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 sample impurities, degradation products of the analyte and 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 was used 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.

INTRODUCTION

Comprehensive sample analysis is essential for determining the presence of unknown or unexpected compounds. These substances could be impurities, degradation products of the analyte or extractables from containers. In addition, identity confirmation and quantitation of these compounds is needed to determine their nature and whether they are below acceptable concentration limits. Evaluation of such complex samples requires multiple complementary detectors. The UV detector accurately quantifies chromophore-containing substance. The charged aerosol detector (CAD) delivers universal detection of non- and semi-volatile compounds. Additionally, its near uniform response allows straightforward quantification without reference standards. Mass spectrometry (MS) offers identity confirmation of the detected compounds. This comprehensive sample analysis platform was expanded further with two ionization modes: HESI and APCI for MS detection.

MATERIALS AND METHODS

A Thermo Scientific™ Vanquish™ Flex UHPLC system, controlled by Thermo Scientific™ Chromeleon™ 7.2 CDS software, was used in two different configurations for chromatographic analysis (Table 1 and Figure 1). In the standard set-up, a single pump delivered the analytical gradient. In the inverse set-up, a two-pump module delivered the analytical and inverse gradients. The inverse gradient resulted in a constant solvent composition for CAD and MS detection, which improved detector response uniformity. Choice between heated electrospray (HESI) or atmospheric pressure chemical ionization (APCI) ionization modes for MS minimized detection problems for poorly ionizable substances. Chromatographic methods are shown in Table 2. Eighteen reference compounds were selected based on literature reports of extractables present in cell culture bags[1,2,3]. Dilutions were prepared in methanol from 1 mg/mL standards at 1, 2, 5, 10, 20 and 50 µg/mL, except for butylparaben, eicosane and tetracosane, which were prepared at 10 – 500 µg/mL. The inner layer of single-use cell culture bags, which comprised ethylenevinyl acetate and different densities of polyethylene, were extracted with 50/50 isopropanol/water (v/v).

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

Module	Standard Setup	Inverse Gradient Setup
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	✓	✓
Thermo Scientific™ ISQ™ EC/EM Single Quadrupole Mass Spectrometer	✓	✓

Figure 1. Schematic display of standard setup and inverse gradient setup. A. The standard setup uses solely 100 µm ID (inner diameter) capillaries (depicted in red). **B.** The inverse gradient setup 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. 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.

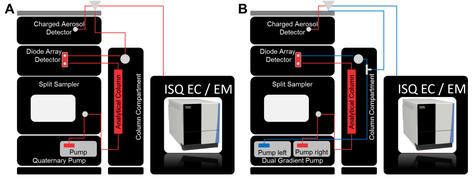


Table 2. Method and detection parameters.

Parameter	Settings					
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 Column, 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 HESI and APCI source settings for 0.167 mL/min (standard setup) or 0.333 mL/min flow rate (inverse gradient setup) Alternating positive/negative mode full scans & SIM scans					

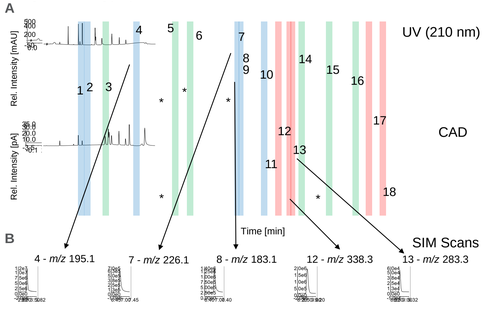
RESULTS

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 because they were too volatile.[4] However, they possessed a sufficiently active chromophore for measurement by UV/Vis detection. Conversely, 5 analytes with poor chromophores were not detected by UV/Vis but were detected by CAD. Two analytes did not show a strong MS signal, likely due to poor ionizability. Sixteen compounds could be clearly detected with the single quadrupole MS (Table 3) by both HESI and APCI. Mass confirmation was based on detection respective *m/z* species in positive or negative mode in full scan and SIM scan at the same elution time as observed by UV/Vis detection or CAD. Five representative extracted SIM scans are shown in Figure 2B. Substances that showed better relative peak areas with APCI were azobenzene and BHT. Samples with better relative peak areas with HESI were BHET, palmitic acid and stearic acid. Extracted SIM scans comparing detectability of BHT and palmitic acid with APCI and HESI are shown in Figure 3.

Table 3. List of reference analytes. Detectability with UV, CAD, MS with HESI source and MS with APCI source is indicated with check marks. Cells in dark blue indicate substances for which the relative peak area using one source was at least 20-fold better than the response using the other source. [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.

