

Extractables and Leachables in Microcentrifuge Tubes – Extensive HPLC/GC/MS Analysis

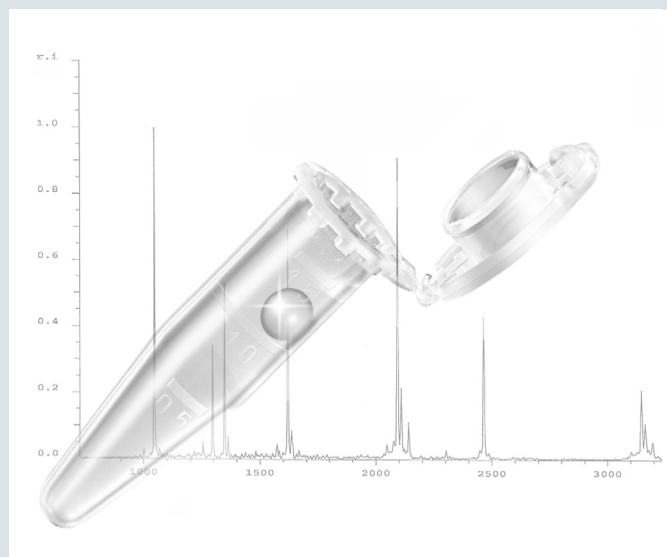
Rafal Grzeskowiak, Eppendorf AG, Hamburg, Germany

Abstract

Substances leaching out of plastic consumables (leachables) are still underestimated in a majority of life science applications. Increasing scientific evidence shows that this heterogeneous and largely undefined group of chemicals may significantly affect experiments and be a source of error in various assay systems.

In this Application Note extensive analyses using HPLC, UHPLC, GC and MS methods have been applied to comprehensively identify and quantify organic substances leaching from standard microcentrifuge tubes into samples after exhaustive extraction with organic solvent (extractables) and organic substances leaching after incubation under typical assay conditions with water (leachables). Both extractables and water soluble leachables analyses identified high amounts of organic substances (up to 1.53 g/kg) migrating into samples incubated in microcentrifuge tubes of various manufacturers. Extractables were identified as typical polypropylene additives: nucleating agents (potentially critical effects on various biological assays) and anti-oxidants (critical), whereas water-soluble leachables could be identified as typical polymerization additives and their by-products: chain-cleaving agents, antistats and nucleating agents/clarifiers.

Samples incubated in Eppendorf Tubes® showed consistently and under all experimental conditions by far the lowest levels of organic substances possibly migrating into samples, and thus having the lowest risk to negatively affect assays.



Introduction

Contrary to common belief, polymers used in the production of pharmaceutical and life science consumables, cannot be considered as pure compounds. Laboratory and medical grade materials used should be seen as a blend of base polymers with a broad range of chemicals added to improve or accelerate the processability of the polymer during production, its long-term stability or to enhance its performance in the end product in various ways.

As recent scientific evidence indicates, part of those processing additives can be released into a sample in direct contact with the polymer over time and become so called "leachables". The bioactive substances leaching out of plastic consumables are still frequently underestimated in the majority of life science applications. Considerable scientific evidence over the past 10 years indicates however, that this heterogeneous group of chemicals may significantly affect experiments and pose a likely source of error in various assay systems [1 - 7]. Growing evidence for consumables hampering experiments leads to change in perception, where high quality consumables are increasingly important and an urgent need arises for a more consequent and detailed analysis of actual (specific) substances migrating from common plastic labware into the sample.

Leaching is a complex chemo-physical process, which can be affected by various factors, most importantly by solvent type, contact time and temperature. Incubation of water samples in microcentrifuge tubes has become a standard experiment and allows showing differences in leachables levels between various manufacturers. General levels of organic compounds leaching into water samples in this experiment may be interpolated to most experimental conditions in life science. Thus far no data on chemical composition and accurate quantification of substances leaching into water samples incubated in standard microcentrifuge tubes was available. In this application note an extensive analysis using state of the art, sensitive analytical methods (HPLC, UHPLC, GC, MS) have been applied to comprehensively identify and quantify organic substances leaching from standard microcentrifuge tubes into samples after exhaustive extraction with organic solvent (extractables) and after incubation with water (leachables relevant for life science). The mentioned analysis was completed by two ISO 17025 accredited organizations routinely performing material analysis for pharma and life-science sector.

Materials and Methods

Extractables: HPLC, FID/MS analysis

A validated and accredited method of exhaustive extraction with dichloromethane was used for analyzing extractables in 1.5 mL standard microcentrifuge tubes from Eppendorf and manufacturers A, S and V. In summary, 1 g of the polymer sample from each tube tested was extracted with 10 mL dichloromethane for 2 days at 40 °C. The dichloromethane extracts were evaporated and re-dissolved in 10 mL of 95% ethanol. The ethanol-dissolved samples were used for HPLC analysis using Shimadzu Class VP, Column: SphereClone ODS 2 (250 x 4.6 mm, 5 µ; Phenomenex); eluent: 90% ethanol (isocratic); mobile phase flow rate: 1.0 mL/min, detector (UV): 230 nm, column oven: 30 °C. Identification / characterization was achieved by coupling the HPLC to a mass spectrometer. The mass spectra that were obtained were identified by comparison with reference spectra in the NIST spectral library.

Water soluble leachables: UV/VIS absorption spectra measurement of incubates

36 of 1.5 mL standard microcentrifuge tubes from each manufacturer (Eppendorf and manufacturers A, S, V) were filled each with 1.5 mL of ultra-pure water and incubated for 30 min at 95 °C in Eppendorf ThermoMixer® C at 600 rpm. After cooling the samples for each individual manufacturer were pooled (36 x 1.5 mL) and 100 µL were used for spectrophotometric measurements using the Eppendorf BioSpectrometer® und UVette®. The rest of the pooled sample of each manufacturer was used for further GC/MS analysis of volatile organic compounds (3.3).

