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Chromatographic benefits of elevated temperature for the proteomic analysis of membrane proteins

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Abstract

Integral membrane proteins (IMPs) perform crucial cellular functions and are the primary targets for most pharmaceutical agents. However, the hydrophobic nature of their membrane-embedded domains and their intimate association with lipids makes them difficult to handle. Multiple proteomics platforms that include LC separations have been reported for the high-throughput profiling of complex protein samples. However, there are still many challenges to overcome for proteomic analyses of IMPs, especially as compared to their soluble counterparts. In particular, considerations for the technical challenges associated with chromatographic separations are just beginning to be investigated. Here, we review the benefits of using elevated temperatures during LC for the proteomic analysis of complex membrane protein samples.

Keywords

Liquid chromatography; Microcapillary; Shotgun; Temperature

1 Introduction

Integral membrane proteins (IMPs) are an important class of cellular proteins that constitutes approximately one-third of the total proteins predicted from the human genome sequence. IMPs also represent the majority of the known pharmaceutical drug targets [1,2]. Despite their biological significance, IMPs still remain under-represented in most proteomic studies, as their hydrophobic nature makes them challenging in all steps of the experimental workflow, from sample solubilization to protein separation to mass analysis (for detailed reviews of the proteomics of IMPs see ref. [3,4]).

Solution-phase chromatographic techniques, such as HPLC and microcapillary LC (μ LC) are important proteomics tools, facilitating the separation and/or affinity purification of protein and peptide species from complex mixtures [4-7]. However, most RP-LC protocols have been tailored for the application to soluble peptide/protein samples, and thus require modification for optimal separation and recovery of hydrophobic species. While the RP-LC separation of IMPs and membrane protein digests is far from perfected, the optimization of one frequently overlooked parameter – namely column temperature – has proven surprisingly critical for the proteomic analysis of IMPs and transmembrane domain (TMD) peptides. Recent reports [8-10] suggest that elevated temperature is *indispensable* for the separation and recovery of

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hydrophobic proteins/peptides. This review will focus on the overall benefits of using elevated temperatures during RP-LC for the proteomic analysis of *complex* membrane samples.

2 Historical benefits of thermostatted LC: Speed, peak shape, and selectivity

Perhaps the first widely recognized benefit of running RP-LC above ambient temperature was that of decreased analyte retention time. However, because shorter retention times were typically associated with poorer resolution, temperature was not a variable often considered for optimization of HPLC. Indeed, it was not until the late 1970s that Snyder and coworkers [11,12] characterized the beneficial effect of temperature optimization on peak resolution.

In the mid 1980s–1990s optimized running temperatures were first applied to the analysis of simple protein and peptide samples, with several groups reporting the use of elevated temperature to increase column efficiency (*i.e.*, maximized peak capacity with minimized analysis time) [13–21]. (Note: for a comprehensive review of the physical parameters of HPLC separation, see ref. [22]). For example, Cohen *et al.* [20,21] reported that elevated column temperature (48°C) was important for reducing the number of protein conformers and thus lessening the effects of peak splitting/broadening.

In another report, Boyes and Kirkland [16] employed a new heat-stable RP material (Zorbax SB, Agilent, Palo Alto, CA) to separate the peptide components of a simple tryptic digest over a range of temperatures. Most silica-based resins are somewhat sensitive to the low pH of the mobile phase due to hydrolysis of siloxane bonds (Si–O–Si). As such, elevated temperature (typically above 60°C) accelerates this breakdown, requiring the use of a resin with sterically protected bonded silica surface for temperatures from 60 to 90°C. Using angiotensin as a standard, the authors note that column efficiency increased markedly up to ~70°C. No significant improvement in peak shape or resolution was obtained above that point, likely do to competing effects such as radial temperature differences (interior *vs.* exterior of the column) and/or incomplete temperature pre-equilibration of the mobile phase, which can be harder to avoid at operating temperatures significantly above ambient. Absence of such thermal gradients is key to optimizing separation, as temperature has a profound effect on mobile phase viscosity (decreases with increase in temperature) and solute diffusion (increases with increase in temperature). At elevated temperature, reduced viscosity and greater solute diffusion typically results in more symmetrical, narrower peaks (provided that the flow rate is sufficient to overcome non-productive diffusion [23]). However, any temperature heterogeneities within the column create regions of disparate viscosities and diffusion, resulting in peak broadening. Thus, the optimized temperature for LC can be greatly affected by how well that temperature can be maintained throughout the entire column. The authors also report that maximum column efficiency for select resins (small pore) was obtained at a higher flow rate at 85 *versus* 35°C, meaning that, with the application of heat, the analysis time could be shortened without loss of resolution [16].

