How Good is SFC for Polar Analytes?

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Supercritical fluid chromatography (SFC) is hardly a new separation method, although it is emerging again after some years in the wilderness. However, while the technology is now fully mature, some important fundamentals are still poorly understood, as is the case for the polarity range of analytes amenable to SFC. This paper presents a non-exhaustive critical review of the analysis of polar compounds using SFC.

Packed column SFC is now gaining popularity again and progressively becoming the first choice of purification technique in many pharmaceutical companies. While it has long been a favoured technique for chiral separations, it is also being used for achiral separations. This is due to the recent introduction of specialised apparatus from different manufacturers, together with a slowly increasing awareness of the significant benefits of the technique. Indeed, SFC provides highly efficient and fast separations thanks to the large flow rates that can be reached with a mobile phase of low viscosity. In addition, the carbon dioxide-based mobile phase is an attractive feature allowing a significant reduction of solvent consumption in the chromatography laboratory.

A long history of misunderstandings

SFC was first introduced in 1962, but did not attract much attention at that time. A little later, during its first rise in the 1980's and early 1990's, there was great hope placed in this emerging technique. Unfortunately, much of this hope was misplaced: it was believed that supercritical fluids would provide greater elution strength than gases, thus allowing for the elution of more polar compounds, without the need for the high temperatures required in gas chromatography, or without derivatisation. Several different fluids were explored initially, but carbon dioxide soon emerged as a favourite, thanks to its mild critical parameters, availability at a low cost, compatibility to most GC and HPLC detectors, relative inertness and absence of toxicity. However, for most compounds aside hydrocarbons the elution strength of pure carbon dioxide is not sufficient. It can be increased with high pressure, but not to a very large extent.

Users at the time were reluctant to use co-solvents, because the compatibility to the preferred detection mode, flame ionisation detection, was impaired and it was noted that column efficiency decreased significantly upon addition of a solvent. In some cases it was also reported that the mobile phase separated into two phases (carbon dioxide and a liquid solvent). Unfortunately, all these elements caused a relative drop in the interest of the academic community during several years.

It is now well known that phase separation can be avoided if backpressure is maintained significantly above the critical pressure [1] or if temperature is maintained below the critical one. However, it is now fully acknowledged that elution strength towards polar compounds can be greatly improved upon addition of a co-solvent [2], and that column efficiency is actually improved upon addition of a polar co-solvent [3]. Industry users then re-discovered the advantages of prep-scale SFC in the mid-2000's, particularly for chiral separations. To this particular application, SFC provides a high productivity compared to preparative HPLC, thanks to high flow rates, limited solvent consumption and concentrated fractions requiring less energy to evaporate the solvent.

Nowadays, SFC practice is much closer to HPLC than GC: HPLC detectors are favoured (UV-visible, evaporative light-scattering detection or mass spectrometry with liquid-compatible ionisation sources); the columns used are essentially HPLC-type packed columns; mobile phase composition comprises a significant proportion (typically 5 to 50%) of co-solvent named modifier (most commonly methanol, ethanol or isopropanol) and often small proportions (0.1 to 2%) of an additive (acid, base, or salt). Finally method

