Agilent hplc column selection guide

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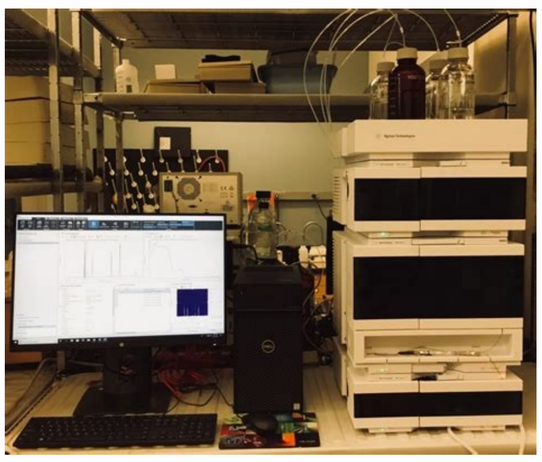

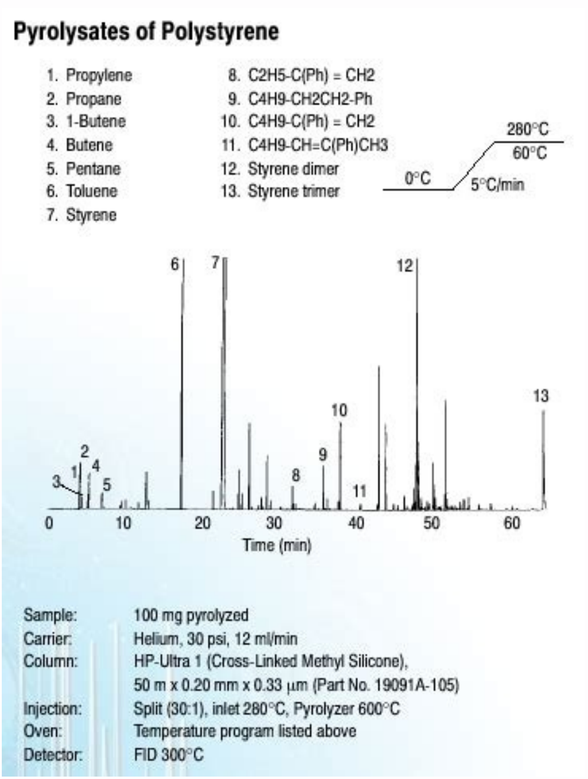

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HPLC methods for purity evaluation of man-made single-stranded RNAs Synthetic RNA oligos exhibit purity decreasing as a function of length, because the efficiency of the total synthesis is the numerical product of the individual step efficiencies, typically below 98%. Analytical methods for RNAs up to the 60 nucleotides (nt) have been reported, but they fall short for purity evaluation of 100nt long, used as single guide RNA (sgRNA) in CRISPR technology, and promoted as pharmaceuticals. In an attempt to exploit a single HPLC method and obtain both identity as well as purity, ion-pair reversed-phase chromatography (IP-RP) at high temperature in the presence of an organic cosolvent is the current analytical strategy. Here we report that IP-RP is less suitable compared to the conventional ion-exchange (IEX) for analysis of 100nt RNAs. We demonstrate the relative stability of RNA in the denaturing/basic IEX mobile phase, lay out a protocol to determine the on-the-column stability of any RNA, and establish the applicability of this method for quality testing of sgRNA, tRNA, and mRNA. Unless well resolving HPLC methods are used for batch-to-batch evaluation of man-made RNAs, process development will remain shortsighted, and observed off-target effects in-vitro or in-vivo may be partially related to low purity and the presence of shorter sequences. The discovery of Chromatography, i.e. separation of a mixture into its components, approximately 120 years ago is credited to Mikhail Tsvet, a Russian-Italian botanist. A major revolutionary step in chromatography was the advent of high-performance liquid chromatography (HPLC) instruments invented 50 years ago1,2. In HPLC, a liquid mobile phase (MP) carries a mixture of compounds through a column packed with particles. As a pump forces the MP through the column, the components of the mixture interact with the stationary phase - the particle’s surface - to different degrees and are separated in the process. HPLC’s first application was the resolution of nucleic acids exploiting IEX normal mode3, even though this type of chromatography was later abandoned. Since then a steady improvement in instrumentation and column packings have yielded methods for analysis and purification of both synthetic materials as well as compounds from biological fluids4,5,6,7. HPLC remains the most widely used analytical technology especially for purity determination and batch-to-batch comparison of compounds poised for pharmaceutical use8. Most chromatography suppliers claim applications for oligonucleotide analysis, but in reality good analytical methods exist for up to 25nt9,10 and become less optimal as the length increases5,6.The synthesis as well as the purification of oligoribonucleotides are intrinsically less efficient compared to the synthesis and purification of their deoxy counterparts, primarily due to the presence of the 2′-OH that needs to be protected during synthesis11,12,13,14,15,16. Protection of 2′-OH and deprotection at the end of the synthesis may not be accomplished at 100% yield, so the overall efficiency of RNA synthesis is compromised compared to DNA. Coupling efficiency in oligonucleotide manufacturing refers to the success rate of a synthesizer adding a new base to a growing nucleic acid chain. This measure is especially important for long oligos, because as the length of an oligo increases, small differences in coupling efficiency have dramatic effects on the yield of the full-length product. Here are two examples to illustrate the compounding effect of less than 100% efficiency in the synthesis of a 100nt vs the synthesis of a 50nt. With an average 99.0% efficiency at each step, the calculated fraction of the 100nt in the final product is 0.366, whereas the calculated fraction of the 50nt is 0.605, almost double. With an average 98.0% efficiency at each step (just 