Boyes and Kirkland also took note of selectivity (peak order, relative spacing) changes associated with varying temperature, an idea which had been advocated several years earlier by Dolan and coworkers [24,25] based on computer simulations. In the mid 1990s, Snyder and coworkers [14,19,26–29] published a detailed investigation into the role of temperature in separation selectivity – specifically in conjunction with gradient steepness – using a variety of protein, protein digest, and synthetic peptide samples. At the time of their publication, the relationship between gradient steepness and analyte retention time was well understood: faster ramping of the percent organic reduced retention time, with the specific shift for each species determined by its particular physical properties. As such, the gradient could be adjusted to optimize selectivity, even for adjacent/overlapping species [14].

However, the relationship between temperature and analyte retention time was less well defined. Based on the studies of small molecules, it was possible to relate the retention factor (k_T) for a given temperature (T) to physical properties of the system: $\log k_T = C\Delta H + D$, where C and D are constants and ΔH is the enthalpy of retention. However, this equation implied that peak selectivity remains largely unchanged regardless of temperature (see ref. [14] for a more complete discussion), and that temperature optimization would not result in peak resolution for coeluting peaks. Such results from small molecule studies were, in part, responsible for the lagging interest in temperature as a selectivity variable. However, the authors explain, it turns out that only neutral molecules tend to obey this equation. For more functionalized species – like peptides and proteins – retention is a more complicated process, affected by (i) interactions with multiple sites on the stationary phase (*e.g.*, both alkyl groups and silanols), (ii) acid–base equilibria, (iii) ionpairing or other secondary equilibria, and (iv) molecular shape [14]. Thus, one could imagine that an amphipathic helical peptide with multiple basic sites would be affected much differently by temperature than a random coil, acidic peptide (*i.e.*, differential protonation, denaturation).

Given that gradient steepness and temperature thus seemed two potentially powerful variables to optimize peptide and protein selectivity, Snyder and coworkers then set out to determine if changes in one parameter were independent of the changes in the other – *i.e.*, if optimizing temperature might lead to peak deconvolution in cases where gradient optimization did not. They first analyzed the tryptic digest of recombinant human growth hormone (rhGH) at 20, 40, and 60°C with a 60 min gradient using Zorbax SB as the RP. As they predicted, significant shifts in peak spacing were observed with changing temperature, with 60°C giving the overall best resolution. The authors then held temperature constant (40°C) and used gradients of 30, 60, or 120 min to separate the rhGH digest. Again, significant selectivity differences were observed, with the longest gradient giving the best peak resolution. Importantly, the peak spacing obtained by increasing temperature could not be replicated by increasing gradient (and *vice versa*), indicating that temperature and gradient slope are indeed orthogonal parameters. It is interesting to note that, by using the optimal temperature of 60°C, good resolution was achieved during a 60 min run as opposed to 120 min run for the optimized gradient. Thus, optimizing temperature rather than gradient can lead to the most efficient analysis [14].

From these early studies of thermostatted HPLC, it is clear that chromatographic analysis of proteomic samples at elevated temperature can be of benefit for reducing analysis time while optimizing both peak shape and selectivity. More recent investigations have uncovered further benefits of elevated temperature, namely (i) the facilitated handling of viscous samples and (ii) the enhanced recovery of hydrophobic species, both of which will be discussed in the following sections.

3 Practical benefits of elevated temperature: Optimized handling of hydrophobic and viscous samples

Because IMPs are embedded in lipid bilayers, samples enriched for IMPs are also necessarily enriched for lipids [30,31]. Unfortunately, the presence of lipids significantly increases sample viscosity, causing sample handling to become problematic during LC [9]. Many lipids bind tightly to RP material and require special gradient compositions to be effectively removed from the column for complete column regeneration [8]. Furthermore, they often coelute with hydrophobic proteins and peptides, obscuring the protein/peptide peak of interest [9]. Therefore, a major and sometimes under-appreciated technical difficulty associated with the analysis of IMPs involves the removal/separation of the contaminating lipids prior to/during proteomic analysis. The hydrophobicity of the IMPs and IMP-derived peptides themselves can be problematic as well – causing aggregation and precipitation – especially in the absence of detergents, which are generally removed prior to LC so as not to interfere with analysis [3].