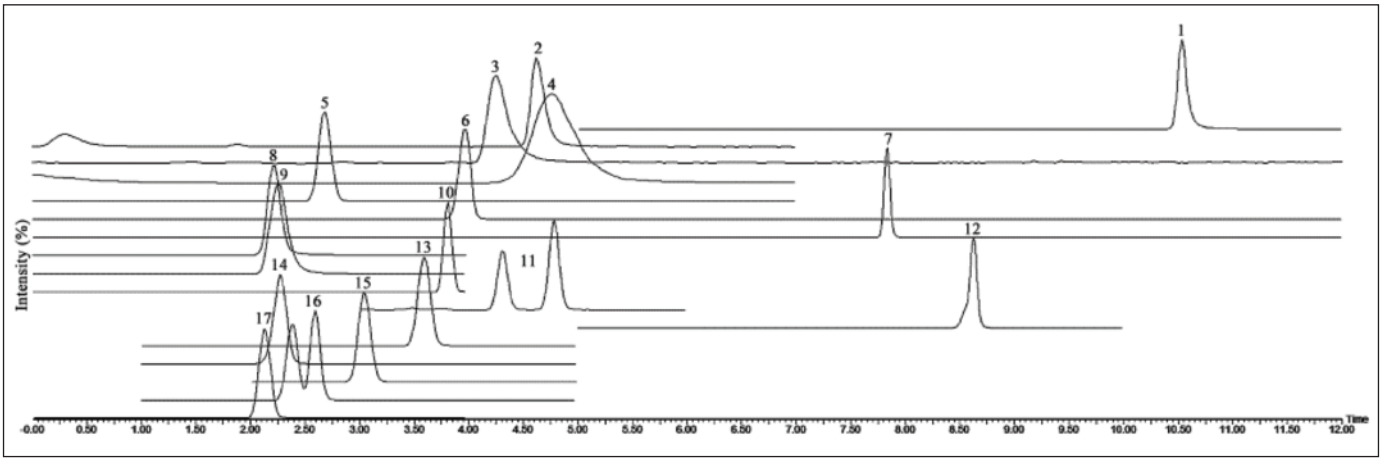


Figure 1: SFC separation 17 polar pesticides. Cosmosil 5CN-MS, gradient elution with methanol + 0.1% ammonium formate, 35°C, 3 ml/min. Reprinted from [53], with permission from Elsevier.

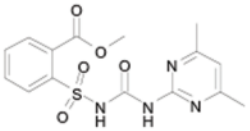
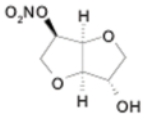
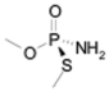
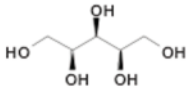
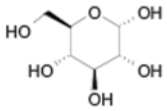
Name	Structure	log $P_{o/w}$	reference
Sulfometuron methyl		1.10	51
Isosorbide-5-mononitrate		-0.15	52
Methamidophos		-0.80	53
Xylitol		-2.50	46
Glucose		-3.24	54

Table 1: Polar neutral compounds successfully analysed with packed column SFC

development is more LC-like with mobile phase composition gradients generally favoured over pressure gradients. True, in these conditions column efficiency is much less than in GC, but usually more than in HPLC, with identical columns but at higher flow rates.

Common practice nowadays is to use a high back-pressure, above the critical pressure of the mobile phase and a temperature below the critical temperature of the mobile phase, thus the resulting fluid is referred to as 'subcritical'. Whatever the composition of the mobile phase employed, its real state of matter should be of little concern, as it was shown in the past that the advantageous features of the fluid remain unchanged as there is a continuum of properties when moving from a supercritical fluid to a liquid. The instrumentation used is no different, only the name of 'supercritical fluid' chromatography would not be appropriate. However, changing now the name of a well-established method might cause confusion.

Surprisingly, the opposite misconceptions to the ones that existed initially are now prevailing, in that most chromatographers who are inexperienced with carbon dioxide-based mobile phases *a priori* believe that most compounds are too polar to be analysed in SFC. Although SFC is generally viewed as a normal-phase technique, only non-polar compounds are supposedly amenable to carbon dioxide – based mobile phases. In the following, significant examples will be presented to question this opinion.

What are polar compounds and how to analyse them?

The definition of polarity is rather vague, and highly dependent on the chemist and the reference in which they are accustomed to working. We must first distinguish between polar neutral compounds, which in our definition might comprise any compound with an octanol-water partition coefficient log $P_{o/w}$ lower than 2, and ionisable or ionic species, regardless of the hydrophilicity of the non-ionic moiety. As a rule-of-thumb, it was suggested that any compound soluble to at least 1 mg/mL in methanol should be amenable to SFC [4]. On the other hand, SFC is usually considered inappropriate to analyse water-soluble compounds [5]. However, the possible direct injection of aqueous formulations in the supercritical mobile phase has been demonstrated [6-8], which should be encouraging as regards the possible polarity range amenable to SFC.

To analyse polar compounds, there are two pre-requisites: the stationary phase must be polar enough to retain them, and the mobile phase must be polar enough to allow for a good compound solubility.

