Greener analytical methods for the determination of nutraceuticals and quality assessment

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## MeIT & Chromaleont Facilities

Sample preparation/Chromatography Spectroscopy/Mass Spectrometry Organic synthesis/Molecular Biology Biotechnology/Fermentation Analytical data processing Software/Libraries



## **Overview**

- > Triacylglycerol Fingerprinting in Table Oils for Assessing Quality and Authenticity
- Profiling of Oxygen Heterocyclic Compounds in Cold-pressed Citrus Essential Oils
- > Analysis of Alkylpyrazines from Maillard Reaction in Foods



### Replacing HPLC methods with SFC-based

- Gain additional selectivity
- Reduce environmental impact
- Increase operators' safety
- Decrease operational costs



## Triacylglycerol Fingerprinting in Table Oils for Assessing Quality and Authenticity



## **Techniques for Triacylglycerol Separation**



## HPLC Techniques for Triacylglycerol Separation

- NARP-LC with C18-bonded silica stationary phases
   retains the analytes according to hydrophobicity or partition number, PN = CN (2\*DB)
- Ag-HPLC on silica phases impregnated with silver nitrate retains the analytes according to the double bond number
- Both methods are well suited for these hydrophobic compounds (no water in the mobile phases)
- Whatever the HPLC method, there are always unresolved pairs of peak: TAGs having the same ECN (NARP-LC), TAGs with the same unsaturation number (Ag-HPLC)
- Both methods make use of toxic organic solvents
- In both methods elution gradients are required, resulting in an additional equilibration time





## **Techniques for Triacylglycerol Detection**

## ELSD, CAD, APCI-MS/ESI detection

- The response coefficients vary depending on mobile phase composition while a mobile phase gradient is always necessary to ensure the elution of all TAGs in LC.
- Quantitation requires multi-calibration, or post-column addition, or post-column gradient compensation, because of the continuous change of the mobile phase composition.

## UV detection

- The UV absorbance of TG is rather weak, requiring measurement at low wavelengths to achieve a measurable response.
- The strong UV absorption of acetone or methylene chloride, which often compose mobile phases in NARP-LC, hinders UV detection.





## **SFC-PDA on Partially Porous Stationary Phases**

SFs are highly compressed gases which combine properties of gases (high diffusivity) and liquids (solvating properties) in an intriguing manner.

SFs offer a range of unusual chemical possibilities in both synthetic and analytical chemistry.



Faster analyses/Longer columns Higher resolution at moderate pressure drops





Pure  $CO_2$ Critical pressure ( $P_c$ ): 73 atm Critical temperature ( $T_c$ ): 31 °C



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## The modifier

- Ethanol: safe, high affinity for CO<sub>2</sub> (SFE-SFC).
- Increases the mixture density, the polarity and also the specific interactions (hydrogen bonding).
- scCO<sub>2</sub> will also tune the dielectric and overall solvating properties of ethanol (permittivity).



<b>x</b> A (CO <sub>2</sub> )	T/K	T/°C	P/bar	%EtOH	phase transition pattern	
1	304.10	30.95	73.8	0	СР	
0.956	298.15	25	61.81	4.4	BP	
0.956	303.15	30	67.46	4.4	BP	
0.956	310.58	37.43	77.73	4.4	СР	
0.956	313.15	40	81.18	4.4	DP	
0.956	323.15	50	93.1	4.4	DP	
0.938	303.15	30	66.78	6.2	BP	
0.938	313.15	40	79.46	6.2	BP	
0.938	318.24	<b>45.09</b>	86.35	<b>6.2</b>	СР	
0.938	323.15	50	93.17	6.2	DP	
0.938	333.15	60	104.95	6.2	DP	
0.863	313.15	40	78.42	13.7	BP	
0.863	323.15	50	93.38	13.7	BP	_
0.863	328.36	<b>55.21</b>	100.89	13.7	СР	ba
0.863	333.15	60	107.64	13.7	DP	P/
0.863	343.15	70	119.49	13.7	DP	
0.769	323.15	50	92.55	23.1	BP	
0.769	333.15	60	106.88	23.1	BP	
0.769	343.15	70	119.84	23.1	BP	
0.769	350.62	77.47	128.04	23.1	СР	
0.769	353.15	80	130.59	23.1	DP	
0.769	363.15	90	138.31	23.1	DP	
0.697	363.15	90	137.34	30.3	BP	
0.697	373.15	100	144.03	30.3	BP	
0.697	376.15	103	146.78	30.3	BP	
0.697	377.17	104.02	<b>146.30</b>	30.3	СР	
0.697	378.16	105	145.89	30.3	DP	
0.646	390.15	117	150.99	35.4	BP	
0.646	392.15	119	151.19	35.4	BP	Yeo e
0.646	393.08	<b>119.93</b>	151.54	35.4	СР	
0.646	395.15	122	151.74	35.4	DP	Al-Ha