Recently, Martosella *et al.* [8] applied elevated temperature LC for the analysis of lipid rafts, a plasma membrane subdomain enriched in cholesterol, glycosphingolipids, and IMPs. Because lipid rafts are detergent insoluble, the first major difficulty encountered involved the production of a solubilized sample amenable to LC column injection. It was determined empirically that 80% formic acid (FA), known to be compatible with HPLC, effectively solubilized the lipid raft sample; however, injection at ambient temperature resulted in immediate sample precipitation within the column. Therefore, it was necessary to perform all injections at 80°C to reduce the sample viscosity and maintain protein solubility for effective column loading.

Our group has also observed the same practical benefits of heat for sample handling during the development of a shotgun proteomic strategy for the targeted analysis of TMDs [9]. The “MEP method” enriches specifically for membrane-embedded peptides (MEPs) by digesting (or “shaving”) intact membranes with a soluble protease (proteinase K) to remove the membrane-associated proteins and protease accessible domains of IMPs. This procedure results in “shaved” membranes enriched in MEPs, which are protected from proteolytic digestion by the intact lipid bilayer. Similar to Martosella *et al.*, we observed optimal sample solubilization with a high concentration of FA (90%), which also facilitated a critical redigestion step at methionines with cyanogen bromide [32]. We also found that μ LC analysis was complicated by high sample viscosity (due to the over-whelming presence of lipids), which made sample loading onto a μ LC column impossible at ambient temperature. Like Martosella’s group, our initial solution was to load at an elevated temperature (~37°C, offline using a high-pressure bomb set on a hot plate). However, this proved to be impractical as a long-term solution, so an alternative strategy was implemented to reduce sample viscosity prior to analysis. By diluting the concentrated FA sample with aqueous buffer, lipids became insoluble and precipitated, while the MEPs remained soluble in the aqueous solution. Therefore, precipitated lipids could easily be separated from MEPs, allowing for autosampler column injections at ambient temperature, and eliminating the majority of the lipid interference during MS analysis [9].

4 Temperature effects on chromatographic elution profiles: Analyte retention and recovery

As described in the previous section, the application of elevated temperature provided a practical solution for sample injection/delivery onto the column for chromatographic separation of two different, highly viscous and hydrophobic samples containing lipid components. However, eluting proteins, peptides, and lipids from the column proved to be problematic as well [8-10].

Prior to their lipid raft studies, Martosella *et al.* had examined an immunodepleted serum sample to characterize the effects of elevated temperature on chromatographic separations and protein recovery in a complex mixture [33]. The serum samples were analyzed using two types of separation media (macroporous RP-C18 (mRP-C18) and standard (300 Å) pore Zorbax SB300-C8; Agilent) at two operating temperatures (26 or 80°C). When protein concentrations in the fractionated eluates were evaluated by protein assay, recovery was found to be 30% lower for both the mRP-C18 and SB300-C8 materials at 26°C as compared with 80°C. This was attributed to irreversible binding of analytes to the column and/or precipitate formation within the column, which decreased column efficiency. Ultimately, it was found that the application of high organic (99.92% ACN/0.08% TFA) held for 4–5 min at the end of each gradient at the ideal temperature of 80°C resulted in complete regeneration of the column surface and maintained complete protein recoveries in sequential analyses. Elevated temperature was found to improve peak selectivity as well, leading to cleaner fractionation as well as increased sample recovery [33].