#	Analyte	CAS	UV	CAD	HESI - MS	APCI - MS	[M]	Mass Found, HESI / APCI	Ion Found, HESI / APCI	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	227.1 / 228.1	$[M-H]^-$ / $[M]^+$	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	205.2 / 206.2	$[M-H]^-$ / $[M]^+$	1 (UV)
10	BHT	128-37-0	✓		✓	✓	220.2	219.2 / 220.2	$[M-H]^-$ / $[M]^+$	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	1194.8 / 1176.8	$[M+NH_4]^+$ / $[M]^+$	1
16	Irgafos 168	31570-04-4	✓	✓	✓	✓	646.5	647.5	$[M+H]^+$	1
17	Eicosane	112-95-8		✓			282.3			50
18	Tetracosane	646-31-1		✓			338.4			50

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 those used for the Single Ion Monitoring (SIM) scans.



Calibration curves for quantification by CAD in the presence and absence of the inverse gradient were compared (Figure 4A). 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 4A. This is further demonstrated in Figure 4B 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. Salt formation also affects response; these factors and methods for identifying semi-volatiles are explained in detail in TN 72806.[4] Comparison of the response uniformity of UV and CAD (Figure 4C) confirms that CAD allows more accurate quantitation of unknowns.

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 5A). Two derivatives of Irgafos 168 were also present in many of the samples (Figure 5B), as previously described.[1] In total, all 19 unknowns and two known substances (stearic acid and erucamide, Figure 5B) 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]

Figure 3. Examples of substances that showed greater peak areas using the APCI source (top row, BHT, SIM scan with positive polarity, for m/z 220.2, CID = 5) or the HESI source (bottom row, palmitic acid, SIM scan with negative polarity for m/z 255.2, CID = 5).

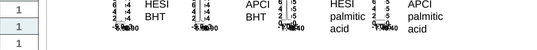


Figure 4. Calibration using a universal calibrant (bisphenol A) with and without the inverse gradient. A. Calibration curves without the inverse gradient. B: Calibration curves with the inverse gradient. C: Reinjection of 20 µL/mL and quantification with and without inverse gradient. The asterisks indicate semivolatiles, which have a lower response and a limit of quantification of only 50 µg/mL. D. Reinjection of 20 µg/mL and quantification by UV and CAD. Butylparaben was identified as a semi-volatile and could not be quantified by the universal calibrant because of its limit of detection of 50 µL/mL.

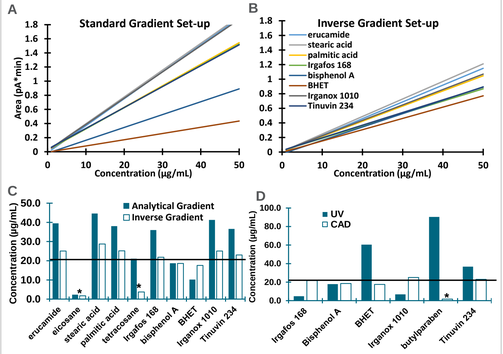


Figure 5. Analysis of cell culture bag lining extracts by MS, UV, and CAD. A. UV (210 nm) and CAD chromatograms of Sample C. Several extractables were detected with both detection modes. **B.** Quantification of two extractables found in several samples using either the calibration curve of the standard 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.)

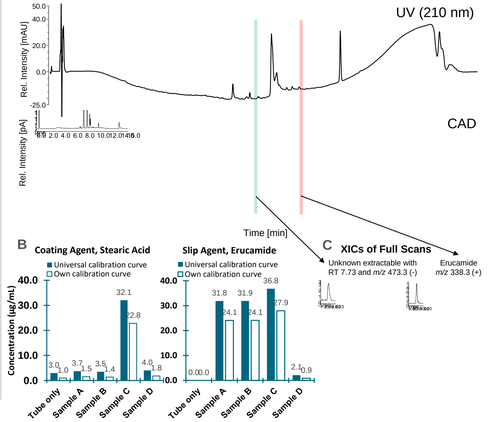


Table 4. Known and unknown extractables from cell culture bags (Samples A, B, C, and D) and from the microcentrifuge tubes used to prepare the samples (labeled with a dash, —). Eleven additional smaller peaks, not shown here, were also detected and quantified by CAD. Abbreviations: bDtBPP = bis(2,4-di-*tert*-butylphenyl)phosphate;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

Choice of Set-up and Source

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/contour plots. If standards exist for all peaks in a sample, a multidetector set-up with only an analytical gradient and quantification by UV/Vis and CAD can be used.

Either the HESI or the APCI sources are suitable for this analysis. Sixteen of eighteen substances in the calibration standard were detected with both sources.

CONCLUSIONS

- UV/Vis and CAD detection complement each other, resulting in comprehensive sample analysis.
- The inverse gradient multi-detector setup enables the use of a single calibrant that allows quantification of compounds that are not available as reference standards or whose identity is unknown.
- Mass spectrometric detection provides additional information on the detected analytes enabling confirmation of known compound identities or tentative identification of unknown compounds. For more complete sample characterization two ionization modes, APCI and HESI, can be applied.

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