SFC stationary phases for polar compound retention

The stationary phase should thus preferably be a polar surface [9]. Bare silica would be the most straightforward [10], but a number of other bonded-silica stationary phases can be of use. Stationary phases designed for normal-phase HPLC (aminopropyl-, cyanopropyl- or propanediol-bonded silica) or for the HILIC mode (triazole, amide...) have been shown to provide adequate retention for polar compounds [11-14]. An example separation with seventeen polar pesticides analysed on a cyanopropyl-bonded phase is shown in Figure 1. Some column manufacturers now favour the production of SFC-devoted stationary phases. Ethylpyridine is one of the favourite phases of SFC chromatographers and now proposed by several manufacturers [15-17]. Octadecylbonded-silica stationary phases have found use on some occasions, but they need to have some polar function (as polar embedded groups or hydrophilic endcapping groups) in order to allow for sufficient retention of polar compounds.

The charge state of ionisable ligands is an important parameter, since the pH control of the carbon dioxide – based mobile phase is impossible. It is however believed that carbon dioxide – alcohol mixtures are acidic, because they react to form alkylcarbonic acid. The estimated pH might be close to 4-5 [18-19]. In such acidic conditions, it is likely that ionisable groups of the stationary phase could be charged: for instance, amino or pyridine bonded ligands could be partly cationic, while residual silanol groups may be partly anionic. Poor robustness might thus be expected.

Permanently charged stationary phases are also the object of current research. Zheng *et al.* [18] reported on the use of a strong anion-exchange stationary phase with propyltrimethylammonium ligands, while others investigated ionic liquid stationary phases based on phosphonium, pyridinium or imidazolium ligands [20-21]. Adequate retention and peak shape was demonstrated for neutral, acidic and basic compounds. According to Zheng *et al.* [18], and based on previous works by Jessop *et al.* [22], it is probable that stationary phases containing basic ligands such as aminopropyl or ethylpyridine might result in an ionic liquid through reaction with the carbon dioxide – alcohol mixture.

SFC mobile phases for

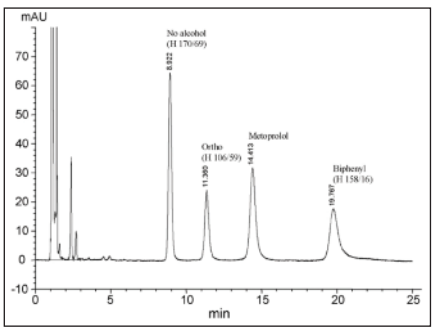


Figure 2: SFC of metoprolol and some related analogues. Kromasil 100-5NH2, 8% methanol, 60 °C, 250 bar, 1.5 ml/min. Reprinted from [61], with permission from Elsevier.

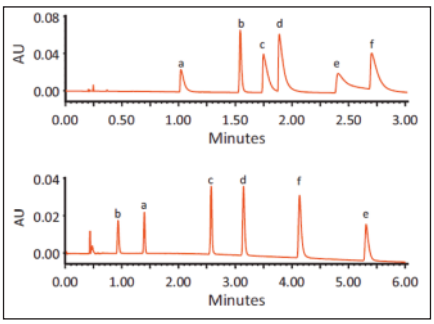


Figure 3: Chromatograms of basic drugs of high pKa values: pethidine (a), buprenorphine (b), dextromethorphan (c), codeine (d), pholcodine (e), and morphine (f). Acquity UPC2 BEH 2-EP, gradient elution with methanol (top), or methanol with 20 mM NH4OH (bottom); 40°C, 150 bar, 2.5 mL/min. Reprinted from [15], with permission from Elsevier.

polar compounds elution

The purpose of increasing the polarity of the mobile phase by adding co-solvents and additives to carbon dioxide is not only to increase the solubility of analytes, but also possibly to mask 'active sites' of the stationary phase that might cause irreversible adsorption.

Initially, a distinction should be made between polar neutral compounds and ionic compounds. Polar neutral compounds can be easily eluted in packed-column SFC, provided the mobile phase composition is adjusted to ensure sufficient solubility of the analytes, as proven with many examples from sugars to polar lipids (Table 1).

To elute ionic and ionisable species, additives are usually considered as an absolute necessity. The truth is that most SFC chromatographers introduce additives in the mobile phase in a *quasi*-systematic fashion in the course of method development. However, several examples prove that charged species can be eluted without any additive. For instance, Geiser *et al.* achieved preparative isolation of hydrochloride salts using simple carbon dioxide – methanol mobile phases [23]. Similarly, Bhoir *et al.* achieved elution of anionic phenytoin and phethenylate sodium salts from an octadecyl-bonded silica phase [24]. Shown in Figure 2 an example separation of basic compounds, analogues of metoprolol, eluted without additives from an aminopropyl-bonded silica phase.