## Operation in SFC: Critical Point Loci for CO<sub>2</sub> – Ethanol Binary Mixture



Yeo et al., J. Chem. Eng. Data 2000, 45, 932-935.

Al-Hamimi, Mayoral, Cunico & Turner, Anal. Chem. 2016, 88, 4336-4345.

## **SFC-PDA on Partially Porous Stationary Phases**



## **SFC-PDA Method for TAG Analysis**



### **PARTITION NUMBER: 40**

LnLnP CN=52, DB=6



LLLn CN= 54, DB= 7



- Column: Ascentis Express C18 (4 x 15 cm × 4.6 mm, 2.7 μm)
- Mobile phase: CO<sub>2</sub> (A), Ethanol (B)
- Oven Temperature: 25 °C
- Elution: Isocratic at 5% B
- Flow rate: 2.0 mL min<sup>-1</sup>
- Injection volume: 0.3 µL
- Detection: 205 nm
- **BPR**: 200 bar, 50 °C



 $\frac{t_r}{t_r}$  $N = 16 \times$ *N* =16,000 to *N*= 63,000

 $Rs = \frac{(t_r)_2}{2}$  $R_s$ =1.096 to  $R_s$ = 2.56

## **SFC-PDA Analysis of Vegetable Oils**







## **SFC-PDA Analysis of Vegetable Oils**







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## **SFC-PDA Analysis of Vegetable Oils**



The elution order of TAGs was mainly governed by hydrophobicity of the solutes: retention increases with PN

Similarly to what observed in RP-HPLC on ODS columns, the apolar character of TAGs becomes dominant at the higher PN values, providing more interaction with the separation medium and diminished solubility in the mobile phase; as a result, the retention increases.

Specific behaviours: the reversal of the retention order between TAGs differing by one palmitic (P, C16:0) or one oleic (O, C18:1) chain. This point resolves most difficulties encountered in NARP-LC for pairs of peaks such as PLO/OLO:

OLP PN= (52)-(2x3)=46 OLO PN= (54)-(2x4)=46



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## **TAG Identification in Vegetable Oils**

A classical retention diagram provides additional suggestions to:

- identify minor compounds that would not have been described previously
- annotate the chromatograms of previously undescribed oils

This diagram is based on the identical energy transfer, i.e. retention variation, related to an identical structural modification between two analytes.

- Increasing the number of methylene groups causes a regular variation in retention.
- The same effect can be observed when varying the number of double bonds.



Journal of Food Composition and Analysis Volume 115, January 2023, 104960



Comparison of the triglyceride composition of vegetable samples with ultra-high efficiency / low-pressure supercritical fluid chromatography – mass spectrometry

Quentin Gros ° b 쯔, Marta Wolniaczyk ° c 쯔, Johanna Duval <sup>b</sup>쯔, Caroline West ° 으 쯔, Shinnosuke Horie <sup>d</sup> 쯔, Yusuke Toyota <sup>d</sup> 쯔, Yasuhiro Funada <sup>d</sup> 쯔, Eric Lesellier ° 쯔



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Fast Triacylglycerol Fingerprinting in Edible Oils by Subcritical Solvent Chromatography by Tania Maria Grazia Salerno, Marianna Oteri, Paola Arena, Emanuela Trovato, Danilo Sciarrone, Paola Donato and Luigi Mondello

Separations 2023, 10(1), 56; https://doi.org/10.3390/separations10010056 - 14 Jan 2023

10

One major difference from NARP-LC is that the solubility of Palmitate chain (P, C16:0) in the subcritical mobile phase is higher than the one of Oleate (O, C18:1).

