Trends in Analytical Chemistry 141 (2021) 116284

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Redefining dilute and shoot: The evolution of the technique and its application in the analysis of foods and biological matrices by liquid chromatography mass spectrometry

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ARTICLE INFO

Article history:
Available online 5 April 2021

Keywords:
Dilute-and-shoot
Liquid chromatography
Mass spectrometry
Sample preparation
Food analysis
Bioanalysis
Mycotoxin
Multi-class

ABSTRACT

With laboratories seeking to expand analytical capabilities and create multi-class, multi-analyte methods, there has been a shift toward generic sample clean-up techniques such as “dilute-and-shoot”. Advantages of this methodology include its simplicity, minimal analyte losses, high sample throughput and number of analyte classes included. The evolution of dilute-and-shoot has permitted its use across a variety of matrices including food and biological and in various scientific fields such as food, forensics and environmental. The versatility of the technique permits the expansion of current fields of research without the usual laborious method development. There can be issues with matrix effects and robust quantitation as analyte number increases. This review provides an overview of the technique combined with liquid chromatography mass spectrometry, highlighting its power in facilitating multi-class analysis. Coupled with increases in instrument performance there is potential to employ this methodology in expanding analytical capabilities in many areas of life science research.

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1. Introduction

In analytical chemistry and in particular in the field of small molecule analysis (approx. < 1200 Da) using liquid chromatography mass spectrometry (LC-MS), the use of “dilute-and-shoot” has gained much traction over the last ten to fifteen years, with it now employed in many types of multi-residue LC-MS analysis [1]. The term itself appears quite self-explanatory, referring simply to:

> “Dilution of a sample or sample extract before ‘shooting’ into an analytical instrument for analysis.”

As a stand-alone methodology, the term implies that this technique is the only clean-up step used before sample analysis. However, dilute-and-shoot (DnS) is sometimes performed after an initial deproteinisation step dependent on the matrix being assayed, with it stated that the protein precipitation step is considered a clean-up process [2]. If this is the case and deproteinisation is carried out before dilution of the sample (supernatant), if could be argued that such methodology cannot be considered DnS. Furthermore, matrices that are not liquid must first undergo an extraction step to facilitate movement of analytes from the matrix to a liquid phase before dilution and analysis on some form of mass analyser. These include matrices such as animal feed, tissues and various (solid) foodstuffs. In these cases, a suitable extraction solvent must be chosen to facilitate migration of analytes from a solid to a liquid phase which is then diluted prior to analysis. One such example is in the recent study conducted by Sulyok et al. (2020) measuring over 500 secondary microbial metabolites in various food matrices using DnS coupled to LC-MS/MS [3]. Therein lies the issue as to what extent a sample clean-up method can be truly classified as DnS. With many publications claiming use of the ‘dilute-and-shoot’ approach but which include additional steps such as solid-liquid extraction or deproteinisation, the use of or definition of the technique appears to have evolved. Therefore, due to the evolution of the technique in the literature over the last decade, we have expounded on the simple definition stated above to include solid matrices.

> “The direct dilution of a sample or dilution of a sample extract before ‘shooting’ into an MS platform for analysis. This also allows for the addition of a solvent to a solid matrix to extract

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https://doi.org/10.1016/j.trac.2021.116284
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untargeted or targeted compounds into a liquid phase before dilution and analysis. Filtration and centrifugation are included in our definition, as they are physical methods that remove solid materials from liquid samples, thereby making the sample more suitable for LC-MS analyses".

In using DnS as a sample clean-up technique, the main purpose of the dilution step is to reduce matrix components which can interfere with the chromatography and ionisation of the target analytes, thereby facilitating greater sensitivity or performance of the analytical method. With that in mind, the dilution factor used is dictated by many factors including; concentration of the analytes expected, the matrix analysed, the selectivity and sensitivity of the MS platform utilised and whether the analysis is quantitative or qualitative, with dilution factors typically ranging from 1:1 up to 1:100. Some examples include a dilution factor of 1:1 (v:v) in the analysis of secondary microbial metabolites in food matrices; a dilution factor of 1:10 analysing metabolites of the mycotoxins deoxynivalenol (DON) and zearalenone (ZEN) in human urine; a dilution factor of 1:50 in the analysis of drugs-of-abuse (DoA) and performance enhancing drugs in human urine; a 1:100 dilution of samples in the analysis of prosthesis-related metals in whole blood [3–6]. One caveat of the last two examples is that the analysis of DoA by Alcántara-Durán et al. (2018) employed nanoflow LC coupled to an Orbitrap MS which improved method sensitivity and permitted a high dilution factor, whereas the study by Bolea-Fernandez et al. (2016) was of prosthesis-related metals, i.e. elements as opposed to intact molecules and which was carried out on Inductively Coupled Plasma MS (ICP-MS). In the main and for the purpose of this review, the focus will be on the analysis of small molecules by way of LC-MS.

Over recent years there has been a shift from single class methods with low analyte numbers toward multi-class, multi-analyte methods. This has been achieved by moving away from use of traditional sample clean-up techniques such as solid phase extraction (SPE) and Immunoaffinity Columns (IAC) to more generic techniques such as QuEChERs (Quick, Easy, Cheap, Effective, Rugged and Safe), with the latter methodology routinely applied in multi-residue pesticide analysis in agricultural commodities [7]. However, even techniques such as QuEChERs still suffer from losses or incomplete recovery of the target analytes when performing multi-class analysis. These analyses typically utilise chromatographic separation to aid detection and can be performed on high performance liquid chromatography (HPLC). Currently however, chromatography is routinely performed on ultra-high performance liquid chromatography (UHPLC or UPLC) coupled to some form of mass analyser. The switch to UHPLC facilitated shorter run times while allowing the same number of analytes to be resolved. Of late, method run times have become longer to facilitate the increase in the number of analytes included in a single run [3,8]. In regards to the mass analyser used, these include low resolution mass spectrometers (LRMS), mainly triple quadrupole instruments (QqQ or MS/MS), and high-resolution instruments such as ion trap or orbital ion traps (Orbitrap), time-of-flight (TOF) and the new generation of hybrid systems such as quadrupole-ToF (QToF) and quadrupole-Orbitrap (Q-Orbitrap), with these HRMS systems offering different resolving powers and therefore selectivities and sensitivities. LRMS instruments are used mainly in routine testing laboratories for high throughput quantitative analysis, whereas HRMS systems are more typically employed for research purposes. Thus, the instrumentation used will be dependent on the type of analysis required, whether it be fully quantitative, qualitative for screening purposes or simply untargeted for "omics" analysis.

The purpose of this review is to highlight the evolution in the use of dilute-and-shoot in analytical chemistry which has now been employed across many different matrices, whilst also indicating that the definition of the term should be extended to reflect this. In addition, we look at how its use is expanding particularly in the area of targeted analysis, mainly due to the advancement of the detection platforms which has allowed it to transcend many different scientific disciplines.

2. Dilute-and-Shoot

2.1. An overview of the technique

DnS refers to the dilution of a sample matrix with a suitable dilution solvent (diluent) for analysis. Therefore, depending on the matrix analysed it can either be a straightforward dilution of a biological fluid such as urine or oral fluid which conform to the 'original' definition of the technique. However, other more complex fluids such as serum, plasma or milk can require an initial protein precipitation step, or dilution of the extraction solvent used in extracting analytes of interest from a solid matrix such as foodstuffs, with the latter in particular included in the extended, updated definition of the technique outlined above. Compared with other traditional sample clean-up techniques which remove unwanted co-extracted matrix components, DnS simply decreases matrix effects while eliminating time-consuming and expensive extraction procedures and can therefore be considered the most straightforward and fastest clean-up method available. One caveat of this is in the application to solid foodstuffs whereby an extraction step is required to facilitate migration of analytes from a solid to a liquid phase, with examples detailed in Table 1.

Use of DnS in analytical chemistry has become quite commonplace due to its simplicity, limited and fast sample preparation, low solvent usage and associated low-costs, as well as reduced waste production, with the benefit of being more environmentally friendly than most other sample clean-up techniques. As well as being a relatively environmentally friendly technique, DnS lends itself well to high throughput routine analysis which has led to its use across various disciplines such as in drugs-of-abuse (DoA) and anti-doping (performance enhancing drugs), forensics and food safety, and can be used across a range of "omics" studies such as metabolomics. It also lends itself to multi-class, multi-analyte approaches as there are little to no analyte losses and its simplicity minimises the chance of errors during the extraction stage. However, there can be issues with the repeatability, reproducibility, accuracy and precision of this technique, especially in relation to the validation of multi-residue methods in foodstuffs using DnS. These aforementioned issues have been addressed somewhat in the studies carried out by Malachova et al. (2015) and Sulyok et al. (2020) [3,9]. Examples of where the DnS methodology has been employed alongside either LC-MS/MS or LC-HRMS are given in Table 1, with examples as to why DnS is such a powerful analytical tool is discussed in more detail later.

2.2. Issues with dilute-and-shoot: terminology and methodology

In terms of the expounded definition of DnS, whereby an extraction solvent is required to facilitate the migration of analytes from a solid matrix into a liquid phase before dilution for analysis, one must be aware that the choice of extraction solvent directly impacts the range of analytes that can be extracted, i.e. the polarity of the extraction solvent used will determine the polarity and therefore range of analytes extracted. It is clear that certain matrices require an extraction step before dilution and an example of this is addressed in the study by Sulyok et al. (2020). The

Table 1
Application of dilute-and-shoot alongside LC-MS/MS across various scientific disciplines and sample matrices.

