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Abstract: TRIzol is used for RNA isolation, but also permits protein recovery. We investigated whether proteins prepared with TRIzol were suitable for two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation mass spectrometry. Proteins from TRIzol-treated SH-SY5Y cells produced 2-DE spot patterns similar to those from an equivalent untreated sample. Subsequent identification of TRIzol-treated proteins using peptide mass fingerprinting was successful. TRIzol exposure neither altered the mass of myoglobin extracted from SDS gels nor the masses of myoglobin peptides produced by in-gel trypsin digestion. These findings suggest that proteins isolated with TRIzol remain amenable to proteomic analyses.

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The Editor

Analytical Biochemistry

Dear Sir/Madam

Submission of manuscript

I submit herewith a "Notes and Tips" article for your consideration, entitled "Proteins isolated with TRIzol are compatible with two-dimensional electrophoresis and mass spectrometry analyses". It is aimed at people who may not be highly experienced in proteomic work and who are trying to sort out sample preparation methods that will suit their experimental situation. TRIzol is especially useful when doing parallel protein/RNA expression analysis with limited sample sizes. Analytical Biochemistry seems to us a very suitable place for this paper.

We have previously submitted this article in a longer format (manuscript ABIO-09-981). This manuscript was rejected as being more suitable for the Notes and Tips section of the journal.We agree and have now re-formatted it. This has taken some time because of new preoccupations and because of the difficulties imposed by distance. Also Dr Young's increasing sophistication in the interpretation of peptide mass fingerprint data has led him to re-analyse one part of the data. There are no significant changes in the data or in the conclusions reached, but there are some minor alterations in the way the data is now is presented resulting from this further examination. The reference list has been updated to include recent relevant papers.

Thank you for your time in dealing with this article.

Yours sincerely

Penelope Truman

Proteins isolated with TRIzol are compatible with two-dimensional electrophoresis and mass spectrometry analyses

Short title: Assessment of TRIzol for protein preparation

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Abstract

TRIzol is used for RNA isolation, but also permits protein recovery. We investigated whether proteins prepared with TRIzol were suitable for two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation mass spectrometry. Proteins from TRIzol-treated SH-SY5Y cells produced 2-DE spot patterns similar to those from an equivalent untreated sample. Subsequent identification of TRIzol-treated proteins using peptide mass fingerprinting was successful. TRIzol exposure neither altered the mass of myoglobin extracted from SDS gels nor the masses of myoglobin peptides produced by in-gel trypsin digestion. These findings suggest that proteins isolated with TRIzol remain amenable to proteomic analyses.

Keywords: 2-DE, mass spectrometry, peptide mass fingerprint, protein extraction, proteomics, TRIzol

Abbreviations:

2-DE, two-dimensional gel electrophoresis

ACN, acetonitrile

CHCA, α-cyano-4-hydroxycinnamic acid

IPG, immobilised pH gradient

MALDI, matrix-assisted laser desorption/ionisation

TCEP, tris(2-carboxyethyl)phosphine

TFA, trifluoroacetic acid

TOF, time-of-flight

TRIzol is a phenol and guanidine isothiocyanate solution used to obtain RNA from biological samples. After nucleic acid extraction, protein can be precipitated from the phenol and ethanol supernatant [1]. Since the methodology allows the stepwise isolation of DNA, RNA and protein, this reagent is useful when genomic, transcriptomic and proteomic information is desired from a single sample [2]. However, we were concerned that TRIzol treatment might produce amino acid modifications to undermine both the quality of the protein isolation and the utility of proteomic methodologies for protein analysis. The suitability of TRIzol-exposed samples for proteomic analyses was therefore evaluated by inspecting the quality of the 2-DE gels and whether subsequent protein identification by peptide mass fingerprinting was affected.