Water soluble leachables: GC/MS analysis of volatile organic compounds (VOC)

A validated and accredited method of purge-and-trap gas chromatography was used for volatile compound analysis of water samples incubated in 1.5 mL standard microcentrifuge tubes from Eppendorf and manufacturers A, S and V. 10 mL of the pooled water samples of each manufacturer (see 3.2) were analyzed by purge-and-trap gas chromatography using gas chromatograph: Thermo Ultra Trace, column: RXI 624 - 60 m - 0.32 mm i.d. - 1.8 μ m film thickness, temperature program: 35 °C (6 min), followed by heating at 10 °C/min to 90 °C, heating at 20 °C/min to 260 °C (10 min), pre-pressure: 120 kPa helium, split: 10 ml/min. Purge-and-trap Conditions (VSP 4000): Sample: 40 °C, purge time: 20 min, purge flow: 20 ml/min, trap temperature: -35 °C, desorption temperature: 240 °C, desorption time: 10 min, water trap: Peltier at -15 °C, trap: micro packed with Tenax®. Calibration was achieved by comparison of the peak area of the leachables with the peak area of the internal standard fluorobenzene. Identification and characterization was achieved by coupling the purge-and-trap gas chromatography to a mass spectrometer. The mass spectra that were obtained were identified by comparison with reference spectra in the NIST® library.

Water soluble leachables: UPLC/MS analysis of non-volatile organic compounds (NVOC)

A validated and accredited method of UPLC/MS was used for non-volatile organic compound analysis of water samples incubated in 1.5 mL standard microcentrifuge tubes from Eppendorf and manufacturer V (representing intermediate leachables levels in previous analysis).

Sample preparation: 72 tubes from each manufacturer were filled with 200 μ L of ultra-pure water and incubated for 30 min at 95 °C, 600 rpm in an Eppendorf Thermomixer. After incubation the samples were pooled in glass vials to nominal total volume of 14.4 mL. For blank sample 50 mL of ultra-pure water was transferred into an inert glass bottle and incubated at 95 °C for 30 min. Subsequently, 10 mL of the water sample extracts were extracted three times (each at pH < 3, at pH 5-9 and at pH > 12) with 10 mL of dichloromethane (DCM).

Analytical procedures: non-volatile organic compounds analysis was performed by UPLC/MS Screening with reporting limit of 500 μ g/L. In summary, 2 mL of the combined DCM extracts (as described in 3.3) were spiked with 20 μ L of an Internal Standard for Injection (ISI) solution (1000 $\pm$ 25 mg/L 2-fluorobiphenyl, 200 $\pm$ 20 mg/L Tinuvin 327, 500 $\pm$ 50 mg/L palmitic acid-d31, and 200 $\pm$ 20 mg/L caffeine-trimethyl-13C3 in methanol). Aliquots of the spiked extracts were subsequently distributed for the UPLC/MS analyses of non-volatile organic compounds.

A summary of all analytical procedures performed in this application note (extractables and leachables) is depicted in figure 1.