Ionisable species (acidic or basic) make up a majority of the compounds analysed in the pharmaceutical industry. Moreover, basic active pharmaceutical ingredients are often present as cationic amine salts. As mentioned above, the estimated pH of carbon dioxide –alcohol mobile phases might be close to 4-5. In such acidic conditions, it is likely that the most acidic compounds would be in their anionic form, while basic compounds would be in their cationic form. In this case, addition of an acidic additive (as acetic acid, trifluoroacetic acid, formic acid or citric acid) with a pK_a below that of the analyte can restore the neutral state of acidic species, and protonate basic compounds. Basic additives (diethylamine, triethylamine or isopropylamine) would restore the neutral state of basic compounds but deprotonate acidic compounds. Influence of the acidic and basic additives on the charge state of the stationary phase would be similar. In many cases, an acidic and a basic additive were used in conjunction. Current practice of packed column SFC often favours ammonium acetate or even ammonia, not only to promote solubility but also for their compatibility to mass spectrometric detection [16, 25-26]. Shown in Figure 3 example chromatograms to illustrate the effect of adding ammonia to the mobile phase when analysing basic compounds with high pK_a values.

There is also the possible problem of analyte reaction with mobile phase components. Indeed, amines are known to react with carbon dioxide to form carbamic acids. However, it appears that any reaction is probably reversible and that the original amines are retrieved after the back-pressure regulator when carbon dioxide is depressurised [27]. There are also alcohol-sensitive compounds which might react with the alcohol solvent (esterification reactions, for instance). Byrne *et al.* have shown that 2,2,2-trifluoroethanol was an interesting substitute to avoid such reactions [28].

Tables 2 and 3 provide examples of acidic and basic species that should be ionic in the usual carbon dioxide – methanol mobile phases and that were successfully analysed in SFC.

Permanently charged analytes are not affected by alterations in the pH, but additives may be useful in achieving their elution. Table 4 presents some analytes with permanent charges that were successfully eluted in SFC.

Whatever the charge state of the analyte, the mechanism through which additives participate in the chromatographic process is still unclear. They were often believed to act as competitors for 'active sites' of the stationary phase [29]. An additive of the same nature as the analyte (acidic additives for acids, basic additives for bases) should therefore be selected. However, Blackwell observed that resolution of neutral chiral analyte enantiomers was also greatly affected by the choice of mobile phase additive, while the competitor theory is difficult to fit to this observation [30]. Also difficult to understand is the improvement in the enantioselectivity of metoprolol (a basic amino-alcohol) when a

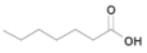
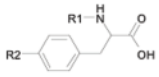
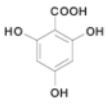
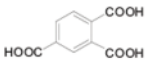
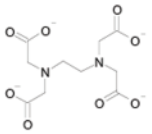
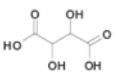
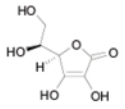
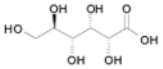
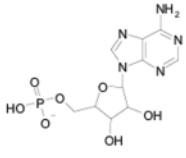
Salicylic acid		2.97	9
Heptanoic acid		2.22	43
Phenylalanine analogues		≈2	38
2,4,6-trihydroxybenzoic acid		1.28	56
Trimellitic acid		1.00	36
Niacin		0.36	42
EDTA		-0.84	50
Tartaric acid		-1.80	50
Ascorbic acid		-1.85	42
Gluconic acid		-2.60	50
Fosetyl		-2.70	53
Adenosine monophosphate		-3.19	49