The effect of TRIzol on protein from human neuroblastoma cells was assessed first. Approximately 10^7 SH-SY5Y cells (CRL-2266, American Type Culture Collection, Manassas, VA) were processed with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, except that the protein was precipitated with ice cold acetone instead of propan-2-ol. After centrifugation at 12000 x *g* for 10 min and removal of the supernatant, the pellet was washed five times with 300 mM guanidine hydrochloride in 95% ethanol and twice in ice cold acetone prior to centrifugation. The dried protein pellet was mixed with 200 µL solubilisation solution (8 M urea, 3 M thiourea, 1% dithiothreitol, 10 mM TCEP, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 1% pH 3-10 ampholytes) for 2 h before centrifugation at 21000 x *g* for 10 min. An equivalent number of control SH-SY5Y cells were directly solubilised and centrifuged in a similar manner for comparison purposes. Protein concentrations were determined by Bradford assay before performing 2-DE analysis (Supplementary Methods).

Comparison of the 2-DE gel images from control and TRIzol-treated SH-SY5Y proteins revealed similar spot patterns (Figure 1). Although there were discernible differences in the intensity of some spots between treatments, the protein extraction by TRIzol was predominantly unbiased. Nonetheless, the representative TRIzol gel displayed vertical streaks originating from the alkaline region of the pH 3-10 IPG strip. When a similar TRIzol-treated sample was focused on a pH 4-7 IPG strip (Supplementary Figure 1), only resolved spots and negligible vertical streaking were observed. These results suggest that 2-DE of SH-SY5Y cells is largely unaffected by TRIzol exposure and some artefacts can be avoided if IPG strips with different pH ranges are used. The utility of TRIzol protein extraction for 2-DE separations has been described [3,4], with several TRIzol samples benefiting from the removal of contaminants that typically interfere with 2-DE [5,6].

Seven spots from a replicate 2-DE gel of TRIzol-treated SH-SY5Y cells (Supplementary Figure 2) were excised for protein identification. Briefly, each gel spot was destained and subjected to trypsin digestion as previously described [7]. After the extracted peptides were analysed by MALDI-TOF MS, peptide mass lists were submitted to database searches for protein identification (Supplementary Methods). All seven spots were confidently identified by peptide mass fingerprinting (Supplementary Table 1), demonstrating the compatibility of TRIzol extracted proteins with subsequent analysis by MALDI-TOF MS. Identification of TRIzol isolated proteins by peptide mass fingerprinting has been demonstrated by several groups [2,8].

Although the identification of several TRIzol exposed SH-SY5Y proteins was successful, there was a possibility that the non-matching peptide masses were adducts produced from TRIzol incubation. An investigation was initiated on a well characterised protein (horse

myoglobin) to ascertain whether the TRIzol methodology induces mass modifications, with myoglobin masses from TRIzol and control treatments determined by MALDI-TOF MS (Supplementary Methods). The average masses obtained from each sample were quite similar, with representative spectra shown in Figure 2. A mean average mass of 16994 Da for control horse skeletal myoglobin (95% confidence interval: 16985 to 17003.1 Da) was calculated from quadruplicate spectra, while 16994.6 Da was obtained from TRIzol-treated samples (95% confidence interval: 16986.3 to 17002.8 Da). These mass measurements were very different to the molecular mass of 16951.5 Da obtained from an earlier myoglobin study [9], which reported the average mass of horse myoglobin without the N-terminal methionine because of cotranslational cleavage. When cotranslational N-terminal acetylation is also considered [10], the recalculated theoretical mass (16993.5 Da) is in close proximity of our myoglobin mass measurements. The remaining three peaks in each MALDI-TOF spectrum (in order of descending *m/z*) correspond well with the respective doubly, triply and quadruply charged masses of extensively cotranslationally modified myoglobin. We found no evidence to suggest TRIzol causes unusual or considerable mass modifications in myoglobin, although we cannot exclude mass changes that occur at a low abundance or those that involve small masses.

To address these issues, gel pieces containing intact myoglobin from TRIzol and control treatments were subjected to peptide mass fingerprint analysis (Supplementary Methods). The sequence coverage from TRIzol-treated myoglobin samples was similar to those obtained by the controls, which ranged from 79 to 84% between all technical replicates (Supplementary Table 2). Importantly, the vast majority of the submitted masses were declared by database searches to match myoglobin tryptic peptides (Supplementary Table

3). These observations suggest that TRIzol treatment does not appear to hinder the identification of myoglobin by peptide mass fingerprinting.

