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A multi-detector Set-up Comprising UV/Vis detection, Charged Aerosol Detection and Single Quadrupole Mass Spectrometric Detection for Comprehensive Quantitative Sample Analysis

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ABSTRACT

Purpose: Reliable verification of the presence of additional compounds in a sample, i.e., of impurities, degradation products of the analyte or extractables from containers.

Methods: A multi-detector HPLC set-up comprising UV/Vis, charged aerosol and mass spectrometric detection was employed. The first two detectors were used for quantitative detection, the mass spectrometer for *m/z*-based confirmation of the analyte identity.

Results: Extracts from single-use cell culture bags were analyzed. 18 known extractables and 19 unknown extractables could be quantified. The charged aerosol detector was used for quantification of all unknowns and for eleven of the known analytes. The UV detector was used for quantification of seven of the known analytes. The mass spectrometer was used for identity confirmation of the detected analytes.

INTRODUCTION

Comprehensive sample analysis is essential for determining the presence of additional compounds in a sample, i.e., impurities, degradation products of the analyte or extractables from containers. In addition, identity confirmation and quantitation of these compounds is often needed to determine their nature and whether they are below acceptable concentration limits. Moreover, compounds may be UV-transparent requiring complementary detection techniques. The charged aerosol detector (CAD) delivers universal detection of non- and semi-volatile compounds making it an ideal second detector. Additionally, its near uniform response allows straightforward quantification without reference standards. The identity of the detected compounds can be confirmed by mass spectrometry (MS). Hence, combining these three detection techniques provides a comprehensive sample analysis platform.

MATERIALS AND METHODS

A Thermo Scientific™ Vanquish™ Flex UHPLC system was used in two different configurations for chromatographic analysis (Table 1 and Figure 1). In the standard set-up, a quaternary low pressure mixing pump delivered the analytical gradient. In the inverse set-up, a pump module that contained two quaternary low pressure pumps delivered both the analytical gradient and the inverse gradient. The inverse gradient resulted in a constant solvent composition during the CAD and MS detection, which improved detector response uniformity. The HPLC system was controlled through Thermo Scientific™ Chromeleon™ 7.2 CDS. Chromatographic methods are briefly described in Table 2. Eighteen reference compounds were selected based on literature reports of extractables present in cell culture bags[1,2,3] and were purchased from Sigma-Aldrich, Steinheim, Germany. Dilutions were prepared in methanol from 1 mg/mL standards (in suitable solvents: hexane, methanol or acetone) at 1, 2, 5, 10, 20 and 50 µg/mL, except for butylparaben, eicosane and tetracosane, which were prepared at 10, 20, 50, 100, 200 and 500 µg/mL. Four different types of single-use cell culture bags, the inner layer of which was made of ethylenevinyl acetate and different density grades of polyethylene, were investigated. Extracts were prepared by rinsing with 50/50 isopropanol/water mixtures.

Table 1. Thermo Scientific™ Vanquish™ Flex UHPLC System Modules in multi-detector set-up.

Module	Standard Set-up	Inverse Gradient Set-up
Vanquish Quaternary Pump (200 µL mixer)	✓	
Vanquish Dual Pump (200 µL mixers)		✓
Vanquish Split Sampler FT	✓	✓
Vanquish Column Compartment H (2-position/6-port valve)	✓	✓
Vanquish Diode Array Detector FG (2.5 µL titanium flow cell)	✓	✓
Vanquish Charged Aerosol Detector	✓	✓
ISQ EC Single Quadrupole Mass Spectrometer	✓	✓

Figure 1. Schematic display of standard set-up and inverse gradient set-up. A. The standard set-up uses solely 100 µm ID (inner diameter) capillaries (depicted in red). **B.** The inverse gradient set-up uses 100 µm ID capillaries (red) and 130 µm ID capillaries (blue). The 100 µm ID capillaries were used for the analytical gradient delivery to the analytical column and the flow cell. Downstream of it 130 µm ID capillaries were used. The inverse gradient was delivered using 130 µm ID capillaries. Flow splitting with a ratio of 2:1 (CAD:MS) was done in a passive way using a standard T-piece (150 µm ID). The split ratio between CAD and MS was achieved by generating double the backpressure on branch leading to the CAD compared to the one leading to the MS. As a result a 100 µm ID capillary (red) was used between the flow splitter and the CAD vaporizer needle.

