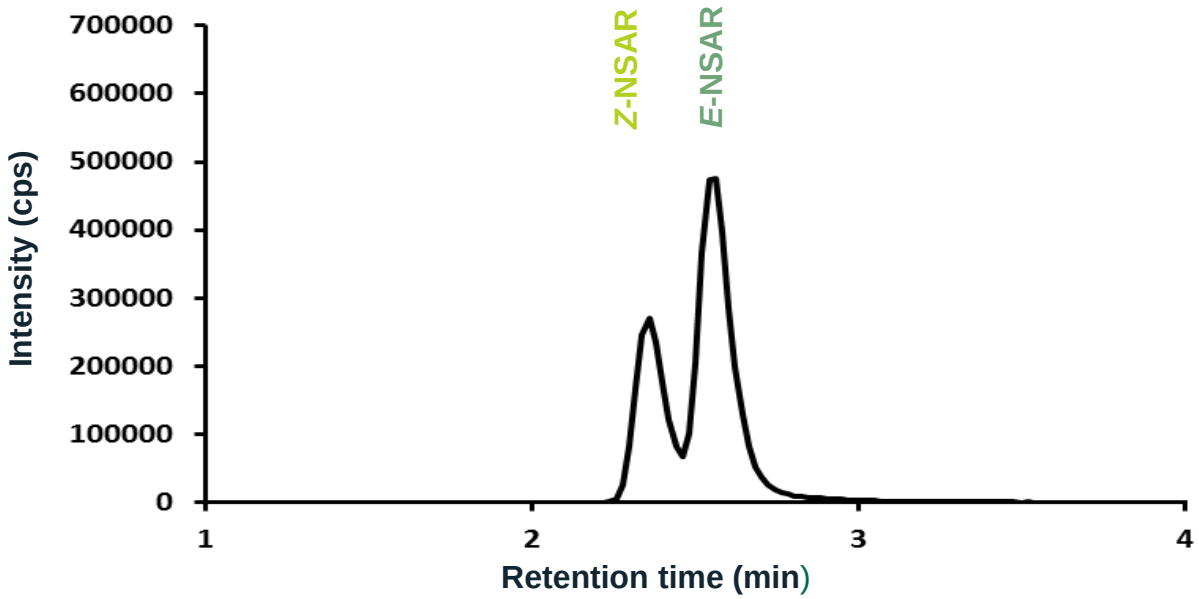
→ **isomers have different ESI-MS/MS response!**

→ **factor of 2 hypothesized**

Method development

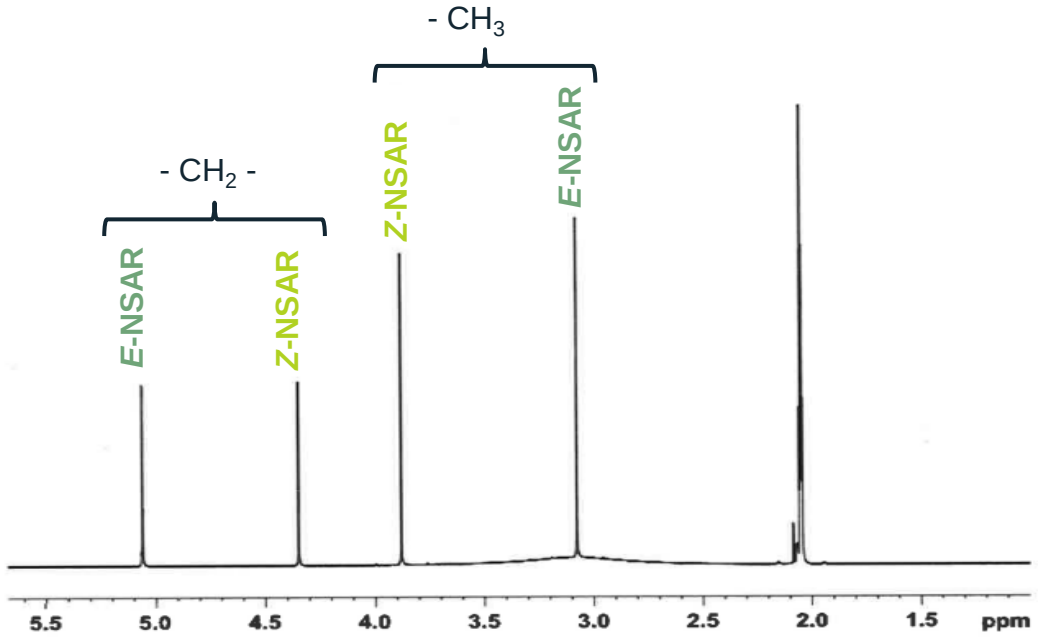
Isomeric ratio investigation

Chromatogram of NSAR at equilibrium



isomers have different ESI-MS/MS response!

^{1}H NMR spectrum of NSAR at equilibrium *

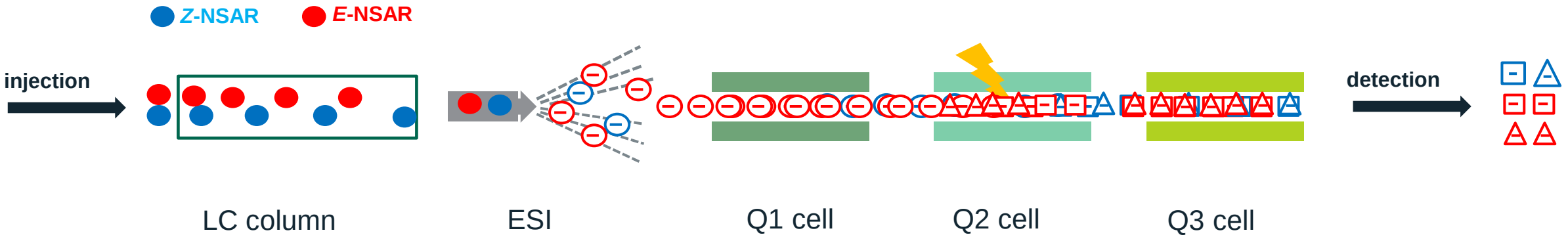


* measurements performed by Prof. Kählig, University of Vienna

factor of 2 confirmed

ESI-MS/MS behavior of NSAR isomers

Why do E- and Z-NSAR have different MS response?