Compound type (number of analytes)	Matrices	Methodology	LC-MS Platform	Analytical conditions	Reference
Mycotoxins (39)	Wheat & maize	0.5 g matrix extracted with 2 mL of extraction solvent ($ACN:H_2O:CH_3COOH$, 79:20:1 v/v/v), 350 μL supernatant diluted with 350 μL of ($ACN:H_2O:CH_3COOH$, 20:79:1 v/v/v) *Dilution factor 1:2*	QTRAP® 4000 equipped with a Turbo Ion Spray ESI source (AB SCIEX) coupled to a 1100 series HPLC (Agilent) ESI^+ & ESI^-: MRM	Gemini C_{18} column (150 mm × 4.6 mm, 5 μm) + C_{18} security guard cartridge (4 mm × 3 mm, 5 μm) @ 25°C, flow rate 1 mL/min, injection volume 5 μL. *MPA*: $MeOH:H_2O:CH_3COOH$ (10:89:1 v/v/v), *MPB*: $MeOH:H_2O:CH_3COOH$ (97:2:1 v/v/v), both containing 5 mM $NH_4CH_3COO^-$.	[27]
Bacterial & fungal metabolites (295)	Apple puree, hazelnut, maize & green pepper	0.5 g matrix extracted with 2 mL of extraction solvent ($ACN:H_2O:CH_3COOH$, 79:20:1 v/v/v). 350 μL supernatant diluted with 350 μL of ($ACN:H_2O:CH_3COOH$, 20:79:1 v/v/v) *Dilution factor 1:2*	QTRAP® 5500 equipped with a TurboV ESI source (AB SCIEX) coupled to a 1290 series UHPLC (Agilent) ESI^+ & ESI^-: sSRM	Gemini C_{18} column (150 mm × 4.6 mm, 5 μm) + Gemini C_{18} guard column (4 mm × 3 mm, 5 μm) @ 25°C, flow rate 1 mL/min, injection volume 5 μL. *MPA*: $MeOH:H_2O:CH_3COOH$ (10:89:1 v/v/v), *MPB*: $MeOH:H_2O:CH_3COOH$ (97:2:1 v/v/v), both containing 5 mM $NH_4CH_3COO^-$	[28]
Mycotoxins & metabolites (>500)	Wheat, maize, figs, dried grapes, walnuts, pistachios & almonds.	0.25 g matrix extracted with 1 mL of extraction solvent ($ACN:H_2O:CH_3COOH$, 79:20:1 v/v/v). 300 μL supernatant diluted with 300 μL of ($ACN:H_2O:CH_3COOH$, 20:79:1 v/v/v) *Dilution factor 1:2*	QTRAP® 5500 equipped with a TurboV ESI source (AB SCIEX) coupled to a 1290 series UHPLC (Agilent) ESI^+ & ESI^-: sMRM *NB*: Two separate runs for ESI^+ & ESI^-	Gemini C_{18} column (150 mm × 4.6 mm, 5 μm) + C_{18} security guard cartridge (4 × 3 mm i.d.) @ 25°C, flow rate 1 mL/min, injection volume 5 μL. *MPA*: $MeOH:H_2O:CH_3COOH$ (10:89:1 v/v/v) *MPB*: $MeOH:H_2O:CH_3COOH$ (97:2:1 v/v/v), both containing 5 mM $NH_4CH_3COO^-$.	[3]
Mycotoxins (10) and pesticides (6)	Rice	5 g of rice was extracted and diluted with 10 mL $ACN:H_2O$ (8:2 v/v) containing 2% CH_3COOH (v/v) then filtered. *Dilution factor 1:3*	QTRAP® 6500 (AB SCIEX) coupled to a 1290 series UHPLC (Agilent) ESI^+ & ESI^-: MRM	Zorbax RRHD C_{18} column (50 mm × 2.1 mm, 3 μm) with pre-column @ 30°C, flow rate 350 μL/min, injection volume 5 μL. *MPA*: H_2O, *MPB*: MeOH both containing 0.5% CH_3COOH (v/v)	[29]
Pesticides (185) and mycotoxins (12)	Wine (red)	A 500 μL aliquot of wine was diluted with 500 μL of MeOH:ACN (1:1, v/v) and filtered. *Dilution factor 1:2*	Xevo® TQ-S (Waters) coupled to an ACQUITY H-Class UPLC® with quaternary solvent manager (Waters) ESI^+: MRM	ACQUITY UPLC® BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) @ 60°C, flow rate 450 μL/min. *Pesticide analysis:* *MPA*: 5 mM $NH_4HCOO^-{}_{(aq)}$, *MPB*: MeOH Mycotoxin analysis: *MPA*: H_2O and *MPB*: ACN, both containing 0.1% HCOOH (v/v)	[17]
Prescription drugs, DoA and metabolites (37)	Urine (human)	100 μL of centrifuged urine diluted with 700 μL H_2O and 200 μL IS_{mix}. *Dilution factor 1:10*	TQ-S Micro (Waters) coupled to an ACQUITY UPLC (Waters) ESI^+: MRM	CORTECS C_{18} column (2.1 mm × 50 mm, 1.6 μm) @ 30°C. *NB*: Injection volume not stated. *MPA*: Water, *MPB*: MeOH (both containing 0.1% HCOOH v/v).	[16]
Mycotoxins and metabolites (37)	Urine (human)	100 μL of centrifuged urine diluted with 900 μL of diluent (H_2O:ACN:HCOOH, 94:5.:1 v/v/v) *Dilution factor 1:10*	QTRAP® 6500 (AB SCIEX) coupled to a 1260 Infinity series HPLC (Agilent) ESI^+ & ESI^-: sMRM	NUCLEODUR® C_{18} Pyramid column (150 mm × 2 mm, 3 μm) with a C_{18} guard column (2 mm × 4 mm), flow rate 600 μL/min, injection volume 10 μL. *MPA*: ACN, *MPB*: Water (both containing 0.1% HCOOH v/v).	[70]
Statins (8)	Urine (human)	500 μL of urine spiked with 60 μL IS_{mix} and made up to 1.5 mL total volume with H_2O. *Dilution factor 1:3*	API 3200 Q-Trap equipped with a Turbo V ESI source (AB SCIEX) coupled to an Agilent 1200 series. ESI^+: MRM	Kinetex C_{18} column (50 mm × 3.0 mm, 2.6 μm) with a C_{18} guard column (4 mm × 3 mm) @ 20°C, flow rate 350 μL/min, injection volume 10 μL. *MPA*: Water (0.1% CH3COOH), *MPB*: ACN.	[92]

(*continued on next page*)

Table 1 (*continued*)