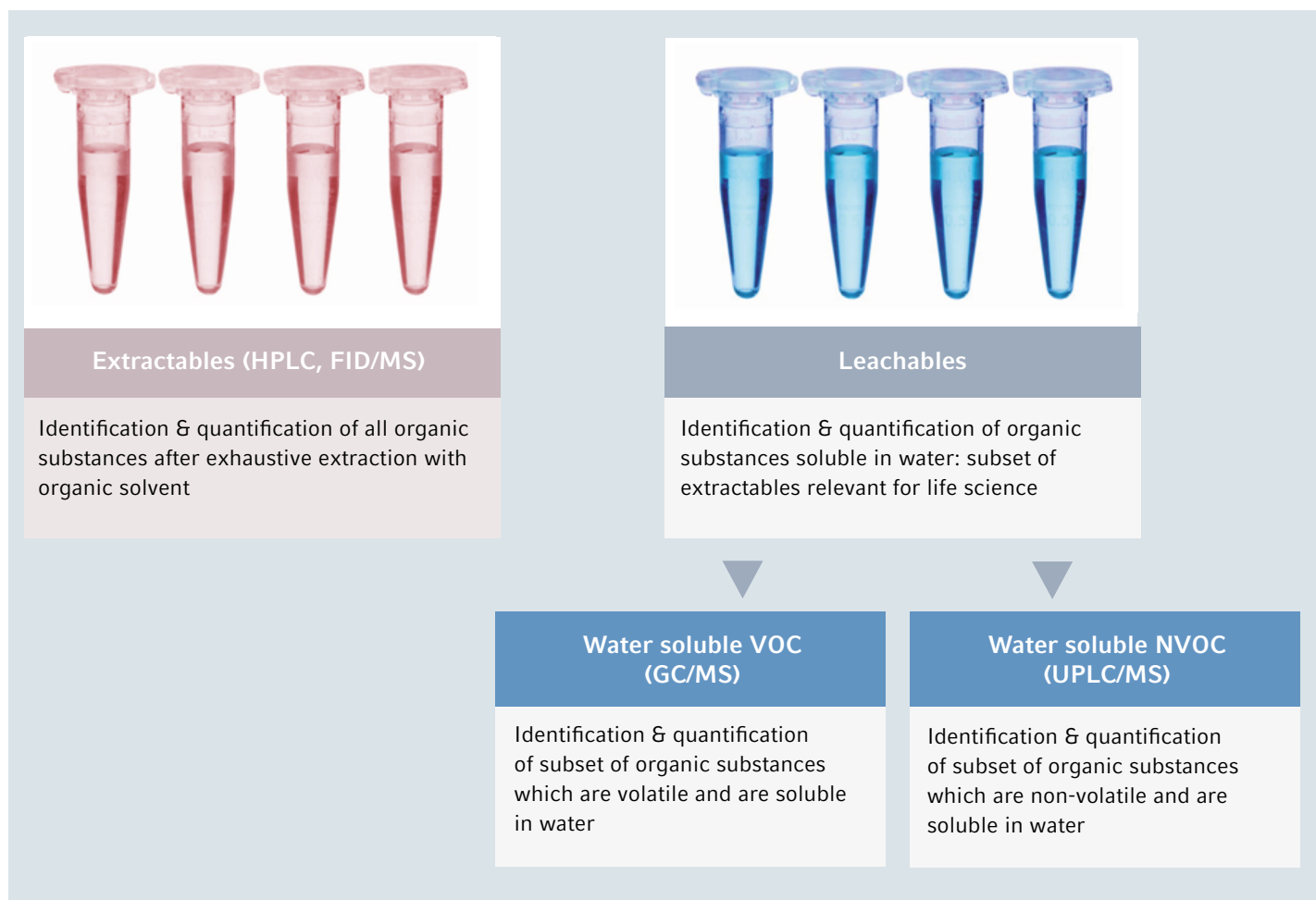


Figure 1: Summary of analytical procedures for extractables and water soluble leachables: volatile organic compounds (VOC) and of non-volatile organic compounds (NVOC)

Results and Discussion

Extractables

The HPLC/MS analysis of samples after exhaustive extraction with dichloromethane (extractables) revealed high levels (up to 450 mg/kg) of various classes of organic substances in the majority of tubes tested (A, S, V), fig. 2.

The extractables could be identified primarily as typical polypropylene additives: anti-oxidants/processing stabilizers and nucleating agents.