Table 2: Acidic compounds successfully analysed with packed column SFC

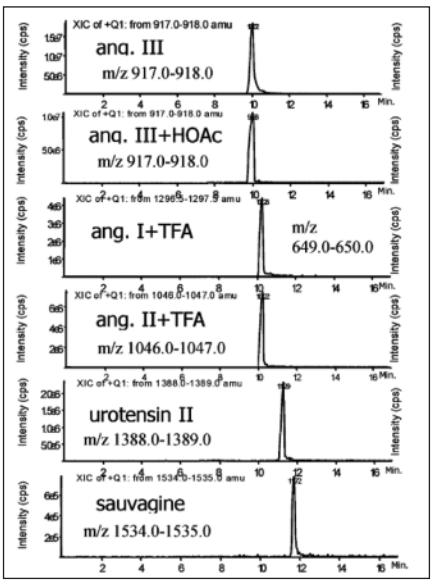


Figure 4: SFC/MS of large polypeptides (angiotensin I, II and III, urotensin and sauvagine). Princeton Ethylpyridine, gradient elution with 13 mM TFA/methanol; 40°C, 120 bar, 2 mL/min. Reprinted with permission from [39]. Copyright 2006 American Chemical Society.

strongly acidic additive (trifluoroacetic acid) was used on a stationary phase made of polysaccharide coated onto silica [31].

In some cases, ion-pairing was shown to occur. For instance, Steuer *et al.* used ion-pairing agents to elute a number of ionic drug substances [32]. Suto *et al.* used an ion-pairing agent to achieve extraction and separation of cationic alkaloids, berberine and palmatine [33]. Gyllenhaal *et al.* separated metoprolol tartrate and metoprolol succinate, and related amino-alcohols with an ion-pairing agent [34]. Patel *et al.* showed that ion-pairing facilitated the elution of peptides [35], while Zheng *et al.* demonstrated an ion-pairing mechanism to elute a number of cationic compounds from a cyanopropyl-bonded silica column with the help of sulfonate salts [18]. However, solubility of the ion-pairing agent in the mobile phase might also be a concern if used to elute ionic solutes.

In other cases, the additive is simply believed to suppress ionisation of the analyte [36-37]. Blackwell showed that retention of acidic phenylalanine analogues correlated well with the pK_a of the acidic additive [38]. Taylor *et al.* succeeded in eluting polypeptides and polypeptide salts with up to 40 residues [39-40]. Some of these compounds did not dissolve in methanol, unless acidic or trifluoroacetic acid (TFA) was introduced. This was an interesting indication as to the best mobile phase composition because those polypeptides required larger concentrations of TFA in the methanol co-solvent (13mM) to elute as sharp peaks. It was believed that TFA acted in protonating the acid and amino functions.

Figure 4 reproduces some of these chromatograms.

Consequently, there is still a poor understanding of why in some cases, additives provide a significant improvement in resolution, while in other cases they simply have no effect or even deteriorate the quality of the separation.

Is water the solution?

Water is possibly the most interesting additive (or co-solvent?) to use in SFC. It is of little use as a single co-solvent because the miscibility of water in carbon dioxide is very limited, but it can be included in a ternary composition comprising carbon dioxide and an alcohol co-solvent. Some old studies paved the way for future SFC practice. Water was often found in mobile phase compositions employed in the early years of packed column SFC, in proportions usually ranging from 0.1 to 10%, with or without any other solvent. An advantage of very small proportions of water without any other solvent was to possibly retain compatibility to flame ionisation detection, and even improve the detector response. Geiser *et al.* used it to improve the separation of free fatty acids [41]. Pyo employed it to achieve SFC separation of vitamins [42], free fatty acids and sulphonamide antibacterials [43]. Thiébaut *et al.* achieved elution of underivatised amino acids [44] and imidazole derivatives [45]. Salvador *et al.* used it to analyse carbohydrates [46]. Strangely, water then seems to have been completely forgotten in the following decennia. It is now appearing again in several papers that have promoted its advantages. While most of them simply advocate small proportions (0.5 to 5%) to enhance peak shape [26,47,48], others introduce it in much larger proportions (5 to 30%) allowing the elution of very polar compounds [49]. Figure 5 illustrates this point. Li and Thurbide [50] demonstrated that isopropanol was the most advantageous solvent, allowing larger proportions of water to