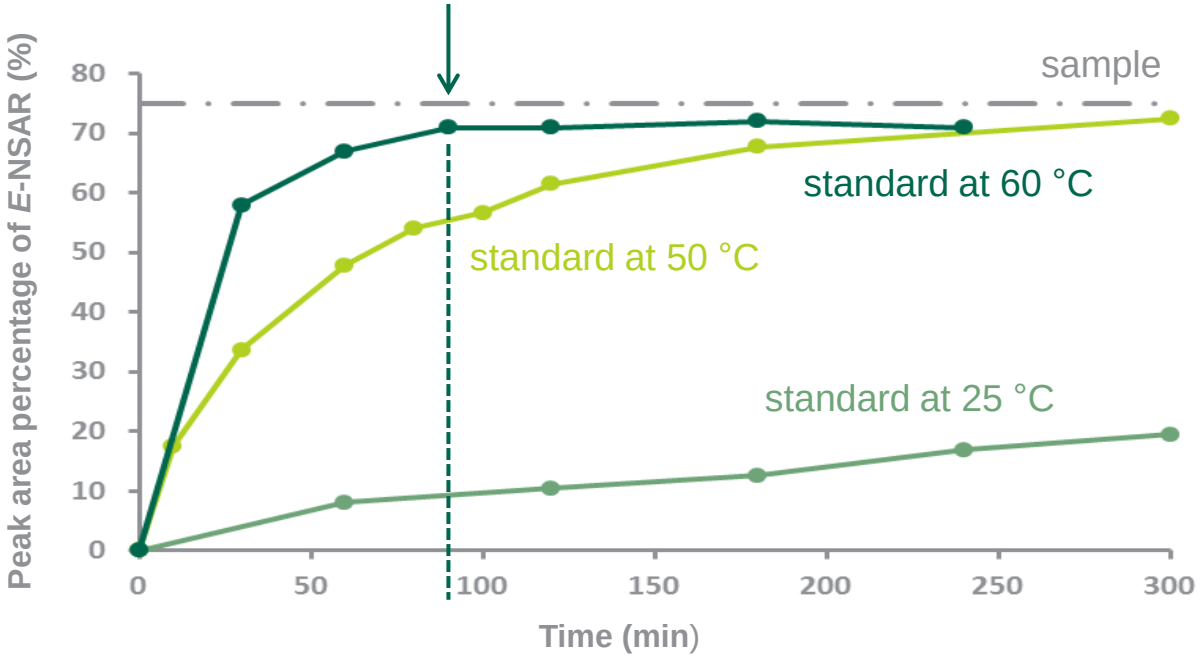
Quantification of NSAR

Approaches to compensate for different ESI-MS/MS response

Correction of the peak areas by the determined factor	Adjustment of the isomeric ratio of the calibration standard to that of the real sample
separation method is required → earlier elution → hampered matrix separation and lower sensitivity	co-elution method can be used → better matrix separation and higher sensitivity
degree of response difference might be instrument dependent	correction is instrument independent

Quantification of NSAR

Preparation of external calibration standard



- external calibration
- calibration standard heated at 60 °C for 90 min
- internal standard correction
  - NSAR-D_3 behaves similarly

Quantification of NSAR

Method validation

	CRP2	CRP3
Intra-day repeatability (% RSD, *n* = 5)	6	5
Inter-day repeatability (% RSD, *n* = 7)	8	5
LOD (ng/g)	4	9
LOQ (ng/g)	14	28
Recovery NSAR (%)	17	9
NSAR-D_3 (%)	18	9

Quantification of NSAR

Results

	Description	NSAR (ng/g)
CRP1	Swedish-style snus pouch	< LOD
CRP2	American-style loose moist snuff	36 ± 8
CRP3	American-style loose dry snuff powder	58 ± 9
CRP4	American-style loose-leaf chewing tobacco	< LOQ
tobacco of 3R4F	Kentucky Reference Cigarette	< LOQ

References

- Y. L. Chow, J. Polo. The nuclear magnetic resonance spectra of *N*-nitroso-*N*-alkyl amino acids. *Org. Magn. Reson*. 1981, *15*, 200.
- J. Wu, W. S. Rickert, A. Masters, P. Joza. Determination of *N*-nitrososarcosine in tobacco and smokeless tobacco products using isotope dilution liquid chromatography tandem mass spectrometry. *Anal. Methods* 2012, *4*, 3448.
- M. Werneth, J. Pani, S. Pummer, M.-T. Weber, L. Hofbauer, G. Pour, H. Kählig, B. Mayer-Helm, H. Stepan. Stereospecific mass spectrometric response of *N*-nitrososarcosine and its impact on quantification in smokeless tobacco products. *Submitted to J. Mass Spectrom.*

I want to thank my team!

Madeleine Werneth

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Special thanks to Hanspeter Kählig, University of Vienna, for NMR measurements

Thank you for your attention!