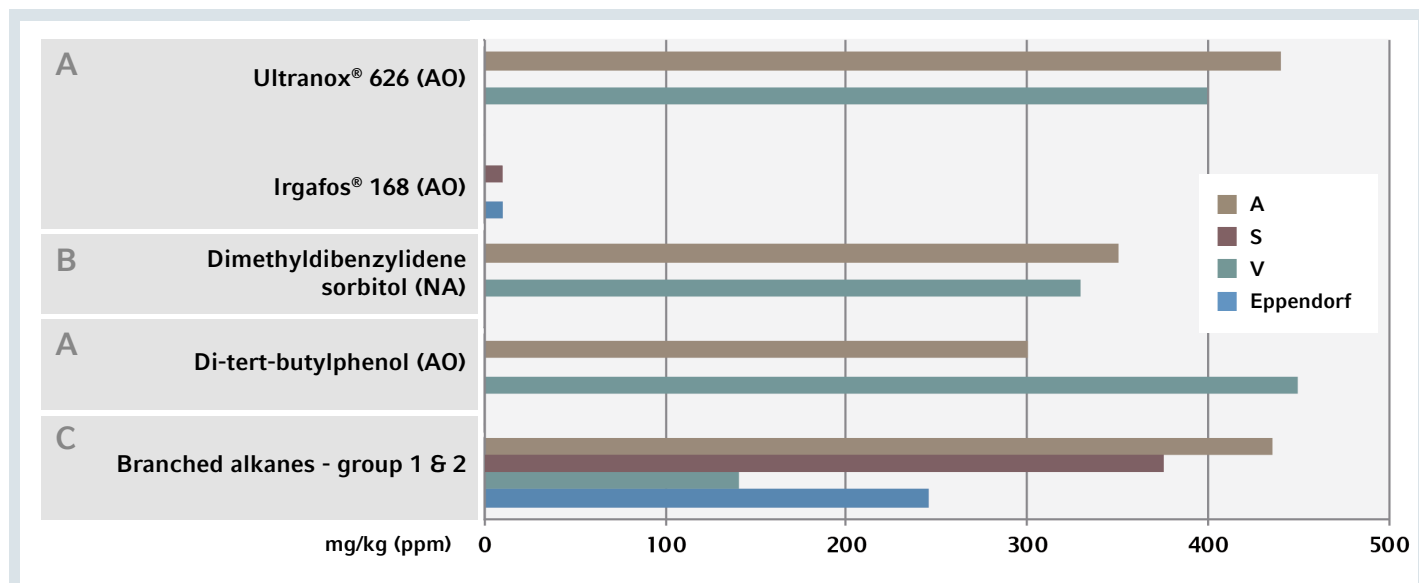


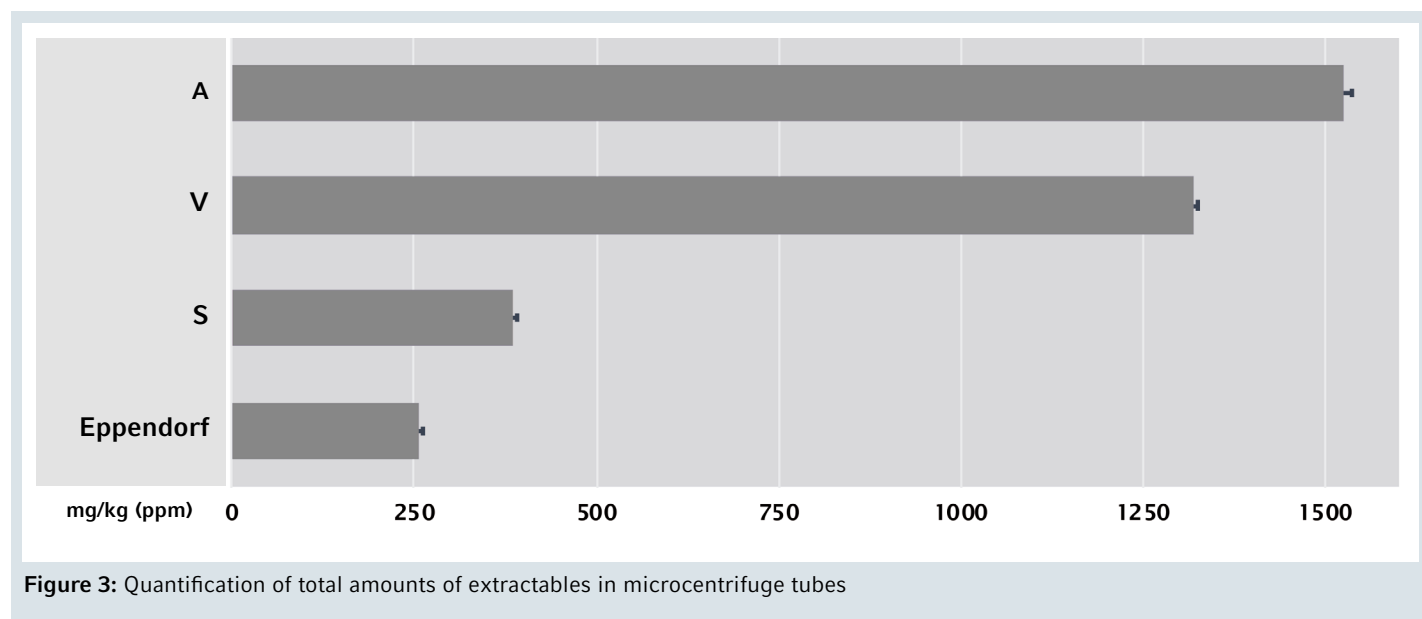
Figure 2: Identification and quantification of extractables in samples incubated in microcentrifuge tubes.

A) AO, antioxidants/processing stabilizers; **B)** NA, nucleating agents; **C)** branched alkanes

Antioxidants (AO) and processing stabilizers are typically complex organic compounds used as free radical and light-protection agents and to stabilize the structure of polymers. They are widely used in most polypropylene types and several AOs were reported to affect various assay systems and rated as critical [5, 8, 9]. The second identified extractable class were nucleating agents (NA), which are widely used to promote crystallization of semi-crystalline polymers (polypropylene). Usage of nucleating agents allows optimization of various polypropylene parameters: crystallization temperature, rate of nucleation, overall rate of crystallization. NAs enhance also flexural modulus and the heat deflection temperature (HDT). NAs are most widely used in polypropylene homo- and random co-polymers (often used in laboratory consumables), where they provide property enhancement, improved molding productivity and increased transparency (clarifiers). Several NAs were reported to interfere with biological assays and rated as potentially critical [5, 8, 10].

A third group of extractables identified in all tubes were branched alkanes, which are typical polypropylene polymerization by-products and are generally regarded as uncritical. Noticeably, the branched alkanes were the only extractables type detected at low levels in Eppendorf Tubes.

The total amounts of extractables are depicted in fig. 3 and indicate high to very high levels of organic substances migrating into the samples from microcentrifuge tubes and also large differences between tested tubes: highest levels were found for A tubes (1,525 mg/kg), followed by V tubes (1,320 mg/kg) and S tubes (385 mg/kg). Samples incubated in Eppendorf Tubes showed very low total extractables levels of 255 mg/kg, which represents 16.7% of the levels observed for A tubes.

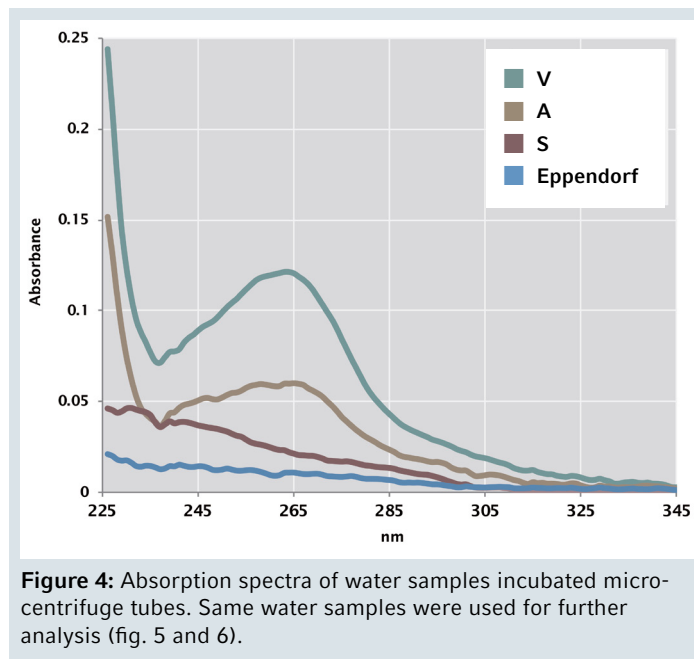


Water soluble leachables – absorption spectra

Leaching is a complex chemo-physical process, which can be affected by various factors, most importantly by solvent type, contact time and temperature. Incubation of water samples is a standard experiment showing differences in general levels of spectrophotometrically active compounds (leachables) in microcentrifuge tubes of various manufacturers. General levels of organic compounds leaching into water samples in this experiment may be interpolated to most experimental conditions in life science.

In fig. 4 the absorption spectra of water samples incubated in standard microcentrifuge tubes are shown. Typical spectra of UV-absorbing leachables could be detected for all water samples and are consistent with previous findings [7, 10]. Eppendorf Tubes showed lowest levels of UV-absorbing leachables ($A_{260}=0.01$) followed by S tubes ($A_{260}=0.02$). Highest levels of UV-absorbing leachables were detected for V ($A_{260}=0.12$) and A tubes ($A_{260}=0.06$). Levels of UV-absorbing leachables approximately correlate with results obtained in extractables analysis: high levels in samples incubated in A and V tubes, low levels in samples incubated in Eppendorf and S tubes (fig. 3).