This different retention and solubility between NARP-HPLC and SFC partly explains the improvement of resolution obtained in SFC, which is not only related to efficiency performance.

Difficult identifications concern TAGs having identical partition number, with the same CN and DB, and saturated TAGs.

## Retention decreases with increasing DB, with straight lines Connecting compounds of a series.

**TAG Identification in Vegetable Oils** 



**TAG Identification in Vegetable Oils** 



## **TAG Quantification in Vegetable Oils**

- The chromatographic quantitation of TAGs is usually based only on the relative areas of chromatographic peaks, neglecting potential differences in the relative responses of TAGs differing in the number of double bonds and acyl chain lengths.
- The obvious advantage of such an approach is its simplicity, but it may lead to significant systematic errors in the determination of TAG concentration.
- Lipid samples contain very complex TAG mixtures, but commercial standards are available only for a limited range of TAGs, mostly single-acid type R1R1R1.
- Less common TAGs are expensive and PUFAs are prone to oxidation, hence quantitation based on the calibration curves for all TGs is practically impossible even if only major TAGs are taken into account.

An approach for the quantitation of complex TAG mixtures with a limited range of authentic TAG standards (single-acid type) was sought in this work.

## **TAG Quantification in Vegetable Oils**

- Working solutions of saturated TAGs (MMM, PPP, MaMaMa, SSS) at 100, 200, 500, 1000, 2000, 5000 mg/L and unsaturated TAGs (PoPoPo, OOO, LLL, LnLnLn) at 100, 200, 500, 1000, 2000 mg/L
- Working solution of OOO, OO, O at 100, 200, 500, 1000 and 2000 mg/L
- Matrix-matched in oil samples (3%) to check for matrix effects (5  $\mu$ L injection)
- Calculation of the RFs (ratio of calibration slopes a(OOO)/a(TAG) and the weighing factors)
- Validation by with GC-MS with LRI
- Comparison with GC-FID results (FAMEs samples on SLB-IL 60, 30 m x 0.25 mm x 0.20  $\mu$ m)



## **TAG Quantification in Vegetable Oils**

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The UV absorbance of TAG at 205 nm results from:

- (a) the ester functions linking the hydrocarbon chains to the glycerol head, which should be the same to all TAGs
- (b) the double bonds in the hydrocarbon chains, which would differ between the TG.

TAGs having different chain length and identical double bonds show close UV absorbance, while TAG with different numbers of double bonds have significantly different absorbance  $(a_{UV})$ .



- 5 calibration points provided better precision than RFs based on a single point only.
- the ratio of calibration slopes a(OOO)/a(TAG) was used for the calculation of RFs of individual TAGs.



## **Detection of EVOO Adulteration**

- The addition of vegetable oils of low commercial and nutritional values to olive oils represents common adulterations that are reflected in the final oil composition and quality.
- Samples: EVOO, EVOO spiked with 5-50% of adulterant oils (corn, soybean, peanut)