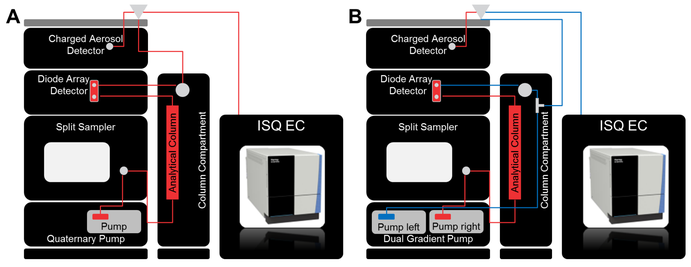


Table 2. Method and detection parameters.

Parameter	Setting					
Eluents	A: 4 mM formic acid in water, B: isopropanol					
Injection Volume	2 µL					
Analytical Gradient	0.5 mL/min	min:	0	10.5	12	12.1	16
		%B:	5	100	100	5	5
Inverse Gradient	0.5 mL/min	min:	0	0.728	11.228	12.728	12.828
		%B:	100	100	5	5	100
Column	Thermo Scientific™ Accucore™ C18, 100 x 2.1 mm, 2.6 µm 45 °C temperature (oven and passive preheater); forced air mode						
UV Settings	10 Hz data collection rate, 0.5 s response time, 4 nm bandwidth, 210, 220, 254, 280, 300, 320 nm and 190-345 nm (3D field)						
CAD Settings	10 Hz, 3.6 filter, 1.0 power function value, 35 °C evaporator temperature						
MS Settings	Easy source settings for 0.167 mL/min or 0.333 mL/min flow rate Alternating positive/negative mode full scans & SIM scans						

RESULTS

The 18 reference standards were analyzed with both set-ups. The CAD and the diode array UV/Vis detector were used to determine peak retention times. Peak detection by CAD complemented that by UV/Vis in that some peaks were only detected by CAD and some were only detected by UV/Vis. By combining these powerful, complementary detectors, all 18 standards could be detected with standard and inverse gradient set-ups. Thirteen were detected with UV/Vis, 11 with CAD, and 6 were detected with both detectors (Table 3). Representative UV/Vis and CAD chromatograms are shown in Figure 2A. Seven analytes could not be detected with CAD, which uses a spray drying technique and requires formation of aerosol particles. These analytes were more volatile making detection by CAD difficult. However, they possessed a sufficient chromophore to allow UV/Vis detection. Conversely, 5 analytes with poor chromophores were not detected by UV/Vis but were detected by CAD due to their less volatile behavior. Five analytes did not show a strong MS signal due to lack of volatility and/or lack of an ionizable moiety.

Fourteen compounds could be clearly detected with the single quadrupole mass spectrometer (Table 3). Mass confirmation was based on detection of the protonated or deprotonated *m/z* species in positive or negative mode in full scan and SIM scan at the same elution time as observed by UV/Vis or charged aerosol detection. Five representative extracted SIM scans are shown in Figure 2B. Bisphenol A could not be detected due to the used eluents. At low pH values the deprotonated species was not detectable. At higher pH values it was clearly detectable (data not shown).

Calibration curves for quantification by CAD in the presence and absence of the inverse gradient were compared (Figure 3A). With the inverse gradient, the overlap of the calibration curves and consequently the uniformity of response, was better than without.

Improved response uniformity of CAD with inverse gradient is apparent from the more similar response curves in Figure 3A. This is further demonstrated in Figure 3B where analytes were quantified using a single calibrant (bisphenol A). With inverse gradient, values for 8 analytes were closer to the target of 20 µg/mL. Three semivolatile analytes (i.e., those with low CAD response) could not be accurately quantified by a single calibrant. Comparison of the response uniformity of UV and CAD (Figure 3C) confirms that CAD allows more accurate quantitation of unknowns. This requires that analytes behave as nonvolatiles, which can be determined by examining the effect of evaporation temperature on CAD response.