The performed absorption spectra method (fig. 4) allows only an approximate sum quantification of subsets of leachables. To precisely characterize the chemical composition and accurately quantify substances leaching into



water samples a further extensive analysis using sensitive analytical methods has been applied to the same samples used for spectrophotometric measurements: a GC/MS analysis of volatile organic compounds (VOC).

Water soluble leachables – volatile organic compounds (VOC)

The GC/MS analysis of volatile organic compounds (VOC) in water samples, revealed middle levels (up to ca. 42 µg/kg) of various classes of organic substances predominantly in tubes A and S (fig. 5).

The majority of VOC identified are chain-cleaving reagents and are typical process additives used primarily to reduce chain length and increase the melt flow index of polypropylene after synthesis. Detailed description of identified VOC is provided in table 1.

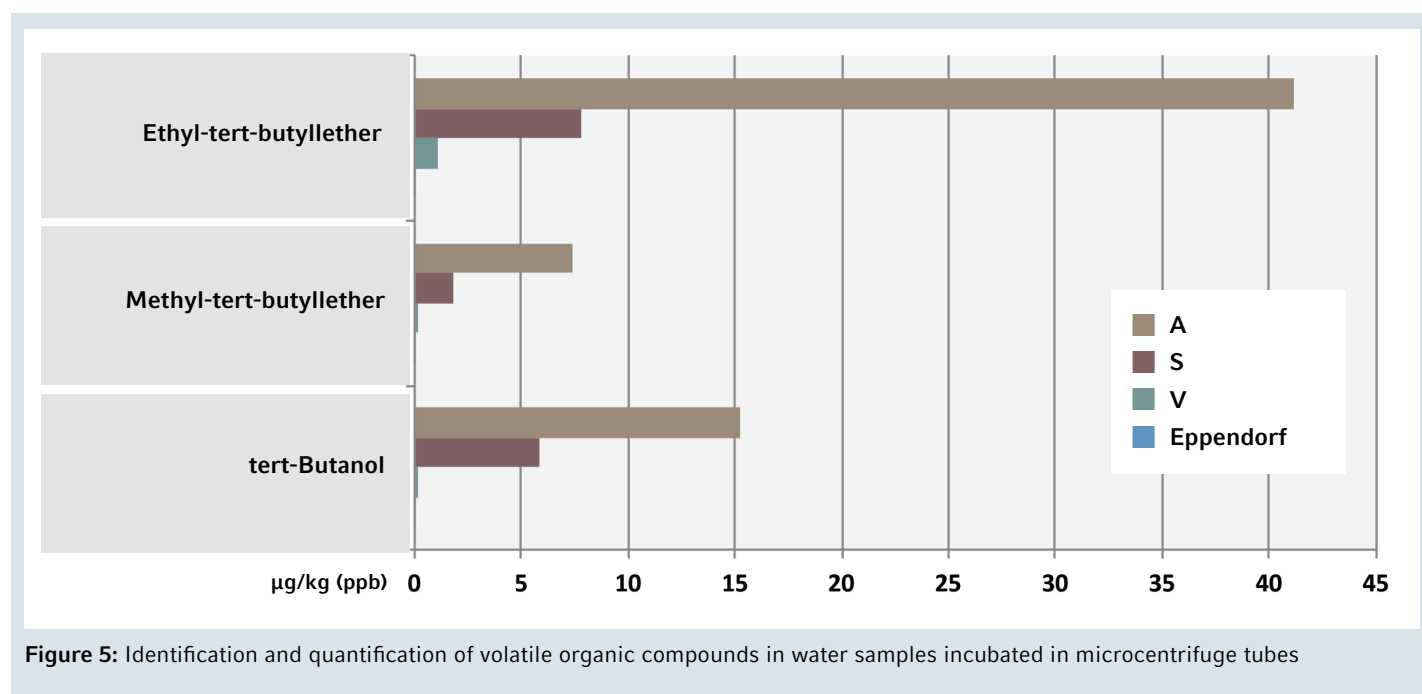


Table 1: Description of volatile organic compounds identified in water samples incubated in microcentrifuge tubes (A, S, V).

Identified compound	Description
Tert-Butanol	Derivative from chain-cleaving agent Di-tert.-butylperoxid
Methyl-tert-butylether	Derivative from chain-cleaving agent Methyl-tert.-butylperoxid
Ethyl-tert-butylether	Derivative from chain-cleaving agent Ethyl-tert.-butylperoxid
Acetone	Derivative from chain-cleaving agent Dicumylperoxid
tert-Pentanol	Degradation product from chain-cleaving agent
tert-Amylmethylether	Degradation product from chain-cleaving agent
tert-Butyl-iso-propether	Degradation product from chain-cleaving agent
Tetramethyl tetrahydrofurane	Probably PP polymerization by-product
Di-tert-butoxymethane	Degradation product from chain-cleaving agent
Orthoformic acid tri-iso-butylester	n.a.

Shaded compounds were found in higher concentrations: > 5 µg/L per sample.

Total amounts of VOC (fig. 6) detected were lower than in the extractables analysis (compare to fig. 3), but also showed large differences between different manufacturers: highest for A tubes (75.6 µg/L), followed by S tubes

(19.6 µg/L) and V tubes (2.8 µg/L). The total amount of VOC measured for Eppendorf Tubes was 0.7 µg/L, which represents only 1.03% of respective levels for A tubes.