TAG	EVOO	EVOO5	EVOO10	EVOO15	EVOO20	EVOO30	EVOO40	EVOO50
LLL	$0.701^{h}$	5.489 <sup>g</sup>	6.555 <sup>f</sup>	$8.594^{\mathrm{e}}$	$11.934^{d}$	13.550°	16.338 <sup>b</sup>	17.882ª
OLLn	0.712 <sup>g</sup>	2.339 <sup>f</sup>	$2.474^{f}$	3.053 <sup>e</sup>	4.116 <sup>d</sup>	4.677°	5.652 <sup>b</sup>	<b>6.2</b> 10 <sup>a</sup>
LLP	0.943 <sup>a</sup>	0.933ª	0.870 <sup>a</sup>	0.888 <sup>a</sup>	0.765 <sup>a</sup>	0.724 <sup>a</sup>	0.757 <sup>a</sup>	$0.824^{a}$
OLPo	1.863 <sup>h</sup>	3.766 <sup>g</sup>	$4.212^{\mathrm{f}}$	5.098 <sup>e</sup>	$6.401^{d}$	7.086 <sup>c</sup>	8.391 <sup>b</sup>	9.263ª
LnOP	1.558 <sup>ab</sup>	1.828ª	1.576 <sup>ab</sup>	1.555 <sup>ab</sup>	1.473 <sup>b</sup>	1.479 <sup>b</sup>	1.477 <sup>b</sup>	$1.570^{ab}$
OOLn	5.869 <sup>h</sup>	8.296 <sup>g</sup>	$9.154^{\mathrm{f}}$	10.263 <sup>e</sup>	12.091 <sup>d</sup>	13.000 <sup>c</sup>	14.679 <sup>b</sup>	15.678ª
OLL	<b>4.011</b> <sup>a</sup>	3.758 <sup>ab</sup>	3.799 <sup>ab</sup>	3.790 <sup>ab</sup>	3.659 <sup>b</sup>	3.609 <sup>b</sup>	3.533 <sup>b</sup>	$3.534^{b}$
POPo	1.799 <sup>b</sup>	2.129 <sup>a</sup>	1.798 <sup>b</sup>	1.822 <sup>b</sup>	1.832 <sup>b</sup>	1.832 <sup>b</sup>	1.836 <sup>b</sup>	1.877 <sup>b</sup>
OLP	10.650 <sup>a</sup>	9.609 <sup>b</sup>	9.581 <sup>b</sup>	9.302 <sup>b</sup>	8.717 <sup>c</sup>	8.459 <sup>cd</sup>	$7.945^{de}$	7.653 <sup>e</sup>
OOPo	0.107 <sup>a</sup>	0.305 <sup>a</sup>	0.121ª	0.106 <sup>a</sup>	0.111ª	$0.104^{a}$	0.102 <sup>a</sup>	0.103 <sup>a</sup>
OLO	20.720 <sup>a</sup>	17.926 <sup>b</sup>	17.851 <sup>b</sup>	16.831°	15.213 <sup>d</sup>	$14.408^{e}$	$12.917^{f}$	11. <b>992</b> g
POP	2.051 <sup>d</sup>	2.647°	2.709 <sup>c</sup>	<b>2.844</b> <sup>c</sup>	3.262 <sup>b</sup>	3.341 <sup>b</sup>	3.777 <sup>a</sup>	<b>3.958</b> <sup>a</sup>
OOP	15.034 <sup>a</sup>	12.366 <sup>b</sup>	11.895 <sup>c</sup>	$10.782^{d}$	9.088 <sup>e</sup>	$8.199^{f}$	6.545 <sup>g</sup>	5.563 <sup>h</sup>
000	30.397 <sup>a</sup>	24.671 <sup>b</sup>	24.086 <sup>c</sup>	$21.908^{d}$	18.302 <sup>e</sup>	$16.513^{f}$	$13.094^{\mathrm{g}}$	$11.005^{h}$
SOP	0.315ª	0.381ª	$0.281^{a}$	$0.270^{a}$	0.257 <sup>a</sup>	0.251ª	0.232 <sup>a</sup>	0. <b>24</b> 1ª
SOO	2.455ª	2.241 <sup>ab</sup>	1.991 <sup>abc</sup>	1.839 <sup>bcd</sup>	$1.547^{cde}$	$1.438^{def}$	$1.213^{\text{ef}}$	$1.036^{f}$

Evaluation of statistically significant differences (ANOVA)





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## Method Features: NARP-HPLC vs. SFC



The experimental pressure drop is much lower in SFC compared to NARP-HPLC NARP-HPLC\* analysis time: > 100 min solvent consumption: > 120 mL (AcN, IPA)

SFC

analysis time: < 35 min solvent consumption: 3.5 mL (bio-EtOH)



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- NARP-HPLC: max of 3 columns (15 cm each)
- SFC: up to 12 columns can be coupled, reaching a total length of 180 cm