Martosella *et al.* [8] applied these improvements in chromatographic separation, protein recovery, and column regeneration at 80°C to the subsequent analysis of the lipid raft sample. As discussed in the previous section, proteins and lipids, solubilized in 80% FA, were injected directly onto the column at elevated temperature (80°C) and pressure. However, repeated injections showed significant peak broadening and inconsistent peak shape in the latter runs, likely due to a buildup of hydrophobic sample components (*e.g.*, lipids, cholesterol, residual detergent) on the column surface. Initial attempts at offline delipidation prior to LC resulted in significant sample loss and noticeable peak broadening (likely due to irreversible protein aggregation/precipitation and/or incomplete lipid removal), and was thus determined to be a nonviable option. As a result, Martosella *et al.* reoptimized the column regeneration protocol specifically for this lipid raft sample. As with the serum analysis, protein elution was accomplished using an increasing gradient of ACN/0.08% TFA. A two-part column regeneration (20%FA/ACN followed by 2-propanol) was then carried out to remove all lipids and other adsorbed components [7,34,35]. By using a combination of elevated temperature and optimized chromatography, protein elution was effectively separated from lipid elution and complete column regeneration was obtained.

Given the success of this elevated temperature methodology, we decided to evaluate whether or not heated chromatography would be beneficial for the analysis of the MEP sample, as, even after we had solved the sample viscosity problem, our room temperature μ LC-MS/MS analysis resulted in very limited peptide recovery. However, most commercially available column heaters cannot easily accommodate μ LC columns, requiring custom-built systems (a solution often employed for the μ LC analysis of small molecules [36,37]). As such, we assembled a simple block heater to house our typical fused-silica microcapillary RP column (12 cm in length, in-house pulled tip; see Appendix: μ LC column heaters on a budget, panel A). To evaluate hydrophobic peptide recovery as a function of temperature, two samples – the MEP sample [9,10] previously discussed and a tryptic digest of an enriched plasma membrane fraction [10] – were analyzed at five temperatures (20, 30, 40, 50, and 60°C) using an increasing gradient of aqueous ACN with 0.1% FA. (Note: The recommended temperature range for most column packing materials is ambient temperature to 40°C for extended use (weeks–years) and up to 50–60°C for short durations (hours–days). Therefore, it is important to check the manufacturer recommendations before incorporating elevated temperatures for proteomic workflow.)

While the overall chromatographic profiles and peptide retention times were found to be similar across the temperature range tested, peptide recovery was not. For the MEP sample, elevation of the column temperature to 60°C resulted in a four-fold increase in protein identifications and a five-fold increase in peptide identifications as compared to results obtained at 20°C. Most importantly, dramatic improvements were observed for the recovery of hydrophobic peptide species, highlighted by an overall increase of the average grand average of hydropathy (GRAVY) score [38] of the recovered peptides (from 0.41 to 0.63) and increased identification of IMPs containing >2 TMDs (from 77 to 87%). Of the identified peptides, 68% recovered at 60°C were found to overlap with a predicted TMD (TMHMM [39]) compared with 63% at 20°C. A more dramatic increase was observed for peptides with very high ($\geq 75\%$) TMD overlap, with 27% recovered at 60°C *versus* only 11% at 20°C [10].

To further improve the comprehension of the analysis of the MEP sample, we designed a second generation column heater to accommodate in-line filter assemblies (Upchurch, Oak Harbor, WA) and facilitate the multidimensional protein identification technology (MudPIT) approach of Yates and coworkers [40–42] (see Appendix: μ LC column heaters on a budget, panel B). Thermostatted MudPIT analysis of the MEP sample (μ LC/ μ LC conducted at 60°C) increased protein identifications by three-fold and peptide identifications by four-fold, as compared to the single dimensional RP- μ LC analysis [43].

As with the MEP analysis, significant improvements were also observed for a standard trypsin digested plasma membrane sample when analyzed at elevated temperature. The following is a more detailed analysis of the tryptic digested data previously reported [10]. Figure 1 summarizes the trends observed from proteomic profiles collected at each of the five temperatures. The most dramatic differences for total peptide and protein identifications were observed with temperature elevation from 20 to 40°C: protein identifications increased by 38% (Fig. 1A), and peptide identifications increased by 36% (Fig. 1B). These % increases may seem modest compared to the MEP analyses described above; however, the gain in total protein and peptide numbers is actually not trivial. Total proteins identified increased by 110 and total peptides increase by 910. Interestingly, while the number of protein and peptide identifications seem to plateau with further temperature elevation from 40 to 60°C (Figs. 1A, B, and D), improvements in the recovery of *hydrophobic* tryptic peptides were dramatic (Figs. 1C and D). The average peptide GRAVYscore increased significantly from 20 to 60°C (four-fold total increase, Fig. 1C), reflecting the improved recovery of hydrophobic peptides as a function of higher temperature. Because the average peptide hydrophobicity (GRAVY score) increases while the total number of proteins and peptides remains the same, this suggests that, unlike the less complex MEP sample, the tryptic peptides recovered at 20°C are not a complete subset of the peptides recovered at 60°C, demonstrating that temperature can have a dramatic effect on the peptide population that is recovered in a complex sample.