On closer inspection of the myoglobin peptide matches, masses obtained from control and TRIzol peptide mass fingerprint spectra were adjudged by database searches to match the peptide GLSDGEWQQVLNVWGK (without the N-terminal methionine). A recent study identified the N-terminal acetylated peptide Ac-GLSDGEWQQVLNVWGK from horse myoglobin [11], but masses that could correspond to this peptide were not detected, raising doubt on the existence of an N-terminally acetylated myoglobin. However, evidence of an N-terminal acetylated peptide was from a mass matching the trypsin miscleavage product Ac-GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR, with one of its two tryptophans oxidised (theoretical MH^+= 3461.745). Although this peptide was identified from only one TRIzol sample, this match provides proof of the extensively cotranslationally modified form of myoglobin and corroborates our previous myoglobin measurements. We hypothesise that this long peptide was not routinely selected by database searches because of its low intensity or poor peak shape.

Because it possesses a similar mass to acetylation, carbamylation (43 Da increase) was considered as a possible explanation for the observed mass of myoglobin. When both N-terminal and lysine carbamylation were also selected as variable modifications, the myoglobin sequence coverage obtained in all samples generally increased to between 81 and 90% (Supplementary Table 4). Despite the additional settings, the number of peptide hits remained the same as before. Several peptide matches from both control (Supplementary Figure 3) and TRIzol (Supplementary Figure 4) spectra were adjudged to represent the doubly carbamylated HLKTEAEMKASEDLK (theoretical MH^+= 1815.891),

its related oxidation product (MH^+= 1831.886) and the doubly carbamylated FDKFKHLKTEAEMK with an oxidised residue (MH^+= 1853.922). However, these masses in the original searches accounted for the myoglobin peptides GLSDGEWQQVLNVWGK (theoretical MH^+= 1815.902), one of its associated tryptophan oxidation products (MH^+= 1831.897) and GHHEAELKPLAQSHATK (MH^+= 1853.962) respectively. We consider the new carbamylation matches to be false positives and cannot find evidence for the widespread presence of this modification in control or TRIzol samples. This suggestion is substantiated by the lower Mascot scores obtained from the carbamylation searches, where they also failed to surpass the 1% significance level. Importantly, the large acetylated peptide was reported with search settings that included carbamylation. Since multiple carbamylations would have increased the mass of myoglobin drastically, the combination of N-terminal methionine cleavage and acetylation remains the most likely reason for the mass difference between the observed and theoretical myoglobin masses.

Overall, no systematic differences between TRIzol-treated and control myoglobin tryptic peptides were detected. We conclude that the TRIzol procedure is compatible with peptide mass fingerprinting for the identification of proteins.

In summary, we have shown in this report that the isolation of proteins with TRIzol does not appear to introduce any artefacts or mass modifications that interfere with intact protein analysis or peptide mass fingerprinting. With only minor changes to the original protocol, this method allows the isolation of complex protein mixtures for further analysis by standard proteomic methods. This is an especially useful application of the reagent when systems biology approaches are desired from limited amounts of starting material.

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The authors have declared no conflict of interest.

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Figure 1: **Young C & Truman P:** Assessment of TRIzol for protein preparation

2-DE of control and TRIzol-treated SH-SY5Y proteins (pH 3-10).