\*Holcapek et al. J. Sep. Sci. 2005, 28, 1315-1333



## Method Features: NARP-HPLC vs. SFC



## **Analytical GREEnness Metric Approach**



- 2. Sample size and number of samples
- 3. In Situ measurements or in laboratory
- 4. Analytical steps
- 5. Automation/minizaturization of analytical method
- 6. Use of derivatization reagents
- 7. Volume of analytical waste generated
- 8. Number of analysis determined
- 9. Energy consumption
- 10. Use of reagents from renewable sources
- 11. Use of toxic reagents
- 12. Safety of the operator





NARP-HPLC method

SubFC method



8

Profiling of Oxygen Heterocyclic Compounds in Cold-pressed *Citrus* Essential Oils



The EFSA Journal (2008) 793, 1-15

Coumarin in flavourings<sup>1</sup> and other food ingredients with flavouring properties

Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC)

(Question No EFSA-Q-2008-677)

Adopted on 8 July 2008





#### IFRA Standards – 50<sup>th</sup> Amendment



★ Coumarin: < 1.5%</p>
★ 5-MOP: < 0.0015%</p>





coumarines

#### furocoumarines



polymethoxyflavones

## **Published Methods for OHC Analysis**

Analytical and Bioanalytical Chemistry (2023) 415:3327–3340 https://doi.org/10.1007/s00216-023-04714-7

#### **RESEARCH PAPER**

Hidden threat lurking in extensive hand hygiene during the Covid-19 pandemic: investigation of sensitizing molecules in gel products by hyphenated chromatography techniques

Tania M. G. Salerno<sup>1</sup> · Emanuela Trovato<sup>1</sup> · Giovanna Cafeo<sup>1</sup> · Federica Vento<sup>2</sup> · Mariosimone Zoccali<sup>3</sup> · Paola Donato<sup>4</sup> · Paola Dugo<sup>1,2</sup> · Luigi Mondello<sup>1,2</sup>

#### RESEARCH ARTICLE

Reduced time HPLC analyses for fast quality control of citrus essential oils

Marina Russo<sup>a</sup>, Ivana Bonaccorsi<sup>a</sup>\* <sup>1</sup><sup>0</sup>, Rosaria Costa<sup>a</sup>, Alessandra Trozzi<sup>a</sup>, Paola Dugo<sup>a,b</sup> and Luigi Mondello<sup>a,b,c</sup>

#### Contents lists available at ScienceDirect Journal of Chromatography A journal homepage: www.elsevier.com/locate/chroma

Linear retention index approach applied to liquid chromatography coupled to triple quadrupole mass spectrometry to determine oxygen heterocyclic compounds at trace level in finished cosmetics

Taylor & Francis o ab.c.d. Francesca Rigano a, Luigi Mondello ab.c.d.\* reutical and Environmental Sciences. University of Messina. Messina. Italy

### HPLC-PDA (LRI), HPLC-MS/MS



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## **RP-HPLC-PDA Analysis**





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**Column: Ascentis Express C18** (5 cm  $\times$  4.6 mm  $\times$  2.7  $\mu$ m)

**Mobile phase**: H<sub>2</sub>O/MeOH/THF 85:10:5 v/v (A); MeOH/THF 95:5 v/v (B)

Elution: 0–4.5 min, 15–28% B; 4.5–7.0 min, 28–60% B; 7.0–11.0 min, 60–85% B, and 11.0–14.0, 85% B.

**PDA:** 190-400 nm, chrom. extr. 315 nm

Flow rate: 2.0 mL min<sup>-1</sup>; Oven temperature: 40 °C; Injection volume: 2 µL



## **RP-HPLC-PDA Analysis**



OHCs in Citrus cold-pressed EO samples

Column: Ascentis Express C18 (5 cm  $\times$  4.6 mm  $\times$  2.7  $\mu$ m)

Mobile phase:  $H_2O/MeOH/THF$  85:10:5 v/v (A); MeOH/THF 95:5 v/v (B)