Compound type (number of analytes)	Matrices	Methodology	LC-MS Platform	Analytical conditions	Reference
Neurotransmitters and metabolites (10)	Urine (human)	Dilute 1 mL of urine with 1 mL of ACN. Mix, centrifuge then dilute 1 mL supernatant with 0.25 mL IS_{mix} and 1.25 mL 0.2 M acetic acid. *Dilution factor 1:5*	6490 TQ MS (Agilent) coupled to a 1290 infinity series LC system (Agilent) ESI^+: MRM	Agilent Poroshell 120 Bonus-RP LC column (2.1 mm × 100 mm, 2.7 µm), flow rate 180 µL/min. *NB*: Injection volume not stated. *MPA*: Water (0.2% HCOOH, v/v). *MPB*: ACN (0.1% HCOOH, v/v).	[31]
(i) Mycotoxin DON and its glucuronide metabolites (ii) Mycotoxins and metabolites (15) (iii) Mycotoxins DON, ZEN and metabolites *NB*: All of the above studies use the same extraction and analysis.	Urine (human)	Dilute 100 µL of centrifuged urine with 900 µL dilution solvent (H_2O:ACN, 90:10 v/v). *Dilution factor 1:10*	QTRAP® 5500 equipped with a Turbo V ESI source (AB SCIEX) coupled to a 1290 series UHPLC (Agilent) ESI^+ & ESI^-: SRM	Atlantis® T3 column (150 mm × 3 mm, 3 µm) with a C_{18} security guard cartridge @ 35°C, flow rate 600 µL/min, injection volume 5 µL. *MPA*: Water, *MPB*: ACN (both containing 0.1% CH_3COOH)	[4,10,11]
ZEN and metabolites (9*) *known metabolites	Urine (porcine)	Urine samples diluted to 0.2 mM creatinine with H_2O then centrifuged. *Dilution factor unknown*	QTRAP® 6500 equipped with an Ion Drive Turbo V ESI source (AB SCIEX) coupled to a 1290 series UHPLC (Agilent) ESI^-: SRM	Kinetex Biphenyl column (150 mm × 3 mm, 2.6 µm) @ 30°C, flow rate 400 µL/min, injection volume 5 µL. *MPA*: Water, *MPB*: ACN (both containing 0.1% CH_3COOH)	[72]
Novel Psychoactive Substances (826)	Urine (human)	100 µL of urine spiked with IS_{mix} and diluted with 400 µL H_2O. *Dilution factor 1:5* *Enzymatic hydrolysis:* 100 µL of urine mixed with 100 µL glucuronidase solution. *Dilution factor 1:2*	Agilent 6460 QqQ MS with Jet Streaming Technology coupled to a 1290 Infinity series LC (Agilent) ESI^+: dMRM	Zorbax RR HD Eclipse Plus C_{18} column (100 mm × 3 mm, 1.8 µm) with pre-column @ 40°C, flow rate 300 µL/min, injection volume 5 µL. *MPA*: 5 mM $NH_4HCOO^-_{(aq)}$ (0.1% HCOOH, v/v), *MPB*: MeOH (0.1% HCOOH, v/v).	[30]
Chemical warfare agent metabolites (6)	Urine (human)	500 µL of urine mixed with 500 µL of H_2O. *Dilution factor 1:2*	QTRAP® 4000 (AB SCIEX) coupled to an Ultimate 3000 LC (Dionex) ESI^+ & ESI^-: MRM	Acclaim 120C_{18} column (150 mm × 2.1 mm, 2.2 µm) @ 30°C, flow rate 450 µL/min, injection volume 20 µL. *MPA*: Water, *MPB*: ACN (both containing 0.5% HCOOH)	[83]
Cannabinoids and acid precursors (8)	Oral fluid, serum and urine (human)	100 µL of sample mixed with 100 µL M3® reagent, 200 µL IS_{mix} and 200 µL acetone:ACN (8:2 v/v) *Dilution factor 1:6*	Xevo® TQ-S (Waters) coupled to an ACQUITY UPLC® I-class (Waters) ESI^+: MRM	ACQUITY UPLC® BEH C_{18} column (50 mm × 2.1 mm, 1.7 µm) @ 50°C, flow rate 400 µL/min, injection volume 10 µL. *MPA*: Water containing 50 mM NH_4HCOO^- pH3, *MPB*: MeOH	[73]
Psychoactive drugs (13)	Oral fluid (human)	500 µL of oral fluid was mixed with 10 µL IS_{mix} and diluted further with 1 mL H_2O. *Dilution factor 1:3*	Xevo® TQ-S (Waters) coupled to an ACQUITY UPLC® I-class (Waters) ESI^+: MRM	ACQUITY UPLC® BEH C_{18} column (75 mm × 2.1 mm, 1.7 µm) @ 50°C, flow rate of 350 µL/min, injection volume 2 µL. *MPA*: Water, *MPB*: ACN (both containing 0.1% HCOOH)	[15]
Broad spectrum drugs of abuse (61)	Urine (human)	200 µL aliquot of urine diluted with 400 µL H_2O, 300 µL β-Glucuronidase solution and 100 µL IS_{mix}. *Dilution factor 1:2*	Xevo® G2 ToF (Waters) coupled to an ACQUITY UPLC (Waters) ESI^+ *Resolution:* 20 000 FWHM @ *m/z* 400 Mass tolerance: ±5 and 20 ppm	ACQUITY UPLC® BEH C_{18} column (150 mm × 2.1 mm, 1.7 µm) @ 50°C, flow rate 400 µL/min, injection volume 20 µL. *MPA*: 5 mM $NH_4HCOO^-_{(aq)}$ (pH 3), *MPB*: ACN (0.1% HCOOH, v/v)	[93]

Anabolic steroids and metabolites (32)	Urine (human)	200 μL aliquot of urine diluted with 200 μL IS_{mix} in MeOH. *Dilution factor 1:2*	Exactive Orbitrap® (Thermo Scientific) coupled to an Accela 1250 LC (Thermo Scientific) ESI^+ & ESI^- *Resolution*: 50 000 FWHM @ *m/z* 100 to 2000 Mass tolerance: ±5 ppm	Varian Omnispher™ C_{18} column (100 mm × 2 mm, 3 μm) + ChromSep guard column (10 mm × 2 mm, 5 μm) @ 35°C, flow rate 250 μL/min, injection volume 25 μL. *MPA*: H_2O and *MPB*: MeOH, both containing 1 mM $NH_4CH_3COO^-$ and 0.1% CH_3COOH v/v.	[94]
Sports drugs and metabolites (27)	Urine (human)	270 μL aliquot of urine diluted with 100 μL of 100 mM NH_4HCOO^-, 700 μL ACN and 30 μL IS_{mix}. *Dilution factor 1:11*	Q Exactive Hybrid Orbitrap® coupled to an Accela LC (Thermo Scientific) and a 1100 series LC (Agilent) ESI^+ & ESI^- *Resolution*: 17 500 FWHM @ *m/z* 100–500 Da Mass tolerance: ±5 ppm	Nucleodur HILIC column (100 mm × 2 mm, 1.8 μm) + Nucleodur HILIC trapping column (20 mm × 2 mm, 3 μm), flow rate 250 μL/min, injection volume 20 μL. *MPA*: H_2O, *MPB*: ACN and *MPC*: 200 mM $NH_4CH_3COO^-_{(aq)}$ (0.15% glacial CH_3COOH, v/v)	[95]
Sport drugs (46)	Urine and plasma (equine)	Plasma: 75 μL aliquot of deproteinated plasma diluted with 75 μL MPA. *Dilution factor 1:3* Urine: 20 μL aliquot spiked with 20 μL IS_{mix} and diluted with 960 μL MPA. *Dilution factor 1:50*	Q-Exactive™ Orbitrap (Thermo Scientific) coupled to an ACQUITY UPLC® (Waters) ESI^+ *Resolution:* 140 00 FWHM @ *m/z* 200 Mass tolerance: ±5 ppm	ACQUITY UPLC® BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) @ 55°C, flow rate 350 μL/min, injection volume 5 μL. *MPA*: 5 mM $NH_4HCOO^-{}_{(aq)}$ (pH 3), *MPB*: ACN (0.1% HCOOH, v/v).	[96]
Pesticide and veterinary drugs (>350)	Meat (beef, pork & chicken)	2.5 mL of H_2O added to 2.5 g of homogenised meat then diluted with 7.5 mL ACN (1% HCOOH, v/v). *Dilution factor 1:4*	Exactive™ Orbitrap (Thermo Scientific) coupled to a Transcend 600 UHPLC (Thermo Scientific) ESI^+ & ESI^- *Resolution*: 25 000 FWHM @ *m/z* 70 to 1000 Mass tolerance: ±5 ppm	Hypersil GOLD aQ C_{18} column (100 mm × 2.1 mm, 1.7 μm) @ 30°C, flow rate 300 μL/min, injection volume 10 μL. *MPA*: 4 mM $NH_4HCOO^-{}_{(aq)}$ (0.1% HCOOH, v/v), *MPB*: MeOH (0.1% HCOOH, v/v).	[80]
Pesticides, mycotoxins, process-induced toxicants & packaging Contaminants (32)	Tea (brew and leaves)	Tea brew: 2 mL diluted with 8 mL H_2O:ACN (3:1, v/v) acidified with 0.1% HCOOH (v/v) then 0.5 mL of the mixture filtered (0.2 μm). *Dilution factor 1:5* *Tea leaves underwent a "dilute, shoot & evaporate approach".*	Xevo® G2-S ToF (Waters) coupled to an ACQUITY H-Class UPLC® with quaternary solvent manager (Waters) ESI^+ & ESI^- *Resolution:* 30 000 FWHM @ *m/z* 200 Mass tolerance: not given	C_{18} PFP column (150 mm × 2.1 mm, 2 μm) @ 30°C, flow rate 400 μL/min, injection volume 10 μL. *MPA*: H_2O, *MPB*: ACN both containing 0.1% HCOOH (v/v) and *MPC*: MeOH	[97]
DoA and sport drugs (81)	Urine (human)	Aliquot of urine diluted with H_2O:ACN (95:5 v/v) and filtered. *Dilution factor 1:50*	Q-Exactive™ Orbitrap (Thermo Scientific) coupled to an EASY-nLC 1000 nano-LC system (Thermo Scientific) ESI^+ & ESI^- *Resolution*: 70 000 FWHM @ *m/z* 195	EASY-Spray PepMap® C_{18} column (150 mm × 75 μm, 3 μm) @ 25°C, flow rate 200 nL/min, injection volume 1 μL. *MPA*: H_2O, *MPB*: ACN both containing 0.1% HCOOH (v/v) *NB*: Nano-flow used as opposed to LC	[5]

ACN: Acetonitrile, *CH_3COOH*: Acetic acid, *DoA*: Drugs of Abuse, *DON*: Deoxynivalenol, *dMRM*: Dynamic Multiple Reaction Monitoring, *ESI:* Electrospray Ionisation, *ESI^-*: Electrospray Ionisation (negative mode), *ESI^+*: Electrospray Ionisation (positive mode), *FWHM*: Full Width at Half Maximum (resolution), *HCOOH*: Formic acid, HILIC: Hydrophilic Interaction Liquid Chromatography, *HPLC*: High Performance Liquid Chromatography, *IS_{mix}:* Internal Standard mixture, *LC*: Liquid Chromatography, *MeOH*: Methanol, *MPA*: Mobile Phase A, *MPB*: Mobile Phase B, *MPC*: Mobile Phase C, *MRM*: Multiple Reaction Monitoring, *MS*: Mass Spectrometry, *MS/MS*: Triple Quadrupole Mass Spectrometry (QqQ), *m/z:* Mass to Charge Ratio, *$NH_4CH_3COO^-$*: Ammonium acetate, *NH_4HCOO^-*: Ammonium formate, *ppm*: Parts Per Million, *QTRAP*®: Triple Quadrupole Linear Ion Trap, *QuEChERS*: Quick, Easy, Cheap, Effective, Rugged, and Safe, *sMRM*: Scheduled Multiple Reaction Monitoring, *SPE*: Solid Phase Extraction, *SRM*: Selected Reaction Monitoring, *UHPLC*: Ultra-High Performance Liquid Chromatography, *UPLC*®: Ultra-Performance Liquid Chromatography, *ZEN*: Zearalenone.
Dilution factor expressed as sample:total (volumes), i.e. 500 μL sample mixed with 500 μL diluent equates to a dilution factor of 1:2.