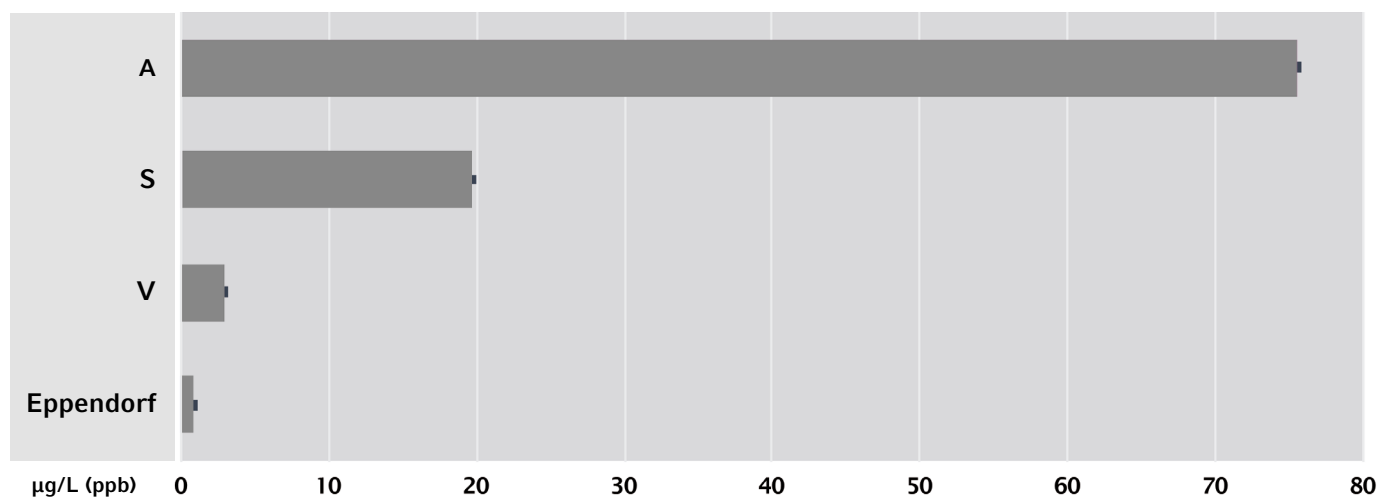


Figure 6: Quantification of total amounts of volatile organic compounds in water samples incubated microcentrifuge tubes

Water soluble leachables – non-volatile organic compounds (NVOC)

The analysis of non-volatile organic compounds (NVOC) has been further narrowed for practical reasons to tubes showing intermediate extractable levels: V tubes, and tubes showing least levels: Eppendorf Tubes. Under the applied

experimental conditions, the UPLC/MS analysis revealed four different compounds detected in water samples incubated in V tubes and no compounds in Eppendorf Tubes as depicted in MS spectra in fig. 7.

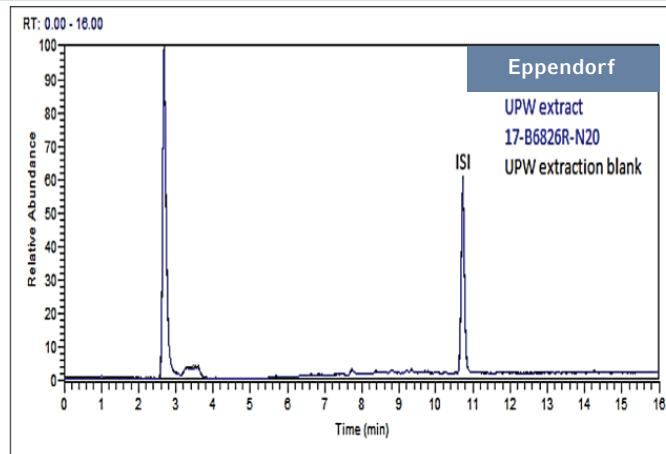
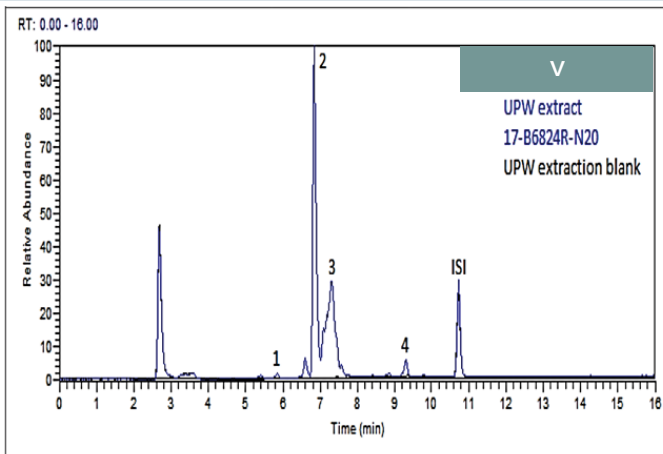
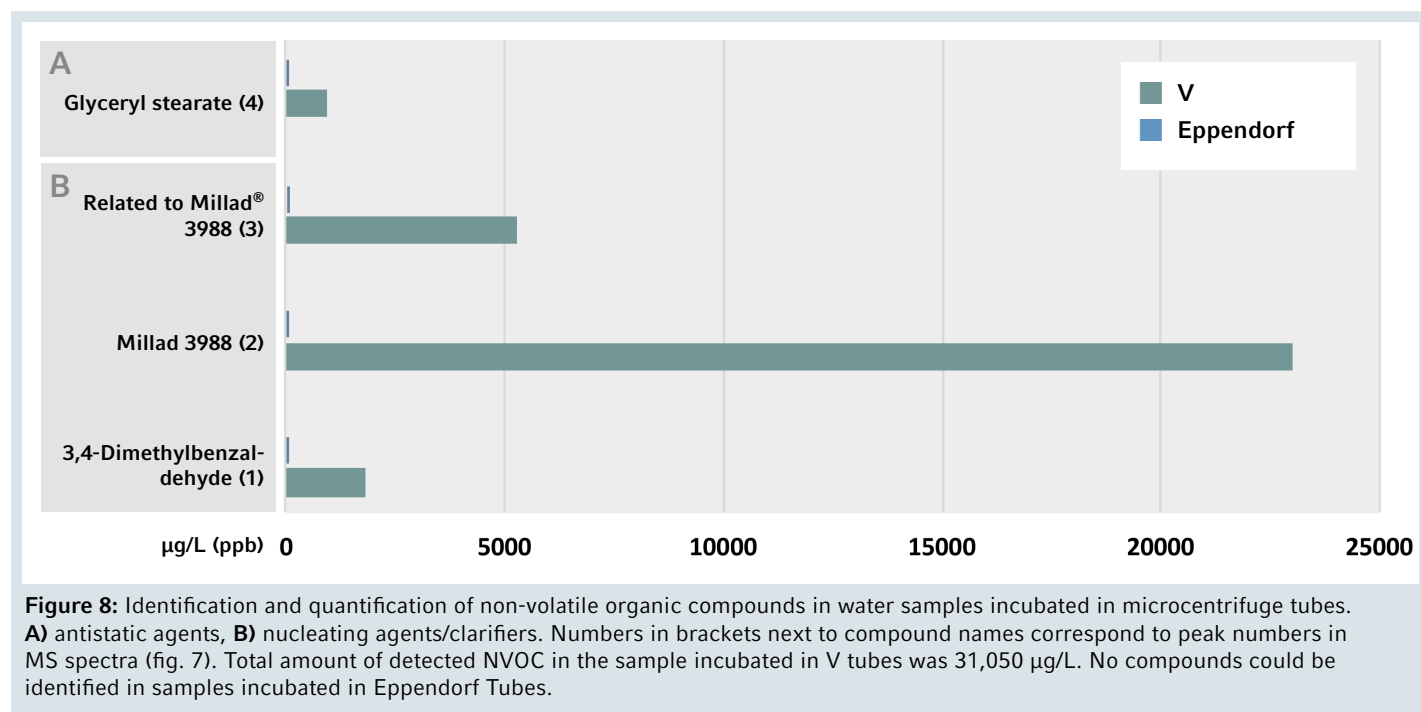