Table 3. List of reference analytes. Detectability with UV, CAD and MS is indicated with check marks. [M] refers to the monoisotopic mass. LOQ refers to the CAD limit of quantification, except where noted as UV, defined as a signal-to-noise (S/N) ratio of 6 or more for the standard at a given concentration, relative to the noise in a blank run. *Bisphenol is detectable as deprotonated anion, but not with used additive / pH.

#	Analyte	CAS	UV	CAD	MS	[M]	Mass Found	Ion Found	LOQ (µg/mL)
1	Phthalide	87-41-2	✓		✓	134.0	135.1	$[M+H]^+$	5 (UV)
2	Phthaldialdehyde	643-79-8	✓		✓	134.0	135.1	$[M+H]^+$	5 (UV)
3	BHET	959-26-2	✓	✓	✓	254.1	255.1	$[M+H]^+$	1
4	Dimethyl phthalate	131-11-3	✓		✓	194.1	195.1	$[M+H]^+$	1 (UV)
5	Bisphenol A	80-05-7	✓	✓	✓*	228.1	227.2	$[M-H]^-$	1
6	Butylparaben	94-26-8	✓	✓	✓	194.1	195.1	$[M+H]^+$	50
7	Tinuvin P	2440-22-4	✓		✓	225.1	226.1	$[M+H]^+$	1 (UV)
8	Azobenzene	103-33-3	✓		✓	182.1	183.1	$[M+H]^+$	1 (UV)
9	2,4-di-t-Butylphenol	128-39-2	✓			206.2			1 (UV)
10	BHT	128-37-0	✓		✓	220.2	219.2	$[M-H]^-$	1 (UV)
11	Palmitic acid	57-10-3		✓	✓	256.2	255.2	$[M-H]^-$	1
12	Erucamide	112-84-5		✓	✓	337.3	338.3	$[M+H]^+$	1
13	Stearic acid	57-11-4		✓	✓	284.3	283.3	$[M-H]^-$	1
14	Tinuvin 234	70321-86-7	✓	✓	✓	447.2	448.2	$[M+H]^+$	1
15	Irganox 1010	6683-19-8	✓	✓	✓	1176.8	1193.8	$[M+NH_4]^+$	1
16	Irgafos 168	31570-04-4	✓	✓	✓	646.5	645.4	$[M-H]^-$	1
17	Eicosane	112-95-8		✓		282.3			10
18	Tetracosane	646-31-1		✓		338.4			10

Figure 2. A. Representative UV/Vis and CAD chromatograms of an analysis of a reference standard mix using the standard set-up. Sample concentration was 50 µg/mL, except 500 µg/mL for butylparaben, eicosane and tetracosane because they are semivolatiles with higher CAD LODs. The numbers refer to the standard names listed in Table 3. Analytes detected only by UV/Vis are highlighted in blue, analytes detected only by CAD are highlighted in red, and analytes detected with UV/Vis and CAD are highlighted in green. Asterisks indicate impurities present in analytical standards. **B.** Extracted ion chromatograms (XICs) of 5 analytes. The numbers in the heading refer to the names in Table 3. The shown *m/z* are the values used for the Single Ion Monitoring (SIM) scans.