Fig. 1. Example of the best practice workflows across different matrices such as foodstuffs (solid and liquid) and biological for both quantitative and qualitative (screening) analysis using LC-MS (MS/MS or HRMS). [1]Dilute and shoot approach; [2]Dilute, evaporate and shoot approach; *Store in darkness overnight to allow solvent evaporation and analyte interaction with matrix; *Representative solvent; IS_{mix}: Internal standard mix.

workflow employed was highly fit for purpose and being classed as DnS is a good example of the ambiguity of the classic DnS definition. In this study, the authors looked at several food matrices with varying degrees of complexity, from wheat and maize to several classes of nuts. A 250 mg aliquot was extracted with 1 mL of extraction solvent and after mixing and centrifugation, a 300 µL aliquot of supernatant was diluted 1:1 (v/v) with dilution solvent giving a dilution factor of 1:2, after which a portion was injected for analysis. Compare this to the DnS protocol employed by Warth et al. (2012) whereby a 100 µL aliquot of sample matrix, in this case human urine, was simply diluted with 900 µL of dilution solvent after centrifugation, giving a dilution factor of 1:10 before analysis [10,11]. Although both methods employ sample dilution, the simplicity of the latter leans more toward the type of application that fits with the DnS concept, i.e. no other extraction or sample clean-up technique was used, with the key differences outlined in the workflows shown in Fig. 1. There are also publications that mention use of DnS as their clean-up approach, however on examination of the methodology employed, some have used an additional clean-up step to reduce matrix interferences further including QuEChERS or SPE and are therefore not DnS [12–14].

A potentially more rigorous definition of DnS is that it is the only technique used prior to analysis by LC-MS, thus the technique only really applies to liquid matrices such as urine, oral fluids and liquid foodstuffs such as wine which can simply be diluted before analysis [4,5,11,15–17]. However, as outlined above, the definition must be extended to include dilution of a sample extract such as that performed in the study by Sulyok et al. (2020). Furthermore, other biological matrices such as blood (whole blood, serum or plasma) must first undergo a step to facilitate removal of proteins due to the fact they can block the column, injector and/or components of the LC system. This protein precipitation step is typically performed by addition of an organic solvent such as acetonitrile, methanol, ethanol or acetone which precipitates the protein content as well as partitioning the analytes of interest from the matrix into the organic solvent layer. However, this protein precipitation step can be considered an additional clean-up process. After deproteinisation and centrifugation, an aliquot of supernatant is diluted for analysis, or no further dilution is performed due to the solvent volume added at the protein precipitation step being sufficient. Therefore, in this case we have to extend the definition of DnS to include this additional step. Examples of this methodology include the analysis of pesticides and carotenoids in human serum using HPLC-DAD and HPLC-MS/MS respectively [18,19].

However, use of DnS can be problematic for detection of blood metabolites found in low concentrations, due to the dilution caused by addition of solvent required for deproteinisation. This can be remedied by drying the supernatant under a gentle stream of nitrogen before reconstitution in an appropriate volume to facilitate detection of lower concentration metabolites. This technique has been referred to as a 'dilute, evaporate and shoot' approach [20]. One such example of this methodology was employed in the study by Devreese et al. (2012) in the analysis of pig plasma for mycotoxins and metabolites and which is routinely employed in this type of analysis [20–23]. One note of caution in this methodology is that the drying step can lead to some loss of analytes, particularly in complex matrices such as blood. Therefore, this methodology does not fall under the expounded DnS definition, and as such should be termed 'dilute, evaporate and shoot'.

2.3. Instrumentation: low resolution (LRMS/QqQ) versus high resolution mass spectrometry (HRMS)

The lack of selectivity associated with DnS is due to its generic sample clean-up and this can be offset by the selectivity afforded by triple quadrupole (QqQ) mass spectrometers that generally afford

higher sensitivity than HRMS systems. There are many MS instruments on the market compatible with HPLC/UHPLC and which have been comprehensively reviewed in relation to their suitability for the intended analytical method [24–26]. As shown in Table 1 whereby DnS was employed alongside LC-MS/MS, the majority of analysts used an AB SCIEX QTRAP [27–29]. With DnS diluting rather than removing matrix, as well as diluting the analytes of interest therein, these systems appear to reduce the influence of co-eluting matrix compounds and offer better sensitivity due to the enhanced selectivity afforded by the QTRAP technology and source design. This is not to state that these are the only QqQ instruments capable of being used alongside DnS, with other QqQ instruments also used successfully alongside DnS such as the Waters Xevo TQ-XS and Agilent 6460 and 6490 Triple Quadrupole LC-MS systems [15,17,30,31].

With improvements in QqQ instrumentation electronics facilitating faster cycle and dwell times, the number of analytes that can be included in a single run can be increased. One example of this is in the analysis conducted by Sulyok et al. (2020) in which more than 500 secondary microbial metabolites were analysed in a single run using a QTRAP 5500 (AB SCIEX), with chromatography performed on HPLC as opposed to UHPLC resulting in a run time of approximately 20 min. One caveat of this method and others with multiple analytes such as that of Steiner et al. (2020), is that with increasing analyte number and subsequent MRM transitions, cycle and dwell times have to be reduced in order to acquire enough data points per analyte for robust quantitation. Further to this, although polarity switching can be performed as rapidly as 5 msec according to some vendors, this along with the settling time would be detrimental to the number of data points acquired at any given time, and therefore, two injections may be required. Another multi-analyte method was that conducted by Kimble and DeCaprio (2019), analysing more than 800 psychoactive substances using an Agilent 6460 MS/MS with Jet Streaming Technology coupled to UHPLC. As with the aforementioned methods, due to the dwell and cycle times afforded by the instrument, the actual number of analytes in a single run was approximately 400, with samples analysed twice to cover all 800-plus compounds. The analysis time for each run was 20 min and therefore 40 min overall [30]. Thus, due to the enhanced selectivity and stability afforded by QqQ platforms, these instruments are generally used for quantitative analysis.

A new generation of hybrid HRMS systems has improved their capabilities, such as the quadrupole-ToF (QToF) and quadrupole-Orbital ion trap and which can now compete with QqQ instruments. These latest HRMS instruments have significantly better sensitivity specifications than previous HRMS instruments, achieved by advances in detection technology and increased resolving power, ultimately leading to a reduction in interferences with matrix compounds. The addition of the quadrupole (Q) to these instruments allows them to perform both qualitatively and quantitatively, with one such study using one of the new generation hybrid HRMS systems carried out by Martinez-Dominguez et al. (2016). In this multi-class, multi-analyte method, they analysed 257 pesticides and mycotoxins using a Q-Exactive Orbitrap both qualitatively and quantitatively. Although their methodology included an extra clean-up step after performing DnS, by way of florisil SPE cartridges, they reported similar results for DnS alone and only added SPE to maintain instrument performance [13].

One drawback in the use of QToF analysers is that polarity switching is not possible or practical and although this can be achieved with an Orbitrap, it is offset by a reduction in the instruments resolving power. However, with use of UHPLC as opposed to HPLC providing faster run times, this allows injection of each sample in positive and negative ion modes with the benefit that ionisation can be optimised for both, therefore increasing the number of detected compounds [32]. Overall, the number of methods employing DnS alongside LC-MS/MS compared to LC-HRMS over the last 10 years is higher as indicated in the number of examples shown in Table 1. One thing to note is that not all articles using the DnS approach mention the methodology in the title, abstract or keywords. Therefore, the number of publications employing the technique are higher than indicated when a literature search is performed with the keywords 'DnS' combined with either 'tandem MS', 'high resolution MS' or 'Orbitrap'. Use of QqQ over HRMS is in part due to the enhanced selectivity afforded by QqQ instruments as well as their sensitivity which compensate for the generic sample clean-up technique. With the matrix being reduced rather than removed, the selectivity of QqQ instruments allows analytes to be identified based on the differentiation between analyte and unwanted matrix, although this is also due to the sensitivity as selectivity alone does not afford adequate detection limits. However, with many of the technical limitations around HRMS instruments having been resolved with advancements in technology, as well as the introduction of the hybrid quadrupole HRMS systems, there may well be a rise in the number of analysts using HRMS for routine laboratory tests.