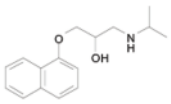
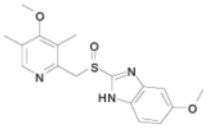
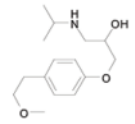
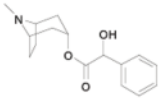
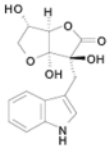
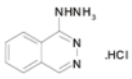
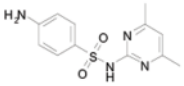
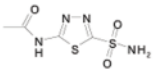
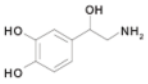
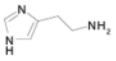
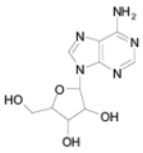
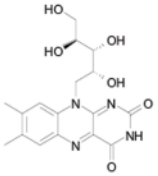
Name	Structure	log $P_{o/w}$	reference
Propranolol		3.48	15, 18, 57, 58
Omeprazole		2.23	59
Metoprolol		1.88	21, 58, 60, 61
Atropine		1.83	62
Ascorbigen		1.45	63
Hydralazine HCl		1.00	25
Sulfamethazine		0.89	64, 65
Acetazolamide		-0.26	47
Noradrenaline		-0.88	66
Histamine		-0.92	66
Adenosine		-1.05	49
Cytosine		-1.40	26, 48
Riboflavin		-1.46	42
Maleic hydrazide		-1.96	53

Table 3: Basic compounds successfully analysed with packed column SFC

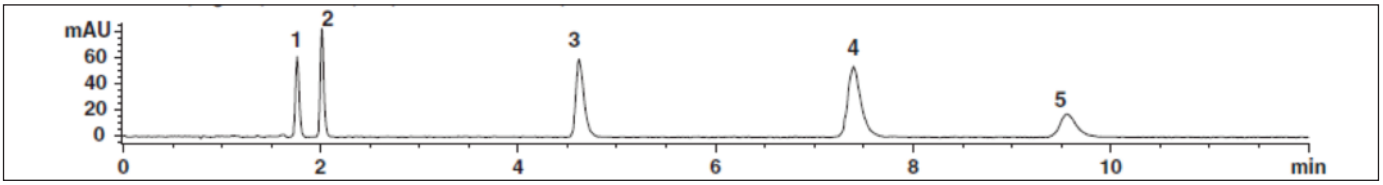


Figure 5: SFC separation of nucleobases: 1. thymine, 2. uracil, 3. cytosine, 4. guanine and 5. adenine. Zorbax Rx-Sil, 1 mL/min ethanol-ammonium formate buffer-formic acid 20 mM pH 3 (95:5 v/v) and 2.0 mL/min CO2; 40°C. Reprinted with permission from [48]. Copyright 2010 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

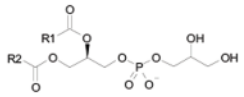
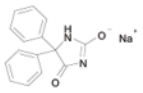
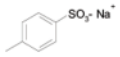
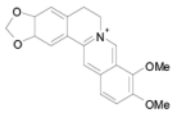
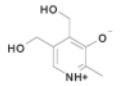
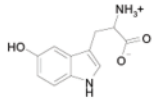
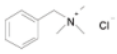
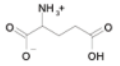
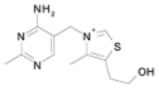
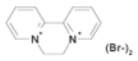
Name	Structure	log P $_{o/w}$	reference
Phospholipids		≈10	67
Phenytoin sodium		2.47	24
Cetylpyridinum chloride		1.70	18
Sodium toluylsulfonate		0.47	68
Berberine		-0.18	33
Pyridoxine		-0.77	42
Tryptophan		-1.06	44, 50
Benzotrimethylammonium chloride		-2.00	18
Glutamic acid		-3.69	50
Thiamine		-4.20	42
Diquat dibromide		-4.60	53

Table 4: Ionic compounds successfully analysed with packed column SFC

be used in the mobile phase (when compared to methanol). While the resulting fluid in such ternary compositions is not a supercritical fluid but a liquid with so-called 'enhanced fluidity', we have pointed out above that technically, the way to practice the technique is no different.

In summary, we hope we have proven here that analysis of polar compounds is not only the future, but also the past and present of SFC. Maybe SFC should not be simply viewed as a replacement for normal-phase and for non-aqueous reversed-phase HPLC, but also for HILIC methods. Now that the technique is fully mature, it would be good to re-visit some of the old studies. It is to be expected that the recent introduction of modern SFC systems by two major manufacturers of chromatographic devices, namely Waters and Agilent, will further the interest for this versatile and highly interesting technique.

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