Figure 7: Non-volatile organic compounds analysis. MS spectra of water samples incubated in Eppendorf and V Tubes. Arrows indicates MS peaks of 4 detected compounds in the samples incubated in V Tubes. ISI: internal analysis standard

Four different compounds detected in water samples incubated in V tubes were further quantified in the range between 1,400 to 23,000 µg/L (ppb) and could be identified as two classes of polypropylene additives: antistatic agents

and nucleating agents/clarifiers (fig. 8). The total amount of detected NVOC in the sample incubated in V tubes was very high: 31,050 µg/L.



Antistatic and nucleating agents/clarifiers identified in water samples incubated in V tubes belong to common polypropylene additives. Antistats (glyceryl stearate) are organic compounds composed of both a hydrophilic and a hydrophobic moiety. The compound migrates to the substrate surface and, via hydrogen bonding with atmospheric water, creates a microscopic layer of water on the surface. Chemical antistats are therefore dependent upon atmospheric moisture for their mechanism to dissipate static electricity and polymerization by-products. Some antistats were reported to may have interfered with biological assays and therefore classified as potentially critical [5, 11].

Nucleating agents/clarifiers promote crystallization of semi-crystalline polymers (polypropylene) and are used to enhance end-product properties, improve molding productivity and increase transparency. Some special nucleating agents are used as clarifiers and were frequently reported in literature to hamper various assay systems and were therefore rated as critical [5, 8, 10, 12]. Noteworthy, one of compounds detected in V tubes, the clarifier Millad 3988 and related compounds, exhibits a strong absorption spectrum resembling nucleic acids. This may partially be linked to UV-absorbing leachables spectra observed using standard spectrophotometric methods. A detailed description of NVOC identified in V tubes is summarized in table 2.

Table 2: Description of non-volatile organic compounds identified in water samples incubated in microcentrifuge tubes (V tubes).

Identified compound	Description
Glyceryl stearate	Additive commonly used during production process to dissipate static electricity and polymerization by-products (antistat). Referred in literature as potentially critical additive
3,4-Dimethylbenzaldehyde	Commonly used in the synthesis of organic compounds such as polymer additives: nucleating agent/clarifier, potentially critical additive
Dimethyldibenzylidene sorbitol DMDBS (Millad 3988)	Nucleating agent/clarifier, commonly used to promote crystallization, improve molding productivity and increase transparency. May partially be responsible for strong UV-absorbing spectra of leachables, which resemble nucleic acids in standard spectrophotometric measurements

Conclusions

The extensive analysis of extractables and water soluble leachables identified high amounts of organic substances migrating into samples incubated in standard microcentrifuge tubes of most manufacturers tested.

The analysis of samples after exhaustive extraction (extractables) revealed primarily typical polypropylene additives: anti-oxidants/processing stabilizers and nucleating agents. Total amounts of extractables were high to very high, with large differences between tubes tested: highest levels were detected for A tubes (1,525 mg/kg), followed by V tubes (1,320 mg/kg) and S tubes (385 mg/kg). Samples incubated in Eppendorf Tubes showed very low total extractables levels of 255 mg/kg and consisted of uncritical polymerization by-products: branched alkanes.

Water-soluble leachables could be identified as typical processing agents and additives used during polypropylene synthesis: chain-cleaving agents, antistatic agents and nucleating agents/clarifiers. Detected total levels of water soluble leachables, volatile and non-volatile organic compounds, were up to 75.6 µg/L and 31,050 µg/L, respectively.