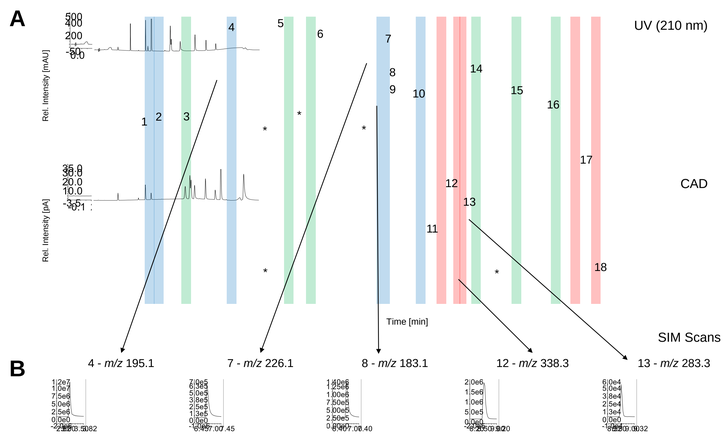
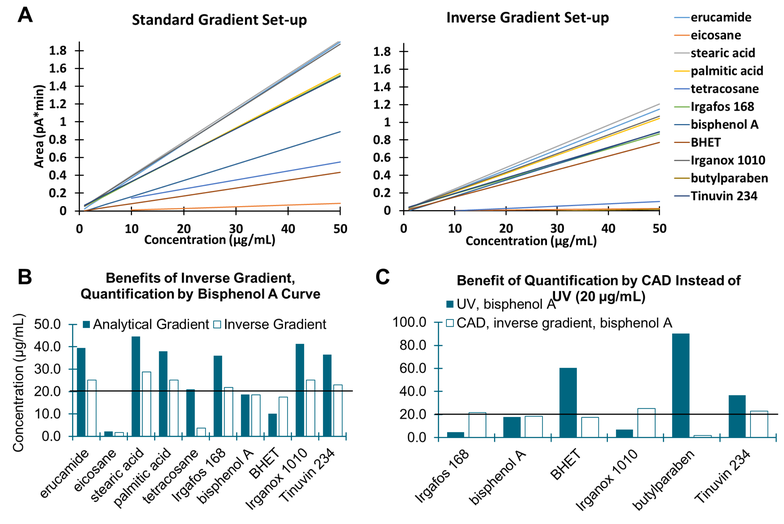


Figure 3. Quantification method development by CAD. A. Calibration curves with and without the inverse gradient. Curves of butylparaben, tetracosane and eicosane do not group with the curves of the other analytes because they are semivolatile. The inverse gradient results in shallower response curves and thus, reduced sensitivity compared to the standard set-up. However, it results in more uniform response and allows for quantification with universal calibrants. **B.** Calibration using a universal calibrant (bisphenol A) with and without the inverse gradient. Using an inverse gradient set-up results in a more uniform signal response in CAD and hence more accurate quantitation with a universal standard. **C.** Comparison of quantification of a reinjected 20 µg/mL standard by UV and CAD using a universal calibrant (bisphenol A). The CAD provides uniform response and thus more accurate quantitation than UV if a universal calibrant is employed.



Sample Analysis

Analysis of cell culture bag extracts revealed high levels of the UV-invisible slip agent, erucamide, in three out of four extracts (Table 4 and Figure 4A). Two derivatives of Irgafos 168 were also present in many of the samples (Figure 4B), as previously described.[1] In total, all 19 unknowns and two known substances (stearic acid and erucamide, Figure 4B) found in the bags were quantified by the universal calibration curve, that for bisphenol A (Table 4). The MS allowed preliminary mass assignments to be made for all extracted substances and for two unknowns to be identified by name based on previous work.[1]

Set-up Choice

When the sample contains unknown substances for which standards do not exist, the inverse gradient multidetector set-up should be used to quantify these substances by CAD. Peak identification should be performed by MS and supported by UV 3D scans. If standards exist for all peaks in a sample, a multidetector set-up with only an analytical gradient can be used. Quantification is performed by the complementary CAD and UV detectors, and MS should be used for peak confirmation.

Figure 4. Analysis of cell culture bag lining extracts by MS, UV, and CAD. A. UV (210 nm) and CAD chromatograms of Sample C. Several extractables were detected with both detection modes. B. Quantification of two extractables found in several samples using either the calibration curve of the standard or a universal calibration curve. C. XICs of two extractables found in several samples. One is an unknown extractable with a peak at 7.73 minutes and a *m/z* of 473.3 in negative mode. The other is erucamide. (Data shown are for Sample C.)