3. Matrix effects

In order to extract analytes from a liquid food matrix or biological matrices such as urine or blood, liquid-liquid extraction (LLE) can be used. Alternatively, solid-liquid extraction is used on solid matrices such as feed and food. In either case, the process using a single or multiple component liquid extraction mixture (organic solvents, water, acids, bases, etc.) serves to migrate the analytes of interest from the matrix into the added liquid phase. The addition of water to the organic (extraction) solvent can help the organic solvent penetrate the food matrix in the case of solid foodstuffs, while also facilitating extraction of some of the more polar analytes. The addition of acid serves to help break bonds between the analytes and some of the matrix components such as proteins and sugars, aiding their extraction [33]. When an extraction technique is chosen, there will always be co-extraction of matrix components giving rise to matrix effects, with the amount dependent on the extraction solvent and complexity of the sample. Some of the most challenging issues in analytical chemistry are matrix effects caused by co-extracted matrix compounds. These are unpredictable and can cause various issues such as: ion suppression (or in some cases enhancement), poor chromatography, false positives due to the presence of isobaric compounds, poor analyte recovery due to chemisorption and interferences with the ion ratio of the product ions in targeted analysis [34,35]. Use of internal standards is one of the most commonly used ways to compensate for matrix effects, however, as various analytes can exhibit different behaviours in the same matrix, ideally an internal standard is required for each analyte. This is not feasible due to the fact they are expensive and also that commercial availability is limited. Standard addition is another way to compensate for matrix effects and is often used in routine analysis. However, it is more suited to single-analyte methods rather than multiple-analyte determination due to it being laborious and costly because of its high consumption of standards [1]. Some of these issues can be overcome by improved chromatography, however, this has limitations as there has to be a benefit of improved chromatography against the need for fast run times, especially in high-throughput laboratories.

Even with the advancement in detection capabilities afforded by LC-MS, there may still be a need for sample clean-up to remove unwanted matrix compounds using more traditional techniques such as SPE, IACs, LLE or QuEChERS when method sensitivity is paramount [26]. Use of these techniques is really only applicable for

targeted methods where the efficacy of the clean-up technique can be assessed. This has limitations in regards to the number of analytes that can be incorporated and can get difficult as the number of analytes increase due to several factors, which include but are not limited to: Differing physiochemical properties, selectivity/specificity of the clean-up technique, instability of some analytes in the working standard solution, ionisation efficiency, availability of reference material and suitability of the clean-up platform chosen. An example of some of the issues encountered when employing DnS for a multi-class, multi-analyte method was highlighted in the study conducted by Malachova et al. (2014), with validation data acquired for 295 of the 331 target analytes due to some of the aforementioned issues. One of the main issues was that of signal suppression/enhancement (SSE) due to matrix effects and which was observed for all four matrices studied. Green pepper was considered the most complex matrix of the four, with only 10% of analytes not suffering from SSE [28]. In this example, samples were run in both positive (ESI^+) and negative ionisation modes (ESI^-). A recent study by Steiner et al. (2020) investigated matrix effects of 80 fungal metabolites, 11 pesticides, and 9 pharmaceutical active agents in complex feed using DnS and LC-MS/MS. Although the majority suffered signal suppression, with polar analytes in ESI^+ particularly susceptible to this, apolar analytes in ESI^- were prone to signal enhancement, both caused by co-extracted matrix compounds [8].

Other matrices such as black tea (highly complex and dry) and rocket (high water and chlorophyll content) have shown to be somewhat problematic in pesticide analysis (39) using dilution of a sample extract [36]. Black tea and rocket indicated that only 4% and 7% of the analytes respectively were free of matrix effects at a 10-fold dilution, whereas the majority were completely free of matrix effects at a 100-fold dilution, indicating that a “dilute-and-shoot” approach may be successful. However, depending on the concentration of analyte(s) present, this dilution factor may prove too much for analyte detection. Therefore, a compromise could be sought, with a 25-fold dilution proving suffice if matrix effects were no greater than 20%. Another pesticide study in fruit and vegetables indicated that with no dilution, the matrices varied in the percent of analytes showing no suppression, defined as being <20%. Leek proved the most problematic, with only 9% of pesticides not supressed and 37% of analytes highly supressed (>50%). Overall, a dilution factor of 15-fold was enough to eliminate matrix effects, permitting quantification with solvent standards for the majority [37]. Therefore, matrix effects and analytical sensitivity will be a major factor in whether DnS can be employed, as well as the number and classes of analytes to be incorporated in the method. In some instances, the complexity of the sample matrix can lead to severe matrix effects and depending on the sensitivity required, DnS may not be suitable and other sample clean-up strategies may need to be employed, such as those listed in section 4.

Taking into account complex matrices such as black tea, rocket, leek and green pepper, as well as other matrices studied such as urine and foodstuffs, all of the aforementioned can lead to signal suppression of the analytes therein, caused by co-elution of unwanted matrix components and the resulting contamination of the MS source and ion optics. Thus, although use of DnS can give excellent results with little to no sample preparation, MS detectors may well require regular venting and cleaning of the source components after multiple injections to maintain instrument sensitivity leading to unwanted instrument downtime. Therefore, when using DnS, the downtime is dependent on the MS system used and source design, with ABSCIEX QqQ instruments appearing to be the instrument of choice for targeted analysis using DnS as indicated in Table 1. Of late, there has been improvements in source design with various vendors implementing different designs or technologies to improve sensitivity while also reducing the necessity for regular maintenance. Examples of this include differential mobility spectrometry (DMS) with SCIEX introducing the SelexION DMS technology and the Agilent Jet Stream Ion Source with the addition of VacShield. These technologies serve to enhance the systems robustness with the bonus of spending less time on maintenance and increasing sample throughput.

4. Sample clean-up techniques to reduce matrix effects

4.1. Solid phase extraction (SPE)

SPE is a useful tool for reducing matrix effects across various complex matrices such as foodstuffs, animal feed, aqueous samples and other biological matrices, and usually affords low LOQs. However, a drawback in its suitability for sample clean-up is the number of analytes incorporated is usually limited due to the selectivity of the stationary phase, with several factors to consider when choosing this such as: varying physiochemical properties of the analytes and the loading, washing and elution steps which can lead to losses or incomplete recovery of analytes. All of the aforementioned can lead to time consuming and laborious method development. Some examples in the use of SPE include the analysis of mycotoxins in pig serum, plasma and urine and in the analysis of freshwater toxins in aquaculture ponds and fish tissues [38–41], the analysis of mycotoxins in baby food, analysis of nerve agents in human urine and the analysis of veterinary drug and pesticide residues in animal feed [12,42,43]. Furthermore, two or more SPE columns can be used in tandem to analyse several compound classes, or an analyte (ZEN) and its metabolites across various matrices [44].

4.2. Micro-extraction techniques: sorbent-based

Similar to SPE, micro-extraction techniques based on solid phases or sorbents are now commonplace for use in sample preparation and clean-up. These techniques include solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) and micro-extraction by packed sorbent (MEPS) [45].

SPME is based on partitioning of analytes between a coated fibre and sample, achieved by placing the SPME fibre above a solution (matrix) to capture the volatile vapour. However, SPME is more routinely employed alongside GC-MS analysing volatile organic compounds (VOCs) or volatile metabolites (VOMs). Use of direct immersion SPME (DI-SPME) has facilitated its compatibility with LC-MS in the analysis of non-volatiles [46]. One main drawback is its use of stationary phases coating the fibre [47], and along with DI-SPME showing poor recoveries in the analysis of polar molecules, the capacity of this technique in the creation of multi-class, multi-analyte methods for analysis by LC-MS is limited. Similar to SPME, SBSE is suited to analysis of medium to apolar molecules across various matrices using GC-MS. When combined with a sorptive phase, multi-residue methods have been developed for the analysis of endocrine disruptors (77) and pesticides (15) in water samples using GC-MS and LC-MS respectively [48]. Analysis of polar molecules by SBSE requires derivitisation which is laborious, although new materials could be developed in order to directly analyse polar molecules by LC-MS, making them comparable to SPE cartridges [48]. Once again however, the limitations for SBSE are similar as those mentioned for both SPME and SPE.

MEPS has been developed as a miniaturised version of SPE and is fully compatible with LC-MS, with the added benefits of being fully automated, rapid and reduced solvent usage in comparison to SPE. Due to its size, MEPS are perfectly suited to small volumes of biological fluids such as plasma/serum, oral fluids and urine [45]. With

their ability to be utilised for small sample volumes and low concentration of analytes therein, they concentrate the analytes of interest whilst simultaneously removing unwanted matrix compounds, giving rise to more sensitive analytical methods. However, as with the other aforementioned techniques, the selectivity of the sorbent limits the number of analytes incorporated.

4.3. Immunoaffinity Columns (IACs)

IACs work on the basis that the stationary phase contains an antibody to capture the analyte(s) of interest and can facilitate analysis at low levels, generally affording low LOQs. Although IACs are very selective, this has drawbacks such as lack of specificity for analytes of different classes or analogues, and they can exhibit some non-specific binding with other proteins. IACs are mainly used in the analysis of one or two compounds due to the cross reactivity profile of the antibody, such as in the analysis of chloramphenicol in food or vitamin B12 in green algae [49,50]. IACs can be utilised in the analysis of several compounds such as in the study of microcystins (15) and the structurally related toxin nodularin, with a comparison between IAC and SPE indicating a lower method detection limit using IAC [51]. They have also been developed with the capacity to bind several mycotoxins with varying physicochemical properties across a range of biological matrices, such as the 11+ Myco MS-PREP IAC (R-Biopharm: https://food.r-biopharm.com/products/11myco-ms-prep/). IACs can facilitate quantification with solvent calibration curves, although this is not always the case as some feed matrices have been shown to cause SSE even with use of IACs [52]. In order to analyse a wider range of analytes, IACs with different specificities can be used in tandem [53], and they can also be used in conjunction with SPE, with the main driver in doing so to increase analyte number [54,55]. However, the selectivity of these techniques do not lend themselves to creation of multi-analyte methods.