Although the trypsin digested sample is not enriched for TMDs and only a small percentage of the recovered hydrophobic peptides overlap with predicted TMDs (2.1% (88 peptides) at 60°C and 1.1% (45 peptides) at 20°C), similar trends are observed. Figure 2 compares the retention times and GRAVY scores of the recovered peptides overlapping with predicted TMDs at five different temperatures. In general, the more hydrophobic peptides that have high percentage overlap with TMDs elute later in the gradient, with no noticeable shift in gross retention times as temperature increases. Similar to the trend shown in Fig. 1 for total proteins and peptides identified, the most dramatic increase in number of peptides overlapping with predicted TMDs is observed from 20 to 40°C (45 vs. 83 peptides, respectively) and then plateaus somewhat at 50 and 60°C (80 and 88 peptides, respectively). However, when considering only the peptides that almost completely overlap with predicted TMDs (peptides with >75% of their sequence overlapping with a TMD (black squares)), as a proportion of all TMD overlapping peptides, there is a noticeable increase for all elevated temperatures, with no apparent plateau (Fig. 2B). This suggests that further recovery of these extremely hydrophobic and long peptides may be possible with modifications to the existing thermostatted platform, such as using longer columns [44-46] or multidimensional separations with heat stable resins (Zorbax SB, mRP-C18, or specialized nonsilica resins [47-49]) at higher temperatures.

These recent reports addressing improved analysis of hydrophobic samples have focused on sample loading and recovery as the main benefits of elevated temperature rather than the more classic benefits of speed, peak shape, and selectivity. Due to the complexity of the peptide samples analyzed, resolution was not significantly improved with heated chromatography, and no improvement in peak shape or analysis time was observed [10]. This is due (at least in large part) to the fact that, for purposes of comparison, we chose to hold the flow rate constant across all temperatures, resulting in a decrease in backpressure (due to decreased viscosity) at elevated temperature. If backpressure is held constant (resulting in an increased flow rate at elevated temperature) instead, then peaks become markedly sharper and the elution profile is shifted and compressed for the heated μ LC runs. While resolution is largely maintained under these conditions, fewer overall proteins/peptides were identified (data not shown). Perhaps future innovations in instrumentation (nanospray ionization optimized for higher flow rates, MS/MS instruments with higher cycle times) or the use of higher resolution instrumentation may

support such higher flow rates, which should lead to faster analysis and improved peak shape along with increased identification of hydrophobic species.

5 Conclusions

The beneficial effects of temperature on peak resolution and selectivity for small molecules and simple mixtures are well established, and reports have shown that heated chromatography is beneficial for complex proteomic mixtures in terms of reduced analysis time and enhanced peak shape and selectivity. Recent analysis of membrane proteomic samples have demonstrated additional important advantages of heated LC: improved sample handling and enhanced recovery of extremely hydrophobic proteins and peptides. Heated LC has a dramatic, positive impact on separation and purification of IMPs (*e.g.*, for top-down proteomics applications [4]), as well as shotgun analysis of IMP digests, as we have demonstrated with our MEP sample. While membrane shaving techniques are used for many different applications (for review see: [3]), only a few groups prior to us have reported proteolytic shaving as a method to enrich for TMDs [50,51] (the most common application of membrane shaving is to examine the exposed soluble peptides generated by digestion [52-57]). However, the benefits of elevated temperature LC should be generally applicable for hydrophobic species regardless of whether the proteomic sample is a TMD-enriched preparation or a conventional tryptic digest.