All of the identified extractables and water soluble leachables belong to commonly used polypropylene additives and as shown in literature [1 - 5] may have negative effects on various assay systems. In addition, identified nucleating agents/clarifiers: 3,4-dimethylbenzaldehyde and dimethyl-dibenzylidene sorbitol (Millad 3988), and related compounds exhibit strong absorption spectrum resembling nucleic acids. This may be associated to UV-absorbing leachables spectra observed using standard spectrophotometric methods.

Noteworthy, samples incubated in Eppendorf Tubes showed consistently and under all experimental conditions by far the lowest levels of extractables and water-soluble compounds migrating into samples. The only class of detected extractables were low levels of branched alkanes, which are typical polypropylene polymerization by-products and are generally regarded as uncritical. No critical polymerization or production additives were detected whatsoever, indicating that irrespectively of experimental conditions (water, organic solvents) Eppendorf Tubes will provide the highest quality preventing contaminations and assay interferences.

Literature

- [1] McDonald GR, Hudson AL, Dunn SM, You H, Baker GB, Whittall RM, Martin JW, Jha A, Edmondson DE, Holt A. Bioactive contaminants leach from disposable laboratory plasticware. *Science* 2008; 322(5903):917.
- [2] McDonald GR, Kozuska JL, Holt A. Bioactive Leachates from Lab Plastics. *G.I.T. Laboratory Journal* 2009; 9-10: 2-4.
- [3] Olivieri A, Degenhardt OS, McDonald GR, Narang D, Paulsen IM, Kozuska JL, Holt A. On the disruption of biochemical and biological assays by chemicals leaching from disposable laboratory plasticware. *Can J Physiol Pharmacol* 2012; 90(6):697-703.
- [4] Schauer KL, et. al. Mass Spectrometry Contamination from Tinuvin 770, a Common Additive in Laboratory Plastics. *Journal of Biomolecular Techniques* July 2013; 24(2):57-61.
- [5] Grzeskowiak R, Gerke N. Leachables: Minimizing the Influence of Plastic Consumables on the Laboratory Workflows. White Paper 026; www.eppendorf.com
- [6] Watson J, Greenough EB, Leet JE, Ford MJ, Drexler DM, Belcastro JV, Herbst JJ, Chatterjee M, Banks M. Extraction, identification, and functional characterization of a bioactive substance from automated compound-handling plastic tips. *J Biomol Screen* 2009; 14(5):566-72.
- [7] Lewis LK, Robson M, Vecherkina Y, Ji C, Beall G. Interference with spectrophotometric analysis of nucleic acids and proteins by leaching of chemicals from plastic tubes. *Biotechniques* 2010; 48(4):297-302.
- [8] Lee TW, Tumanov S, Villas-Bôas SG, Montgomery JM, Birch NP. Chemicals eluting from disposable plastic syringes and syringe filters alter neurite growth, axogenesis and the microtubule cytoskeleton in cultured hippocampal neurons. *J Neurochem* 2015; 133(1):53-65.
- [9] Hammond M. et al. A cytotoxic leachable compound from single-use bioprocess equipment that causes poor cell growth performance. *Biotechnol Prog.* 2014; Apr: 332-7.
- [10] Grzeskowiak R, Huebler D. Is That Really DNA in Your Tube? Comparative Analysis of UV-Absorbing Leachables in Micro-Test Tubes. Application Note 396; www.eppendorf.com
- [11] Belgaiedb S. et al. Characterization of plastic packaging additives: Food contact, stability and toxicity *Arabian Journal of Chemistry* 2017; 10 (2).
- [12] Schiffer C, Müller A, Egeberg DL, Alvarez L1, Brenker C, Rehfeld A, Frederiksen H, Wäschle B, Kaupp UB, Balbach M, Wachten D, Skakkebaek NE, Almstrup K, Strücker T. Direct action of endocrine disrupting chemicals on human sperm. *EMBO Rep.* 2014; 15(7):758-65.

Ordering information

Description	Order no. international
Eppendorf Safe-Lock Tubes , 1.5 mL, Eppendorf Quality™	0030 120.086
Eppendorf Safe-Lock Tubes , 2.0 mL, Eppendorf Quality™	0030 120.094
Eppendorf Tubes® 5.0 mL, Eppendorf Quality™	0030 119.401
Eppendorf Conical Tubes 15 mL , sterile, pyrogen-, DNase, RNase and DNA-free	0030 122.151
Eppendorf Conical Tubes 50 mL , sterile, pyrogen-, DNase, RNase and DNA-free	0030 122.178
Deepwell Plate 96/2000 µL , wells clear, 2000 µL, PCR clean	0030 501.306
Deepwell Plate 96/1000 µL , wells clear, 1000 µL, PCR clean	0030 501.209
Deepwell Plate 96/500 µL , wells clear, 500 µL, PCR clean	0030 501.101

Your local distributor: www.eppendorf.com/contact

Eppendorf AG · Barkausenweg 1 · 22339 Hamburg · Germany

eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com

Irgafos® is a registered trademark of BASF SE, Germany. Millad® is a registered trademark of Milliken and Company, USA.

Ultranox® is a registered trademark of Addivant Switzerland GmbH, Switzerland.

NIST® is a registered trademark of National Institute of Standards and Technology, USA. Tenax® is a registered trademark of Buchem B.V..

Eppendorf®, the Eppendorf Brand Design, Eppendorf ThermoMixer®, BioSpectrometer®, UVette® and Eppendorf Tubes® are registered trademarks of Eppendorf AG, Germany.

Eppendorf Quality™ is a trademark of Eppendorf AG, Germany.

All rights reserved, including graphics and images. Copyright © 2018 by Eppendorf AG.