4.4. QuEChERs

QuEChERs was introduced in 2003 and has rapidly expanded from use in pesticide analysis to applications in food, pharmaceutical, environmental and biological analysis. Due to its success, two international standard organisations adopted versions of the original technique for quantitation of pesticides in both fruit and vegetables; the European Committee for Standardisation (CEN) and the Association of Official Analytical Chemists (AOAC) International [56]. The method combines liquid extraction and partitioning using salts, followed by a clean-up step using dispersive SPE (dSPE). As well its simplicity, it is relatively cheap in comparison to other techniques as well as being rapid.

QuEChERs has been used extensively in pesticide residue analysis in fruit and vegetables, with 451 pesticide residues analysed along with a Q-Exactive Orbitrap, permitting both identification and quantitation [7]. Work has been conducted to evolve the method to areas other than pesticide analysis in agricultural commodities, with these discussed in two comprehensive reviews looking at its evolution, applications and trends [56,57]. Suffice to say, it is now used in the analysis of mycotoxins, antibiotics, veterinary drugs and herbicides across a variety of matrices. One such example is the analysis of 630 multi-class food contaminants using UHPLC-QToF including, veterinary drugs, food-packaging contaminants and mycotoxins, although the majority were pesticides (426) [58]. Although a very effective and evolving technique, it like other clean-up techniques has disadvantages due to incomplete or low recoveries of the suite of analytes due to the choice of dSPE chosen, as well as being mainly pesticide-centric.

4.5. Liquid-liquid extraction (LLE)

Used as an extraction technique for some liquid matrices, LLE is considered to be a sample clean-up step. Apart from facilitating protein precipitation in some matrices, the partitioning separates compounds based on their solubility in two different immiscible liquids, such as water and an organic solvent, with analytes typically extracted in the organic layer leaving matrix components in the other, with some matrix co-extracted in the organic layer and *vice versa*. As mentioned previously, the choice of extraction solvent dictates the polarity of the analytes extracted and therefore presented for analysis. This is particularly important for untargeted qualitative analysis or metabolomic studies using LC-HRMS. In general, most of these extractions are conducted on biological matrices with examples given below.

4.5.1. LLE applied to blood samples (plasma or serum)

This involves addition of an organic solvent for both deproteinisation and migration of analytes from matrix to the solvent, which may be acidified. This technique has limited advantages due to only partial removal of matrix components which are responsible for signal suppression [21]. Furthermore, in most instances instead of dilution, the sample is dried and reconstituted to concentrate the analytes of interest, with the caveat that it also concentrates any matrix present too. This technique, referred to as 'dilute, evaporate and shoot' has benefits similar to DnS where the same extraction procedure can be employed for targeted and untargeted analysis due to minimal or no analyte losses. Examples include the studies carried out by Catteuw et al. (2019), Devreese et al. (2012) and Lauwers et al. (2019) on pig plasma [21,22,59] and others conducted in human plasma [60,61].

4.5.2. LLE applied to urine samples

Unlike blood, urine can simply be diluted for analysis as it does not usually require deproteinisation. In some instances, the addition of an organic solvent is needed to facilitate migration of the analytes from the polar, aqueous urine phase into the organic phase, whilst removing some matrix, before drying and reconstituting the organic layer. Examples of this include the analysis of mycotoxins where the urine was extracted with acetonitrile/water/formic acid (52/45/3, v/v/v) or ethyl acetate in human and pig urine, respectively [23,62]. In both cases, use of both targeted and untargeted methods can be employed, however, some caution has to be observed for the latter extraction if analysing by HRMS for metabolites or potential biomarkers as some analytes had low recoveries in ethyl acetate at varying pH levels.

4.6. Dilute-and-shoot

In comparison to the aforementioned techniques bar LLE, DnS is a generic analytical method with its advantages and disadvantages outlined in Table 2. The evolution of DnS has facilitated the creation of multi-analyte methods which were previously limited due to the differing physiochemical properties of the analytes in question and the sample clean-up technique chosen. One can note that with the evolution of DnS to include foodstuffs, the initial solid-liquid extraction can lead to SSE due to co-extraction of matrix, as well as low recoveries of some of the target analytes based on the solvent chosen and possible chemisorption. However, due to the absence of stationary phase and chemistry therein, the technique is considered not selective and permits the creation of multi-class, multi-analyte methods. Although in comparison techniques such as SPE, microextraction (SPME, SBSE and MEPS) and IAC can generally achieve lower detection limits, they are hampered by their respective selectivities and specificities, limiting the number

Table 2
Comparison of the various sample clean-up techniques employed in LC-MS, highlighting the main advantages and disadvantages of each and indicating their suitability for multi-class, multi-analyte methods.

TECHNIQUE	Advantages	Disadvantages
SPE	• Matrix removal: Cleaner extracts • Analyte concentration: Enhanced sensitivity • Used across most matrices • Semi-automation possible • Reproducibility • Selectivity and specificity • High recovery of target analytes • Multi-well formats available	• Moderately expensive in cost/sample • High solvent usage • Labour intensive • Skilled operator required • Method development required • Moderately complex • Loss of analytes • Time consuming • Selectivity and specificity
IAC	• Matrix removal: Cleaner extracts • Analyte concentration Enhanced sensitivity • Specificity • Reproducibility • High recoveries of target analytes • Used across most matrices	• Expensive in cost/sample • High solvent usage • Labour intensive • Skilled operator required • Method development required • Losses of analytes • Time consuming • Specificity
QuEChERS	• Matrix removal: Cleaner extracts • Selectivity • High to moderate recovery of target analytes • Relatively simple • Inexpensive in cost/sample • Reproducibility • Reduced solvent consumption • Quick	• Some analyte losses • Slightly laborious • Method development required • Unspecific •
LLE	• Removes proteins (solvent dependent) • Minimal method development • Inexpensive • No equipment required • High throughput	• Some analyte losses • Knowledge of chemistry needed • Solvent consumption • Emulsions possibly formed • Losses due to phase separation • Unsuitable for polar analytes
Dilute and Shoot	• Simple • Minimal or no method development • Inexpensive • No equipment required • Minimal solvent usage • High-throughput • Broad specificity • Minimal to no analyte losses • Suitable for multi-analyte, multi-class methods • Rapid	• Dilution of matrix: Higher detection limits • Dilution of analytes

Increasing selectivity, specificity, complexity, solvent usage and cost (↑, from Dilute and Shoot to SPE)

Increasing suitability for multi-class, multi-analyte analysis (↓, from SPE to Dilute and Shoot)

of analytes incorporated. QuEChERs has led to the development of multi-analyte methods, especially in pesticide analysis, but due to the selectivity of the dSPE there is a limit to the number of different classes incorporated in comparison to DnS. Furthermore, a recent study by Steiner et al. (2020) analysed > 1200 biotoxins, pesticides and veterinary drugs in complex feed using LC-MS/MS and compared QuEChERS and DnS on their final extract. They concluded that the QuEChERS approach showed limited improvement in relation to matrix effects, whereas a straightforward 1:1 dilution (DnS) of the sample extract gave the best results in regards to reducing matrix effects and resulting method sensitivity [63]. This indicates that although DnS dilutes rather than removes matrix components in comparison to techniques such as SPE and QuEChERS, it may not be inferior to these techniques. In the following section, we will look at the application of DnS across various matrices and scientific disciplines, detailing its evolution to allow an increase in the number and class of analytes incorporated in a single method.

5. Dilute-and-shoot: applications

One of the main challenges in analytical chemistry is to strike a compromise between matrix effects and method sensitivity. This is where DnS can be extremely powerful, because it reduces matrix effects and analyte exclusion, providing a platform for the creation of multi-class, multi-analyte methods. Dilution of a sample or extract can help abate matrix effects, depending on the dilution factor, and higher dilution factors usually lead to an overall reduction in method sensitivity. However, with little or no sample workup there is massive potential for increased sample throughput as well as lower solvent and consumable consumption.

Due to the lack of analyte selectivity afforded by DnS, use of LC-MS/MS is usually preferred over LC-HRMS due to the increased selectivity of the former. This is shown in Table 1 with examples of DnS coupled to LC-MS/MS given across many different analyte classes, disciplines and matrices. In comparison, use of DnS coupled to LC-HRMS has fewer examples as indicated in Table 1, with its use mainly limited to the analysis of prescription drugs, DoA and sports drugs in urine. Although the application of DnS coupled to QqQ instruments is favourable, the MS platform is usually the limiting factor in terms of the number of compounds that can be incorporated in one run. In this instance however, use of HRMS such as TOF, Orbitrap or their quadrupole-hybrids can be considered a powerful alternative [12]. It has been stated that use of DnS is more commonly employed for low-complexity matrices and high-abundance analytes, such as honey and wine [64], however, below are examples of where DnS has been applied to complex matrices.