1.0% less), the corresponding calculated fraction for a 100nt is 0.13 and for a 50nt is 0.36. The “theoretical” 13% yield to make a 100nt with an 98.0% average step efficiency is consistent with literature claiming 5.5% yield, after purification, for the optimized synthesis of a 110nt RNA oligo17. With synthetic oligos the highest HPLC peak is very likely to correspond to the desired oligo. Identification of the final product is conducted using IP-RP HPLC analysis directly followed by mass spectrometric (MS) detection18. It should be noted that product identification by mass determination does not require resolution, and therefore identification is independent of relative abundance. However the effectiveness of purification/isolation of the desired product correlates with the product’s abundance in the crude mixture5,6.In addition to the synthetic efficiency issue, the longer oligoribonucleotides, let us say longer than the 50nt, exhibit self-structure within the otherwise linear polymer19,20. Such self-structure could be due to short intramolecular double stranded regions between distant sequences, to regional stem-loop folding, to Hoogsteen base-pairs in G-rich sequences, and/or to a wide array of base-stacked conformations. Self-structure leads to the presence of several conformers of comparable stability. Conformers may bear scientific interest regarding in-vitro and in-vivo activity, but they are an additional obstacle to good separation of the mixture into the desired oligo and its impurities. Hence analysis of longer RNAs necessitates denaturing conditions. Such conditions are (i) aqueous MP at pH 12 (see later), (ii) high temperature, and (iii) the presence of an organic cosolvent, such as methanol (CH3OH) or acetonitrile (CH3CN), in the MP. Additional additives are formamide and urea, favored by molecular biologists, but not by HPLC analysts. All these additives/conditions act by disrupting base-pairing and base-stacking interactions, and therefore practically linearize the nucleic acid. Linear, non-structured, polymers may elute as sharp peaks by HPLC, and thus resolve from closely related impurities.Credit for the development of column packings and methods to resolve oligonucleotide mixtures, in this author’s opinion, should be given to the late Dr. Leslie E. Orgel of the Salk Institute and his coworkers for the research they spearheaded in support of the “RNA world” hypothesis21,22,23 (first described by Alexander Rich in 1962, and later coined by Walter Gilbert in 1968). In the 1970s-80s the primary mode of chromatography was normal phase3,24, eventually completely replaced by reversed-phase. In the 1970s Orgel and coworkers discovered the non-enzymatic, template-directed synthesis of oligoribonucleotides25. Using phosphoimidazolide activated ribomononucleotides, as building blocks, and homo- or hetero-polymeric nucleic acids, as templates, they demonstrated formation of the complementary strand as a series of oligos up to the 40nt26. In order to investigate length and linkage, they took Kel-F packing, coated it non-covalently with Adogen, a mixture of tetraalkylammonium compounds, and packed it in HPLC columns (named RPC5)27,28. This was the first packing known to resolve oligos up to the 40nt based on length and internucleotide bond linkage (2′-5′, 3′-5′ as well as pyrophosphate)26. For the next two decades oligonucleotide analysis was accomplished using packing material from Orgel’s Laboratory, and a MP made out of aqueous 10 mM NaOH (pH 12.0, see also Results below) and $NaClO_4$ for salt gradient elution29,30,31. The RPC5 packing was not user-friendly, and ultimately replaced by commercially available ones, that barely claim to match its resolving power.Several HPLC interaction modes have been used successfully for the separation of an oligonucleotide mixture. While initially the mode of choice was normal phase IEX, in 1987 we introduced reversed-phase mode for separation of oligos with N 60. Some of the advantages of the DNAPacIEX are: usability in a pH range of 2.5

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