**Elution**: 0–4.5 min, 15–28% B; 4.5–7.0 min, 28–60% B; 7.0–11.0 min, 60–85% B, and 11.0–14.0, 85% B.

Flow rate: 2.0 mL min<sup>-1</sup>

**PDA:** 190-400 nm, chrom. extr. 315 nm

Oven temperature: 40 °C

Injection volume:  $2 \ \mu L$ 



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## **SFC-PDA Analysis**





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Column: Ascentis Express HILIC ( $25 \text{ cm} \times 2.1 \text{ mm} \times 2.7 \mu \text{m}$ ) Injection volume:  $1 \mu \text{L}$ Mobile phase: CO<sub>2</sub> (A); EtOH + 0.6% water and 0.07% TFA (B) Elution: 0-1 min, 0-3% B; 4-6 min, 3-10% B; 11 min, 20% B Flow rate: 1.0 mL min<sup>-1</sup> PDA: 190-400 nm, chrom. extr. 315 nm BPR: 120 bar Oven temperature: 40 °C



**Nexera-UC** 



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SFC-PDA chromatogram of cold-pressed LIME essential oil



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## **SFC-PDA Analysis**



SFC-PDA chromatogram of cold-pressed LEMON essential oil

## **Analysis Costs**

## **HPLC-PDA**

Analysis time: 19 min

Mobile phase A: H<sub>2</sub>O/MeOH/THF, 85:10:5, v/v

Mobile phase B: MeOH/THF, 95:5, v/v

Solvent consumption: 38 mL

Mobile phase A consumption: 21 mL  $(17.85 \text{ mL H}_2\text{O} + 2.1 \text{ mL MeOH} + 1.05 \text{ mL THF})$ 

Mobile phase B consumption: 17 mL (16.15 mL MeOH + 0.85 mL THF)

### Solvent cost: 2.03 €/analysis

Water 33.70 € (1L) Methanol 58.50 € (1L) Tetrahydrofuran 197 € (1L)

Waste disposal: 38 mL, 0.095 €

## SFC-PDA

Analysis time: 15 min

Mobile phase A: CO2

Mobile phase B: EtOH/TFA, 95:5, v/v

Solvent consumption: 15 mL

Mobile phase A consumption: 14 mL (14 mL CO2)

Mobile phase B consumption: 1 mL (0.95 mL EtOH + 0.05 mL TFA)

Solvent cost: 0.084 €/analysis Compressed CO2 165 € (1,500 L) Bio-ethanol 16.40 € (1L) Trifluoroacetic acid 132 € (100 mL)

Waste disposal: 1 mL, 0.0025 €



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## Method Features: NARP-HPLC vs. SFC



- 1. Sample treatment and preparation
- 2. Sample size and number of samples
- 3. In Situ measurements or in laboratory
- 4. Analytical steps
- 5. Automation/minizaturization of method
- 6. Use of derivatization reagents
- 7. Volume of analytical waste generated
- 8. Number of analysis determined
- 9. Energy consumption
- 10. Use of reagents from renewable sources
- 11. Use of toxic reagents
- 12. Safety of the operator

## **Analytical GREEnness Metric Approach**









## Analysis of Alkylpyrazines from Maillard Reaction in Foods



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## **Challenges in Pyrazine Analysis**



## **HPLC-PDA Analysis on a C18 Column**



Column: C18, 150 x 3.0 mm, L x I.D., 2.7  $\mu$ m Elution: 2-60% acetonitrile into water in 30 min, at 0.8 mL/min Detection: UV at 272 and 215 nm (1 uL injection).



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VERSI

## **HPLC-PDA** Analysis on a PGC Column



**Column:** SupelCarbon LC PGC, 150 x 3.0 mm, L x I.D., 2.7 μm Elution: 2-60% acetonitrile into water in 30 min, at 0.8 mL/min Detection: UV at 272 and 215 nm (1 uL injection).



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### **SFC-PDA Analysis on a PGC Column**



## **Concluding Remarks**

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SFC significantly reduces the usage of organic solvents and wastes by using SF  $CO_2$  as the mobile phase.



Compliance of SFC with the principles of green chemistry: prevention of waste, use of safer solvents and reduced costs.



Renewable raw materials enable the reduction of the environmental impact with performance comparable to conventional petrochemical-based solvents.



Flow, composition, column temperature, and column outlet pressure are all independently controlled.



Limited pressure drops allow for faster analysis and/or higher separation efficiency.



## **Acknowledgements**





Merck





My research group

# Thank you all for your attention!

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