5.1. Urine, other biological fluids and liquid foodstuffs

With an increase in the number of drugs used to enhance sporting performance and in use of both illicit and prescription drugs, there is potential for laboratories to create multi-class, multi-

analyte methods for routine analysis. For example, a decade ago different screening procedures were utilised for analysis of different drug classes including diuretics, beta-2-agonists, stimulants and narcotics, whereas use of DnS coupled to sensitive MS platforms has facilitated the development of a multi-method that encompasses them in one analytical run [65]. Furthermore, due to most xenobiotics undergoing phase II metabolism including glucuronidation and sulfonation, the presence of these metabolites can act as indicators these compounds have been ingested, allowing an increase in the detection window, especially beneficial for drugs known to be metabolised quickly. DnS couples matrix removal with minimal analyte losses to significantly improve urine analyses, providing accurate and timely results.

An example of the efficacy in multi-class drug analysis is the study by Guddat et al. (2011) looking at several drug classes, covering more than 60 compounds in total with the fully validated method permitting detection at levels lower than the required limits. The group were also able to detect glucuronidated metabolites of a plasticiser commonly found in blood transfusion bags. This enabled screening for these metabolites with their presence at abnormally high levels indicating illegal use of blood transfusions to enhance sporting performance, while avoiding the time-consuming hydrolysis [65]. Another study by Dahlin et al. (2019) analysed drugs used in clinical pain management including 37 clinically relevant prescription drugs, DoA and glucuronide metabolites. The method employed a 1:10 dilution and was successfully validated to the Food and Drug Administration (FDA) bioanalytical guidelines for 23 of the analytes, with the other 14 validated qualitatively [16]. Generally, clinical testing is routinely performed by immunoassays (IAs) which are mainly considered as screening or qualitative only. Although IAs give fast turnaround times and are inexpensive when compared to LC-MS, they are less sensitive, can give false negatives and most are not specific for a particular drug but rather for broader drug classes. The simple methodology embraces reduced testing costs and enhanced patient care by allowing quick turnaround times, while also providing clinicians with specific detection and quantification of multiple clinically relevant analytes.

A study by Kong et al. (2017) used a DnS approach alongside LC-MS/MS in the analysis of 113 DoA and their metabolites in human urine. Their method required only 400 μL of sample and showed minimal matrix effects, corrected for by use of isotopically-labelled internal standards. However, this practice is not economically viable for high numbers of analytes due to the prohibitive cost. Their simple methodology was successfully applied in the identification and quantification of various DoA in 17 forensic urine samples [66]. Another example is the analysis of the sports drug higenamine and its metabolite by Gruzca et al. (2018). Apart from improving the original methodology, another benefit was the appearance of a second peak not present using the original methodology. This peak was characterised as a sulfo-conjugate of higenamine, an unknown metabolite which was indicated to last longer than the native drug itself, aiding its detection [67]. Further examples in the use of DnS in detection of these class of compounds are the analysis of more than 80 DoA and sports drugs and the analysis of more than 800 novel psychoactive substances (NPS), both in human urine [5,30]. In the latter example by Alcántara-Durán et al. (2018), they used a dilution factor of 1:50, five times more than the usual dilution factor employed. This increase in sensitivity was afforded by use of both nanoflow LC coupled to a Q-Exactive Orbitrap HRMS, with nanoflow LC providing improved ionisation efficiency. In the study by Kimble and DeCaprio (2019), they improved on previous methodologies for detection of NPS in biological matrices, with only a few methods capable of detecting more than 100 in a single run and the majority detecting fewer than 50, with their methodology increasing the number to over 800 analytes.

Another field where the use of DnS with urine is utilised regularly is the analysis of mycotoxins and their metabolites such as glucuronides, sulphates, glutathione's, and other routes of bodily detoxification. This has allowed scientists to explore toxicokinetics and metabolism through biomarker studies, facilitating research into understanding human and animal exposure to mycotoxins and to investigate mitigation strategies. Biomarker-driven research has been proposed as a successful method to assess exposure to xenobiotics such as mycotoxins through analysis of both parent compounds and metabolites in biological matrices such as urine. Recent advances in MS detectors in both QqQ and HRMS platforms have facilitated increased identification and quantification which will help track chronic exposure to mycotoxins, with some listed as carcinogenic and which are linked to some cancers [68,69]. Examples of these mycotoxin-biomarker studies include that conducted by Gerding et al. (2015) investigating mycotoxin excretion patterns across Bangladesh, Germany, and Haiti. They analysed 23 mycotoxins and metabolites in human urine using a 1:10 dilution and LC-MS/MS [70]. This approach has also been used in several studies conducted by Warth et al. (2012, 2013 and 2014) investigating human exposure to mycotoxins through a multi-biomarker approach, utilising the urinary excretion profile to ascertain exposure. In all the aforementioned studies, urine was diluted 1:10 and analysis performed using LC-MS/MS, with some studies looking at several different mycotoxin classes [4,10,11,71]. There have also been studies carried out in swine using the DnS approach as in those conducted by Binder et al. (2017), whereby urine and faeces were analysed after administration of ZEN and some of its 'masked' plant and fungal metabolites. Using a multi-biomarker approach with a 1:10 dilution and LC-MS/MS analysis, they identified what they considered ideal biomarkers to indicate chronic exposure of animals to naturally-contaminated feed with ZEN [72]. A study by Cao et al. (2018) developed a multi-mycotoxin LC-MS/MS method for the simultaneous determination and quantitation of carcinogenic mycotoxins in human biological fluids. In this instance, urine was diluted approximately 1:4 (DnS) whereas plasma was analysed using a 'dilute, evaporate and shoot' approach [20]. As discussed in the paper, the previous LOD using this approach was considered too high for most analytes (» 10 ng/mL), possibly due to the sensitivity of the instrument employed. However, in this study the use of an API 4500 MS (AB SCIEX) afforded LODs and LOQs of 0.03–0.41 ng/mL and 0.11–1.0 ng/mL respectively in urine. This sensitive multi-mycotoxin method could therefore be employed in monitoring chronic exposure of mycotoxins in humans and in particular those considered carcinogenic.

Another matrix that can simply be diluted for analysis is oral fluid, especially applicable in the field of forensics, with one study by Malaca et al. (2019) looking at psychoactive drugs such as morphine, codeine, cocaine and Δ-9-tetrahydrocannabinol, with 13 analytes in total. The method required only 0.5 mL of sample and a dilution factor of 1:3, with chromatographic separation taking only 6 min. The sample clean-up required 4 min, making the total analysis time per sample 10 min [15]. Another study using OF was conducted by Pichini et al. (2019) studying cannabinoids, their acid precursors and some metabolites. The methodology required only 100 μL of sample with the validation parameters used indicating its suitability for use in clinical trials in cannabis preparations [73]. Milk, and in particular bovine milk is another matrix that can simply be diluted, although it requires an initial protein precipitation step to prevent blocking the injector and/or chromatographic components. Draher et al. (2016) investigated nitrogenous protein adulterants with a dilution factor of 1:10. They compared DnS and SPE and although found greater precision and accuracy with SPE,

the DnS approach was deemed suitable for semi-quantitative determination of these adulterants in routine lab analysis [74]. Furthermore, the US FDA developed a DnS methodology based on that described by Mol et al. (2008) for the analysis of 30 veterinary drugs in raw milk using HRMS in both ESI^+ and $ESI^−$, with the method then expanded to 76 compounds to include mycotoxins and pesticides [75].

5.2. Animal feed and foodstuffs

Animal feed is an area that DnS has been heavily employed over the last decade, in particular for mycotoxins with the number of analytes in a single analysis increasing over time. This has in part been due to the increased performance of MS platforms, with improvements in the systems electronics facilitating reductions in the dwell, cycle and polarity switching times, allowing the inclusion of more analytes. The application of DnS in this field first appeared in 2006 when Sulyok et al. (2006) used this methodology in the analysis of 39 mycotoxins using LC-MS/MS and a dilution of 1:1 (v:v), although the paper did not refer to the technique as DnS. The move to this generic sample clean-up technique was driven by a desire to simultaneously analyse as many different classes of mycotoxins as possible in one run. However, with different classes having differing physiochemical properties, the drawback was that existing clean-up technologies such as SPE would result in losses or incomplete recovery of some analytes. In order to circumvent this but still reduce matrix effects, the group decided to simply dilute the sample extract. This proved successful with negligible ion suppression shown with wheat, however, significant ion suppression was seen for approximately one third of analytes in maize. Due to polarity switching not being fast enough at the time for the number of analytes included, each sample was run twice, once in positive ionisation mode (ESI^+) and once in negative ionisation mode ($ESI^−$) [27]. In 2008, a study by Mols et al. extended it to 172 analytes, including pesticides, plant toxins, veterinary drugs and mycotoxins. The technique used was slightly different to the “mycotoxin multi-analyte method” employed by Sulyok et al. (2006). Instead of diluting the final extract 1:1 (v/v), they performed an initial extraction with water (5 mL) followed by dilution by addition of 15 mL of acetonitrile giving an overall 1:4 dilution factor [76].