While there are only a few reports to date of the benefits of elevated temperature for IMP analysis, hopefully the importance of temperature will soon gain wider recognition in the proteomics community. Thermostating capability is already widely available on commercial HPLC systems, and, we hope to have demonstrated, relatively easy to incorporate into any μ LC platform. Routine implementation of heated LC should enhance IMP purification efforts as well as the information content of shotgun proteomics, resulting in more comprehensive analysis of membrane proteomes.

Appendix: μ LC column heaters on a budget

Column temperature is commonly controlled by block heaters, circulating heated air, or water baths [58]. While commercial column heaters are widely available through HPLC pump manufacturers, currently they are not available for microcapillary columns. Block column heaters for microcapillary columns can be constructed in-house easily and economically. Two main factors should be considered when designing microcapillary column heaters: (i) The column heater should be slightly longer than the column to allow for adequate temperature equilibration of the liquid phase; (ii) the temperature along the length of the column heater should be constant. (For a more complete discussion of column heater control, see ref. [58]). Our general design sandwiches the column between two grooved aluminum plates outfitted with a cartridge heater. Aluminum was chosen to reduce overall cost; however, stainless steel could be substituted. Three variations of this design (A, B, C) have been employed to accommodate individualized proteomic applications. These column heaters provide a straightforward and inexpensive way to incorporate thermostating capabilities to any μ LC platform, and the integration of heated chromatography should greatly aid in the comprehensive proteomic analysis of membrane proteins. (Schematics are available at www.membraneproteome.uchsc.edu.)

(A) Single-phase chromatography: Column heater design for 12 cm column with pulled tip (block heater is 15 cm in length). A microcapillary column is positioned within a $\sim 380\ \mu\text{m}$ diameter groove machined into the bottom block (shown in the lower panel). The bottom block also contains a cartridge heater and thermocouple for temperature control feedback.

(B) Multiphase chromatography or MudPIT (with optional adapter for in-line filter assembly): Column heater modified to accommodate multi-phase columns where a larger diameter RP loading column (1 cm resin) is attached to a 12–15 cm SCX/RP biphasic column via in-line filter assembly (Upchurch). This block heater is 22 cm in overall length. As can be seen in the lower panel, there is a cutout for the filter union, otherwise the construction is similar to (A).

(C) Long (> 15 cm) columns: There are practical limitations in the total length of the design shown in (A) and (B). Therefore, a box column heater was design to accommodate columns of any length. Instead of direct contact with the metal, the column is coiled and suspended in an enclosed air chamber with thermocouple (shown in the lower panel). Two cartridge heaters in the bottom block maintain temperature. The tip and tailing end of the capillary are held in place with electrical tape. Chamber is of adequate size to accommodate a filter union if necessary. Chromatography is highly reproducible, and peaks shifts between runs at 60°C are minimal (less than 20 s).

Note: All column heaters shown in the upper panels are clamped at an angle to optimize electrospray.

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Abbreviations

FA	formic acid
IMP	integral membrane protein
MEP	membrane-embedded peptide
mRP	macroporous RP
MudPIT	multidimensional protein identification technology
TMD	transmembrane domain
μLC	microcapillary (nanoflow) LC

Figure 1.

Comparison of proteomic profiles resulting from the shotgun analysis of a tryptic digest of an enriched membrane sample as a function of temperature. (A) Elevated temperature increases the number of non-redundant protein identifications. (B) Elevated temperature increases the number of unique peptide identifications. (C) Elevated temperature increases the average peptide GRAVY score. (D) Relative gains in protein identifications, peptide identifications, and GRAVY scores with increasing temperature as compared to 20°C. * $p < 0.01$ compared to 20°C, Student's *t*-test.

Figure 2.

Comparison of retention times for TMD peptides. (A) GRAVY scores (scale on left y-axis) of tryptic peptides predicted to overlap with predicted TMDs (as predicted using TMHMM [39]) are plotted against their chromatographic retention times during μLC analyses carried out at 20, 30, 40, 50, and 60°C . Gray circles designate peptides with $\leq 75\%$ of their sequence overlapping with a TMD. Black squares designate peptides with $> 75\%$ of their sequence overlapping with a TMD. The μLC gradient is superimposed as a dashed line with the %B on the right y-axis. (B) Relative gains in the identification of peptides that have $> 75\%$ overlap with a predicted TMD with increasing temperature as compared to 20°C .