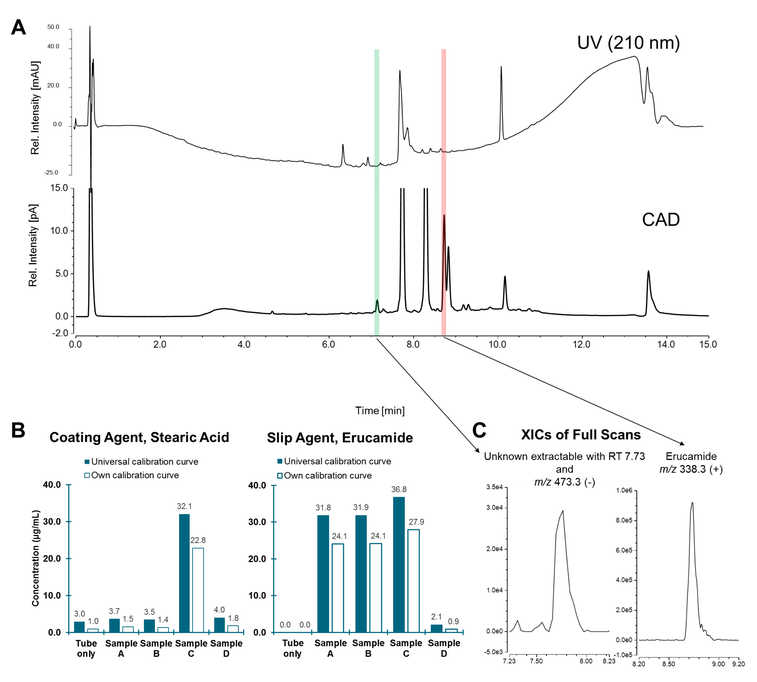


Table 4. Known and unknown extractables from cell culture bags (Samples A, B, C, and D) and from the microcentrifuge tubes used to prepare the samples (labeled with a dash, —). Eleven additional smaller peaks, not shown here, were also detected and quantified by CAD. Abbreviations: bDtBPP = bis(2,4-di-tert-butylphenyl)phosphate; TBPP-ox = oxidized Irgafos 168; IPA = isopropanol; RT = retention time.

RT (min)	UV	CAD	MS	Amount (µg/mL)					Detected Mass	Possible Identity
				–	A	B	C	D		
7.14		✓	✓	5.5	5.2	5.2	5.6	5.2	325.3 (+)	unknown
7.29	✓	✓	✓	x	1.9	1.7	3.1	x	374.3 (+)	unknown
7.66		✓	✓	5.9	4.6	8.9	3.8	6.2	375.4 (-)	unknown
7.73	✓	✓	✓	149	144	111	144	136	473.4 (-)	bDtBPP, $[M-H]^-$
8.28	✓	✓	✓	124	131	95.6	132	115	403.4 (-)	unknown
8.72	✓	✓	✓	x	31.8	31.9	36.8	2.1	338.3 (+)	erucamide, $[M+H]^+$
8.83		✓	✓	3.0	3.7	3.5	32.1	4.0	283.3 (-)	stearic acid, $[M+H]^+$
10.17	✓	✓	✓	1.9	15.8	16.2	15.2	8.2	663.5, 685.5, 723.5 (+)	TBPP-ox, $[M+H]^+$, $[M+Na]^+$, $[M+H+IPA]^+$
10.88	✓	✓	✓	1.4	1.9	2.0	1.7	3.1	279.2, 366.2 (+)	unknown

CONCLUSIONS

- UV/Vis and CAD detection complement each other, resulting in comprehensive sample analysis.
- Mass spectrometric detection provides additional information on the detected analytes. This allows confirmation of known compound identities or tentative identification of unknown compounds.
- The standard multi-detector set-up is suitable for quantitation of known compounds and for amount estimation of unknown components.
- The inverse gradient multi-detector set-up allows quantification of compounds which are not available as reference standards or whose identity is unknown.

REFERENCES

1. Dorival-García, N.; Bones, J. Monitoring leachables from single-use bioreactor bags for mammalian cell culture by dispersive liquid-liquid microextraction followed by ultra high performance liquid chromatography quadrupole time of flight mass spectrometry. *J. Chromatogr., A* **2017,** *1512,* 51–60.
2. Norwood, D.L.; Jenke, D.; Manolescu, C.; Pennino, S.; Grinberg, N. HPLC and LC/MS Analysis of pharmaceutical container closure system leachables and extractables. *J. Liq. Chromatogr. Relat. Technol.* **2009,** *32,* 1768–1827.
3. Asa, D.; Carreiro, D. Extractable and leachable measurements by universal HPLC detection. The Application Notebook – September 2007, 65. Accessed May 2018 at www.chromatographyonline.com/parabens-topical-preparations-using-spme-gc-ms.

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TRADEMARKS/LICENSING

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