In 2014, a study on the validation of a quantitative LC-MS/MS method covering 295 bacterial and fungal metabolites including all regulated mycotoxins was published by Malachová et al. (2014) [28]. This study looked at four ‘model’ food matrices and how use of DnS could be employed across each without the need for validating individual food matrices. With 295 analytes validated, this was a major step in the number incorporated in a single analytical run in comparison to previous multi-methods published in the area [77,78]. Of the four matrices investigated, the most complex (green pepper) proved problematic with only 10% of analytes not suffering from SSE and only 21% of the 295 analytes showed acceptable recoveries in the range of 70–120% as laid down by SANCO, leading to the highest LOQs for the analytes involved. This highlighted some of the issues with DnS when used with complex matrices with high numbers of analytes suffering from SSE and poor recoveries of some. A study by Sulyok et al. (2020) extended this approach further by validating a DnS LC-MS/MS method for more than 500 mycotoxins and metabolites in seven different food matrices. Employing DnS facilitated the analysis of multiple analytes in a single method due to no other sample clean-up technique being suitable for as many different classes of analytes with varying physicochemical properties. Other examples where use of DnS in foodstuffs has been successfully employed is in the analysis of rice for both mycotoxins (10) and pesticides (6), or in the analysis of mycotoxins (42) in cocoa beans [29,79]. One note on the latter example is that although they used DnS, they also used a modified form of QuEChERs to reduce co-extractives through addition of sodium chloride. Although this method is not strictly DnS, it may prove useful as the extra step was simply salt addition, adding little to the cost or technicality with the advantage of reduced matrix effects and increased method sensitivity.

In 2014 the group of Gómez-Pérez et al. developed an LC-HRMS multi-method for the simultaneous determination of veterinary drug residues and pesticides in meat matrices using a DnS approach, with more than 290 out of the target 350 analytes meeting the desired recovery and precision values. Due to the fatty nature of the matrix, further clean-up steps were employed, including dSPE and SPE. However, the number of compounds with an adequate recovery was less than when using DnS only and therefore use of these was dropped [80]. In 2020, a study conducted by Monteiro et al. developed a “mega-method” analysing veterinary drug residues, pesticides and environmental contaminants in beef by LC-MS/MS in tandem with GC-MS/MS, covering a total of 262 analytes. Regarding the LC-MS/MS sample analysis, the group claimed a QuEChERS-based approach, however with no salts or dSPE added in the extraction procedure, their methodology equated to the expounded definition of DnS [81].

5.3. Other analytical areas and “omics” studies

Over the last 5 years, the use of DnS has been employed in the detection of a wide range of analyses across many different scientific disciplines. These include the analysis of drug seizures permitting detection of the drug and the cutting or bulking agents added. The previous methodology did not allow for the detection of cutting agents or adulterants due to their physiochemical properties, however, switching to DnS alongside LC-HRMS allowed detection of both [82]. It also has been used in the analysis of honeybee venom (HBV), measurement of urinary neurotransmitters and their metabolites to probe the pathology of several diseases, identification of illegal insulin which use has led to lethal incidents in the past, and in the detection of vesicant chemical warfare agent metabolites in human urine as a monitoring or screening tool [31,83–85].

Furthermore, there is the potential for the use of DnS for non-targeted analysis or “omics” studies, such as metabolomics, either using a targeted or untargeted approach. This is very much dependant on the sample clean-up step taken prior to analysis, with use of solid phase microextraction and/or extract and concentrate both utilised. The use of solid phase microextraction in sample preparation would lead to a ‘targeted’ metabolomics approach due to the selectivity of the stationary phase, whereas the latter technique, defined as “dilute, evaporate and shoot” being more suitable for a fully untargeted metabolomics approach. Depending on the matrix analysed, there may be the requirement for an initial solid-liquid extraction, or deproteinisation step. In either case, after addition of the solvent of choice and centrifugation, in most cases the resulting supernatant is evaporated to dryness and reconstituted, usually in a smaller volume than the original sample so as to concentrate the sample. This is preferred so as not to dilute the global profile of analytes/metabolites present, with the concentrations of analytes expected not at high enough levels for an untargeted approach. This is also in part due to the lack of selectivity afforded by HRMS instruments and the fact that a complete profile is sought rather than a targeted class of analytes. This approach can be defined as “dilute, evaporate and shoot”, with the methodology employed in most cases for biological matrices such as blood (serum/plasma) and urine. This “dilute, evaporate and shoot” approach has been used in biomarker discovery in

pancreatic cancer, lung cancer metabolomic studies and in building spectral library's from urine samples [86–88], whereas a straightforward DnS approach has been employed in the analysis of fresh orange juice to study chemical contaminants [89]. The "dilute, shoot and evaporate" approach is in essence very similar to DnS and has been a powerful tool for metabolomics approaches across various foodstuffs in determining contaminants, as well as in food safety and quality analyses, with examples covered in reviews by Li et al. (2020) and Lopez-Ruiz et al. (2019) [90,91].

6. Conclusions and future trends

DnS is a less expensive and more environmentally-friendly sample clean-up technique that facilitates high sample throughput. The evolution and application of DnS in analytical chemistry has allowed it to be used across various scientific research applications. An optimal practice workflow for the "dilute-and-shoot" approach is indicated in Fig. 1 for the main matrices covered in this review, and has been extended to include the "dilute, evaporate and shoot" approach for the analysis of blood. This is a proposed best practice workflow based on the literature surveyed and is very much dependent on the sensitivity requirements of the methods and MS platforms available. With newer hybrid HRMS systems, there is the ability to carry out both qualitative (screening) and quantitative analysis in the same run. Furthermore, samples could be qualitatively analysed by LC-HRMS and any potential 'hits' quantified on LC-MS/MS, allowing users to identify specific contaminants/adulterants and therefore decide which targeted methodology should be employed. It also allows retrospective data analysis for emerging threats with the knowledge that a global profile of analytes should be present due to minimal analyte losses due to use of DnS as the sample clean-up.

Dilution of the final extract reduces matrix effects and subsequently increases LOQs, but this can vary depending on the analyte/matrix combination. There does not appear to be a linear relationship between the dilution factor applied and to what extent the LOQ will increase. With that in mind and with a wide range of dilution factors employed, usually between 2 and 50, it is difficult to predict what dilution factor should be used for other analytes in varying matrices and therefore if DnS can in fact be used [36]. However, from the literature surveyed, 1:2 and 1:10 appear to be the dilution factor of choice more often than not for feed and urine studies respectively. The trade-off in not achieving LOQs as low as when using more selective sample clean-up techniques is the benefit in building multi-methods which can include many analytes over many different classes, with the number of analytes increasing year on year. A recent example of the increasing number is the analysis of >1200 biotoxins, including fungal metabolites, pesticides, veterinary drugs, plant toxins and bacterial metabolites by Steiner et al. (2020). They analysed a sample extract using a 1:1 dilution, which gave the best results in regards to matrix effects reduction and resulting method sensitivity. This example highlights how employing the expounded definition of DnS can be used in the future to create multi methods that cover a wide variety of analyte classes. A further example of the future application of DnS is in creation of methods for mixed organic chemical residues and contaminants to encompass as many different residues, metabolites of and contaminants [75]. These multi-methods can potentially be extended to include natural toxins such as mycotoxins, phytotoxins such as pyrrolizidine alkaloids, freshwater toxins, contaminants including pesticides, veterinary drug residues and toxins produced during food processing and packaging. One of the challenges of increasing analyte number is that method performance drops in terms of the number of data points across each peak and therefore the subsequent loss in reliable, robust data for accurate quantification and validation. This is due to the increased number of MRM transitions required within a particular retention window, with cycle and dwell times, as well as polarity switching having to be taken into consideration in order to acquire enough data points per analyte. These factors eventually limit the number of analytes assayed in one run when using QqQ instruments for accurate quantification. This can be improved by use of HPLC columns with 5 μm particle size as opposed to UHPLC columns with sub 2-μm particle size, which although give broader peaks, allow more data points per peak, yet which also increases analysis run time, as well as running the samples twice in both ESI^+ and ESI^- mode [3,63].

Multi methods could also be adopted in place of several single-class methods and could be applied to clinical or forensic toxicology where "drugs of abuse" (DoA) can be combined into one multi-method including those that are illegal, both naturally occurring and synthetic, alongside therapeutic drugs which can be misused for recreational purposes. In this instance, a strict DnS approach of urine could be employed, although depending on the levels of the parent present and how quick they are metabolised, sensitivity may be an issue. Nonetheless, use of LC-HRMS with DnS for DoA, especially with the improvements in analytical capabilities of HRMS instruments may be more beneficial due to being able to retrospectively check the data produced for new and emerging drugs.

There are also limits to the use of DnS in that some methods such as pesticide residue analysis in fruit and vegetables already have established, validated methodologies and there is no point in trying to modify these well-established, validated and accredited procedures. However, methods such as this could be extended to include other contaminants such as mycotoxins, veterinary drugs and antibiotics, with a switch from QuEChERs to DnS facilitating this without the laborious method development that would normally be required, with an example of the study carried out by Martinez-Dominguez et al. (2016), with screening and quantitation afforded by use of LC-HRMS. Although DnS has been successfully employed in numerous research fields, there is the potential for its scope to be widened if nanoflow LC was routinely incorporated in some cases alongside MS detection. This would potentially permit further reductions in matrix effects by facilitating further dilution of the sample while still enabling analysis of analytes at low levels in complex matrices that may not have been possible before unless specific, selective techniques such as SPE or IAC were employed. Therefore, as the frontiers of LC-MS platforms continue to evolve resulting in instruments with increased resolution, selectivity and sensitivity, the use of dilute-and-shoot has and will continue to become more commonly used.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by Agritox, a project funded by the Interreg Atlantic Area Programme through the European Regional Development Fund [EAPA 998/2018] and by the InvestNI funded Agri-Food Competence Centre project Food Fortress [11-12-17-001].

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