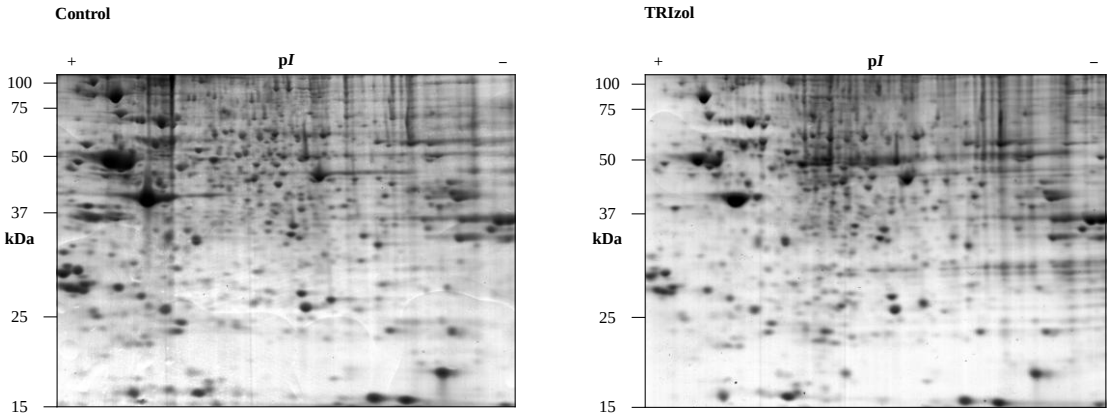
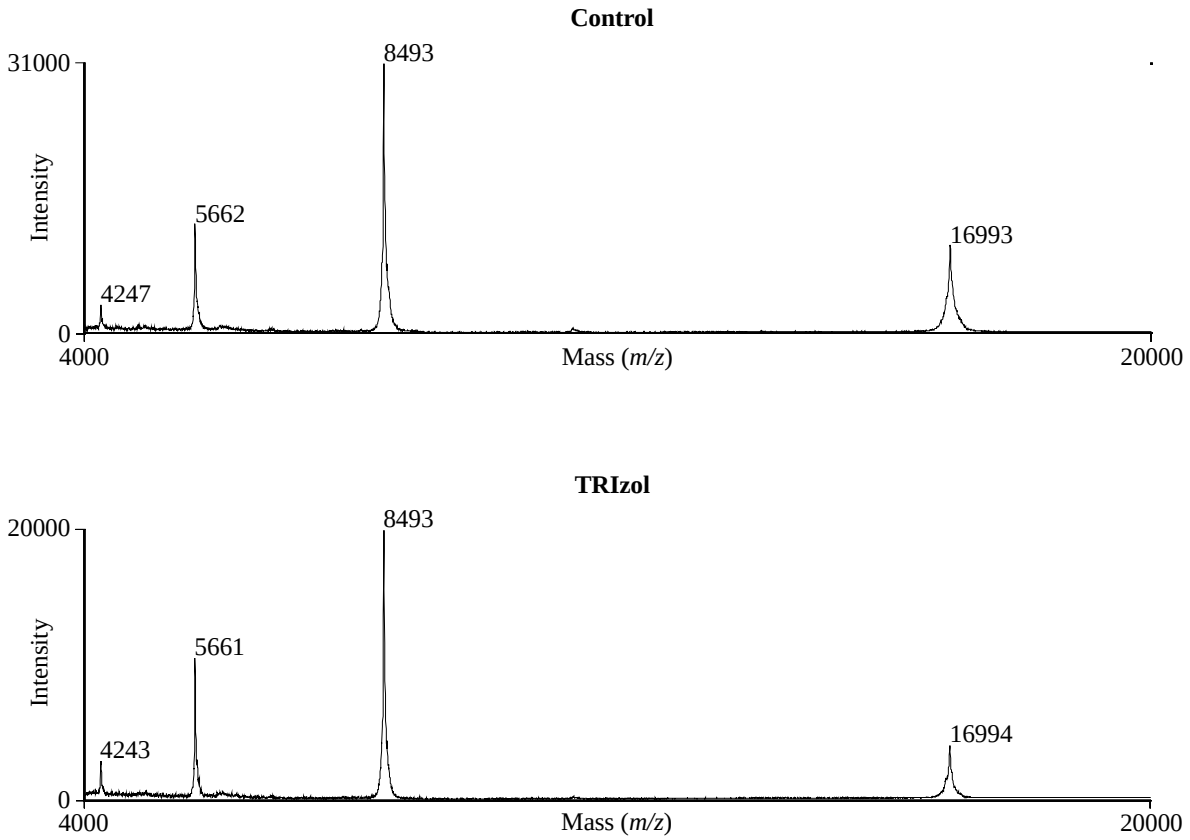


Figure 2: Young C & Truman P: Assessment of TRIzol for protein preparation

MALDI-TOF spectra of control and TRIzol-treated myoglobin.



Supplementary Figure 1

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Supplementary Table 1

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Supplementary methods

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